Non-invasive assessment of ventilation maldistribution in lung disease using multiple breath inert gas washouts.

Alex Horsley

PhD
University of Edinburgh
2009

Thesis Abstract

Clinical research in cystic fibrosis (CF) requires study endpoints that are sensitive to airways disease, repeatable and non-invasive. Despite significant advances in the treatment of CF, lung function assessments continue to rely on the forced expiratory volume in 1 second (FEV_1). Although simple to perform, it lacks sensitivity, is difficult for younger subjects, and changes over time. An alternative method of assessing lung physiology is to derive measures of ventilation heterogeneity from inert gas washout tests. In early lung disease, measures of gas mixing appear to be more sensitive than spirometry. In addition, since only tidal breathing is required, they are more physiological and are more straightforward for younger subjects. Widespread use has been impaired by the lack of a robust and cost effective gas analyser technology.

The work presented in this thesis concerns the adaptation, validation and then use of a novel gas analyser (Innocor) in a clinical system for the performance of multiple breath washouts. Lung clearance index (LCI), a simple measure of ventilation heterogeneity, has been calculated from washouts in 52 adults with CF and 50 healthy controls. LCI was more sensitive to disease than FEV_1 in CF, being elevated in 11 of the 12 CF patients with normal spirometry. In healthy subjects, LCI has been shown to be repeatable and reproducible, with a narrow range of normal that is stable over a wide age range.

In a separate study of 19 patients, LCI has also been shown to improve with treatment of an exacerbation in CF. Correlation with changes in other biochemical (serum CRP, peripheral blood white cell count, sputum IL-8, sputum neutrophil) clinical (symptom score) or structural (computed tomography) markers was poor. Short term change in LCI has also been demonstrated in CF patients in response to chest physiotherapy, although there was considerable heterogeneity of response in terms of both LCI and volume of lung ventilated by tidal breathing (as measured by washout functional residual capacity).

In addition to LCI, multiple breath phase III slope analysis has been performed on washouts of CF patients and healthy controls, and this has been compared to other measures of lung physiology. Proposed measures of convective and diffusive gas mixing have been shown to be unreliable in CF. These studies have also been the first to demonstrate multi-centre use of washout tests as endpoints.

The technology described here offers the possibility of a simple and reliable system for performing multiple breath washouts, though at present it is not available commercially. The studies have added to the understanding of the utility and reliability of washout tests, as well as some of their limitations. It is hoped that in future LCI will be an important clinical endpoint in therapeutic intervention studies in CF, and that it will also offer new ways to follow changes in lung physiology in other diseases.

CONTENTS

Abbreviations

Abbreviation	Meaning
ACBT	Active Cycle of Breathing Technique (chest physiotherapy)
ASL	Airway Surface Liquid
BAL	Bronchoalveolar Lavage
C_{end}	End tidal marker gas concentration at the end of a washout
CEV	Cumulative Expired Volume
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Regulator
CI	Confidence interval
C_{init}	End tidal marker gas concentration at the start of a washout
CRP	C-Reactive Protein
CV	Coefficient of Variation
DF508	Delta F 508, the most common CFTR mutation in Caucasian populations
D_LCO	The diffusing capacity of the lung for carbon monoxide, a measure of integrity of the alveolar membrane
D_LCO / V_A	Diffusing capacity corrected for alveolar volume, also known as K_{CO}
ENaC	Epithelial sodium channel
FEF	Forced Expiratory Flow, usually suffixed by a number to indicate the percentage of FVC that flow was measured between
FEV_1	Forced Expiratory Volume in 1 second
FRC	Functional Residual Capacity
FVC	Forced Vital Capacity
GL67	Genzyme corporations Lipid 67
GTA	Gene Therapy Agent
IL	Interleukin
K_{CO}	See D_LCO / V_A
LCI	Lung Clearance Index
MBNW	Multiple Breath Nitrogen Washout
MBW	Multiple Breath Washout

Abbreviation	Meaning
MS	Mass Spectrometer
PCL	Pericilliary Liquid
PE	Pulmonary Embolus
R_{aw}	Airways Resistance
RV/TLC	Ratio of Residual Volume to Total Lung Capacity
S_{acin}	A measure of ventilation heterogeneity due to diffusion-convection interaction in the lung compartment defined by the acinar airways
SBW	Single Breath Washout
S_{cond}	A measure of convective ventilation heterogeneity in the lung compartment defined by the conducting airways
SD	Standard Deviation
SEM	Standard Error of the Mean
SF_6	Sulphur Hexaflouride
Sn_{III}	Normalised phase III (alveolar) slope
TLC	Total Lung Capacity
TO	Lung volume Turnover
V_D	Dead space
V_{exp}	Volume expired
V/Q	Ventilation / Perfusion ratio
V_T	Tidal Volume

Statement

This thesis has been composed wholly by myself, and has not been submitted for any other degree or professional qualification.

I have been the principal investigator in the work presented in this thesis, and have been involved in the planning, execution and analysis of all studies. Where data was obtained as part of a collaboration with other researchers, I have indicated this in the text.

Some of the results of the studies described in this thesis have been previously presented elsewhere; a list of publications is presented.

This work has been wholly funded by the UK Cystic Fibrosis Gene Therapy Consortium.

Alex Horsley

Acknowledgements

I wish to thank many people for their invaluable help and assistance in the completion of this thesis. In particular, I am grateful to Alastair Innes, my supervisor, for his enthusiasm, advice and support during this work. I also owe a debt of gratitude to Per Gustafsson, whose extensive knowledge in this area I have gratefully tapped into on a number of occasions, and without whose input this might all have been so different. I have also been grateful for the support and input I have received from Chris Boyd, Steve Cunningham and Andy Greening.

This work was carried out at the Western General Hospital, and could not have been completed without the assistance of the Welcome Trust Clinical Research Facility. I am also obliged to Mags Imrie, Javier Leiton and James Gibson for the processing and analysis of laboratory samples; and to Andy Robson and Jill Macleod for access to the Respiratory Function Laboratory. I have worked particularly closely with Kenny Macleod, who has provided data on measurements he performed in paediatric subjects.

I am profoundly grateful to the all the patients and healthy volunteers who have taken part in the studies contained within this thesis – it goes without saying that without their patience and willingness to participate none of this would have been possible. I would also like to acknowledge the CF Trust, and the CF Gene Therapy Consortium, who have provided the funding for this research.

Publications arising from this work

Work included in this thesis has been published in *Thorax*, in *Respiratory Physiology and Neurobiology*, and has been presented at national and international conferences as both poster and spoken presentations.

Lung clearance index in the assessment of airways disease (Review). Horsley A. *Respiratory Medicine* 2009; **103** (6): 793-9.

Effects of cystic fibrosis lung disease on gas mixing indices derived from alveolar slope analysis. Horsley AR, Macleod KA, Robson AG, Lenney J, Bell NJ, Cunningham S, Greening AP, Gustafsson PM, Innes JA. *Respiratory Physiology and Neurobiology* 2008; **162** (3): 197-203.

Lung clearance index is a sensitive, repeatable and practical measure of airways disease in adults with cystic fibrosis. Horsley AR, Gustafsson PM, Macleod KA, Saunders C, Greening AP, Porteus DJ, Davies JC, Cunningham S, Alton EW, Innes JA. *Thorax* 2008; **63** (2): 135-40.

Conference Abstracts

S_{cond} is an early and sensitive measure of airways dysfunction in cystic fibrosis. Horsley A, Macleod K, Bell N, Cunningham S, Innes JA. *European Respiratory Journal,* 2008, **32**; supl 51, 540s

Functional and structural changes in the cystic fibrosis lung following antibiotic treatment for exacerbation**.** Horsley A, Aziz Z, Macleod K, Saunders C, Meister M, Tyler P, Dewar M, Gray R, Voase N, Cunningham S, Greening A, Davies JC, Alton E, Hansell D, Innes JA. *Thorax*, 2007, **62**; supl. III, A31

Lung clearance index improves with treatment of an exacerbation in cystic fibrosis. Horsley A, Saunders C, Gray R, Macleod K, Dewar M, Voase N, Greening A,

Cunningham S, Davies J, Alton E, Innes JA. *European Respiratory Journal,* 2007, **30**; supl 51, 450s

Changes in lung gas mixing after physiotherapy in adults with cystic fibrosis. Horsley A, Ridley S, Beattie C, Macleod K, Greening A, Innes JA. *European Respiratory Journal,*2007 **30**; supl 51, 223s

Interpretation of inert gas washout phase III slope analysis in cystic fibrosis. Horsley A, Gustafsson P, Macleod K, Robson A, Lenney J, Greening A, Cunningham S, Innes JA. *European Respiratory Journal,* 2007, **30**; supl 51, 338s

Lung clearance index is superior to moment ratios in the analysis of inert gas washouts in cystic fibrosis. Horsley A, Macleod K, Greening A, Innes JA. *European Respiratory Journal,* 2007, **30**; supl 51, 338s

Lung Clearance Index improves with treatment of an infective exacerbation. Horsley A, Macleod K, Saunders C, Gray RD, Dewar M, Voase N, Porteous D, Boyd C, Hyde S, Gill D, Geddes D, Greening AP, Cunningham S, Davies JC, Alton E, Innes JA. *Pediatric Pulmonology*, 2007, **42**; suppl 30, 335s

Compact and portable lung clearance measurements using a photoacoustic analyzer. Horsley A, Greening A, Gustafsson P, Innes A. *European Respiratory Journal,* 2006, **28**; suppl 50, 834s

Lung Clearance Index is a more sensitive measure of airways dysfunction than FEV_1 in adults with CF. Horsley A, Greening A, Cunningham S, Innes JA. *Pediatr Pulmonology,* 2006, **41**; suppl 29, 66s

Compact & portable gas mixing measurements using a photoacoustic analyzer. Horsley A, McCullagh A, Greening A, Innes J, Gustafsson P. *Journal Cystic Fibrosis,* 2006, **5**, suppl 1, 50s

Awards

Work from this Thesis has been subject to the following awards and recognition:

1. Methven prize, Scottish Thoracic Society, Winter Meeting 2006, for best abstract
2. Croom Lecture prize, 2007, Royal College of Physicians of Edinburgh
3. British Lung Foundation Travel Fellowship award for attendance at the European Respiratory Society conference, 2007
4. Invited speaker at a symposium at the British Thoracic Society Winter Conference, 2007

Chapter 1- Introduction

Overview

This thesis concerns the technical validation, adaptation and use in a clinical setting of a novel gas analyser technology to measure multiple breath inert gas washouts in patients with airways disease. The research was initiated by the Cystic Fibrosis Gene Therapy Consortium as part of an ongoing programme into the development of minimally invasive biomarkers of cystic fibrosis (CF) disease activity. In this regard, multiple breath washouts are particularly attractive as potential study endpoints, since they are non-invasive and are believed, particularly in early airways disease, to be sensitive to processes occurring in the small airways. The majority of the work presented in this thesis was carried out in patients with cystic fibrosis or in healthy controls. However, the value of this technique is not limited to CF, and the final chapter describes results obtained from other patient populations.

The first part of this introduction concerns the genetics and pathophysiology of CF lung disease, and the nature of the gene therapy programme whose requirements have driven this research. The final part of the introduction describes the nature of CF airways disease and considers the current literature on multiple breath washout tests.

Cystic Fibrosis

Cystic fibrosis is the most common lethal autosomal recessive disease of Caucasians. The condition has a carrier frequency of 1:25 and affects 1 in 2500 live births. The faulty gene was identified almost 20 years ago, and encodes an epithelial ion channel known as the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan, Rommens et al. 1989). CFTR is a member of a class of proteins termed the ATP-binding cassette (ABC) family (Gadsby, Vergani et al. 2006). Unlike other members of the family however, the CFTR functions as a chloride channel, and is regulated by protein kinase A in a c-AMP dependent fashion (see Figure 1.1). CFTR forms part of a multi-protein assembly in the apical plasma membrane, and is also responsible for the regulation of a number of cellular processes. In particular it acts to down-regulate the activity of the transepithelial sodium channel (ENaC) in the apical epithelial membrane. In addition to this, it also interacts with calcium-activated chloride channels and potassium channels (Kunzelmann, Schreiber et al. 2000). These other proteins are potential modifiers of the CF phenotype, and may help to explain the wide variability in clinical phenotype even amongst those with identical CFTR mutations.

Over 1500 CFTR mutations have been described (http//www.genet.sickkids.on.ca/cftr), but the most common mutation in the CF gene is a deletion of phenylalanine at position 508, known as ΔF508. This is largely responsible for the high incidence of CF in the Caucasian population, where it accounts for over two thirds of CF mutations (Bobadilla, Macek et al. 2002). The mutations are grouped into six classes depending on their effect on gene expression (see Figure 1.2) (Rowe, Miller et al. 2005). These classifications are not merely academic since clinical severity is affected by the amount of residual CFTR activity. For instance, the presence of a class IV mutation (e.g. R117H) is associated with pancreatic sufficiency, and class V mutations may be associated with particularly mild phenotypes (e.g. congenital bilateral absence of vas deferens, with no other clinical manifestations of CF). Patients whose genotypes include class I or II mutations (i.e. the more severe mutations, where there is no or little CFTR expressed

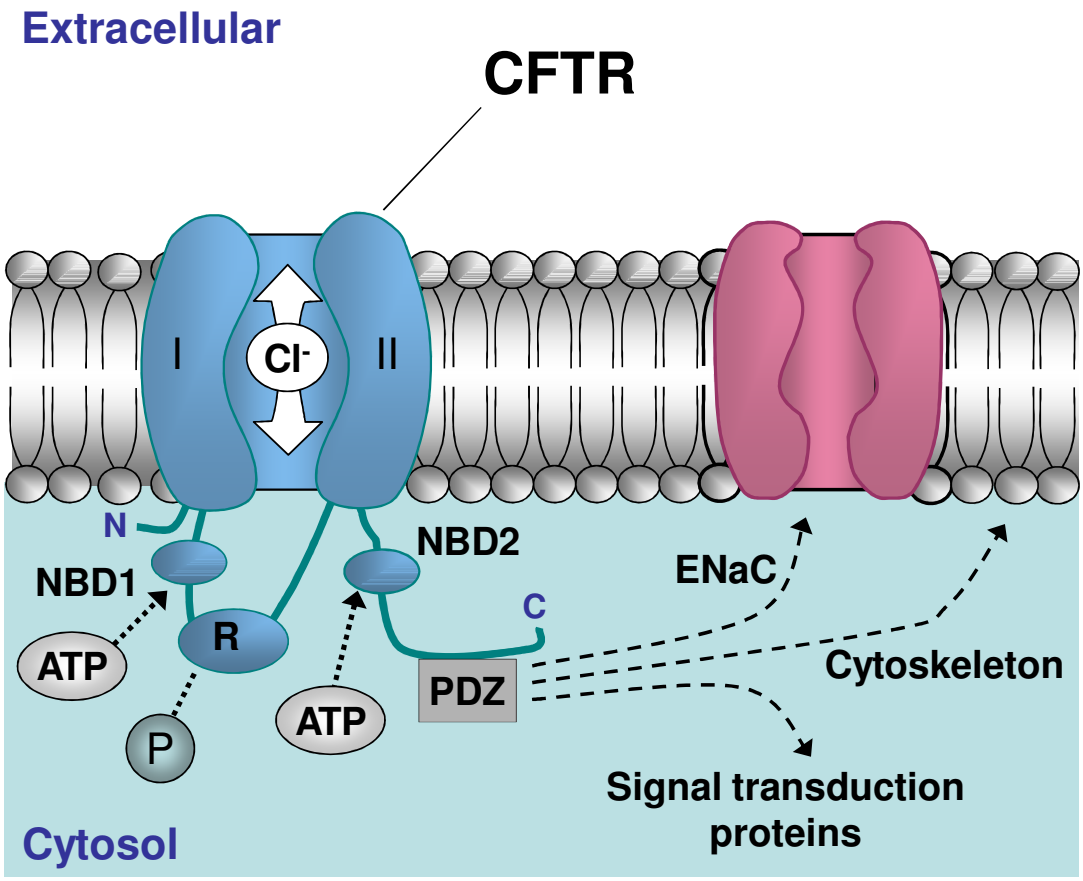


Figure 1.1: Structure of the cystic fibrosis transmembrane regulator (CFTR) molecule. The protein is expressed in apical cell membranes where it functions as a chloride channel. The channel is made up of two membrane spanning domains (I and II), each consisting of 6 membrane spanning alpha helices. Two nucleotide binding domains (NBD1 and NBD2) mediate ATP hydrolysis, and it is on one of these (NBD1) that the most common CFTR mutation (ΔF508)) occurs. Phosphorylation of the R domain permits a conformational change and opening of the channel. CFTR interacts through its C terminus with an array of different cellular proteins, including cytoskeleton, cell signaling mechanisms, and other ion channels - in particular the epithelial sodium channel ENaC (in purple).

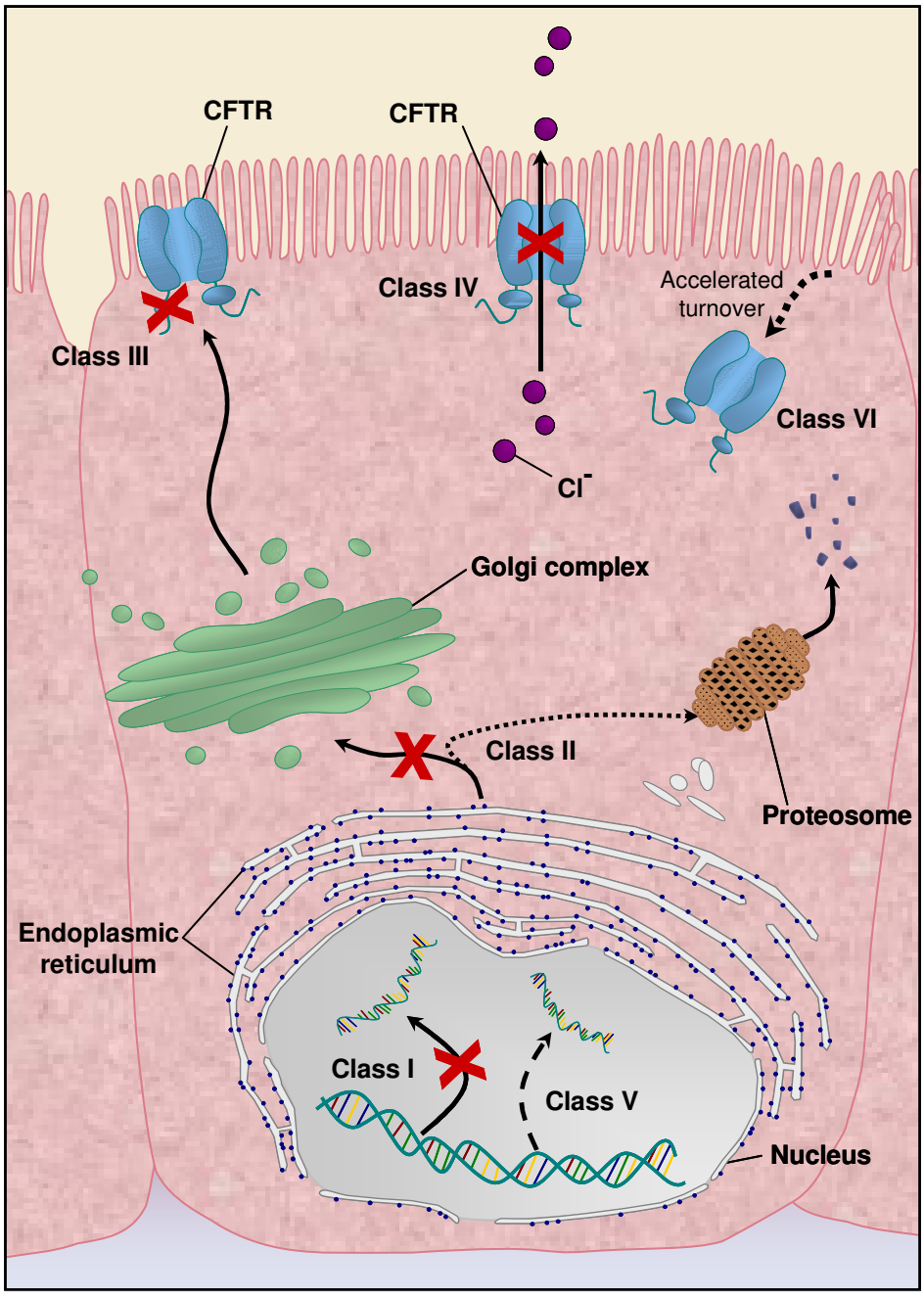


Figure 1.2: Classes of CFTR mutation and their effect on gene expression. Class I mutation – transcription errors; Class II – defective protein maturation and trafficking; Class III – disordered regulation; Class IV defective channel gating; Class V – splicing abnormalities; Class VI – accelerated turnover. The first three of these are associated with particularly diminished or absent CFTR activity and more severe clinical phenotypes. Adapted from Rowe et al. (Rowe, Miller et al. 2005).

at the cell surface) on both alleles have, on average, more rapid deterioration in lung function, and lower survival rates, than patients who have class IV or V mutations (i.e. those possessing some residual CFTR activity) on at least one allele (de Gracia, Mata et al. 2005). Even amongst those with identical CFTR genotypes however, there is considerable phenotypic variability. The reasons for this are multi-factorial, and include not only the activity of modifier genes but also social and environmental factors, such as access to specialist healthcare and compliance with treatment regimes (Schechter, Shelton et al. 2001; Padman, McColley et al. 2007).

Pathophysiology

Lung disease in cystic fibrosis

Pulmonary manifestations of CF present as chronic bacterial bronchitis that progresses to chronic suppurative bronchiectasis and ultimately respiratory failure and death. In the lung, CFTR is detectable on the apical membrane of ciliated cells within the gland ducts and in the superficial epithelium of healthy individuals (Kreda, Mall et al. 2005). In CF, the submucosal glands and distal airways are obstructed by thick tenacious secretions. The clinical syndrome suggests a failure of the normal airway defence mechanisms against bacterial infection. There are two main hypotheses that have been proposed to link the clinical findings with the known actions of the CFTR chloride channel.

The first of these hypotheses, known as the **high salt hypothesis**, was proposed by Smith et al. (Smith, Travis et al. 1996). They observed that cultures of CF airway epithelia in vitro lacked the innate anti-pseudomonal activity present in cultures of healthy cells. Furthermore, this activity was dependent on a low molecular weight soluble factor, human β-defensin-1, which could be inactivated by high concentrations of NaCl (Goldman, Anderson et al. 1997). Such high ASL salt concentrations had been demonstrated in vivo and in vitro (Joris, Dab et al. 1993; Smith, Travis et al. 1996). The theory proposed that loss of the CFTR Cl^- channels

prevented Cl^- from accompanying Na^+ absorption, as in CF sweat ducts (Quinton 1990), with consequent inhibition of defensin activity. There is impaired clearance of bacteria deposited on the airway surface during normal respiration, and neutrophils and macrophages are recruited, ultimately disrupting normal airway function and leading to the familiar picture of chronic infection and inflammation.

However, this theory is not supported by in vivo observations on airway surface liquid (ASL) height, nor by the effects on ASL volume of ionic and non-ionic osmolytes (Tarran, Grubb et al. 2001). The alternative, and increasingly widely accepted, explanation for the clinical effects of CFTR deficiency is known as the **low volume hypothesis** (Matsui, Grubb et al. 1998). It is proposed that the absence of CFTR leads to over-activity of ENaC (since CFTR down-regulates ENaC in the wild type). Excessive sodium is absorbed, and chloride follows through non-CFTR chloride channels. In support of this, a mouse model with excessive ENaC activity has a lung phenotype similar to that of human CF (Mall, Grubb et al. 2004). There is a net absorption of ions and fluid, which leads to a shrinking of the ASL layer, dehydration of mucous secretions and collapse of epithelial cilia. The effects on the mucus layer and the cilia are felt to be crucial in the development of CF lung disease. In health, the mucus layer is a gel consisting of around 1% salt, 1% high molecular weight secreted mucins (MUC5AC and MUC5B), and 98% water (Verdugo, Tam et al. 1983). At its inner surface, between the mucus layer and the epithelial cell membrane, lies a thin pericilliary liquid layer (PCL). This layer provides a low viscosity solution in which the cilia can beat rapidly (8-15Hz). It also prevents adhesion of the mucus to the epithelial surface, thereby lubricating the movement of mucus during cough clearance (Zahm, Pierrot et al. 1989).

The regulation of the PCL volume is complex, and is dependent on the integrity of both CFTR-dependent chloride secretion and CFTR regulation of ENaC. Blockage of chloride secretion with bumetanide, or addition of an artificial active Na^+ channel in the form of nystatin, leads to excess ion and water absorption (Boucher 2007). Healthy airway epithelia in culture maintain an ASL depth of 7µm, but CF airway epithelia continue to absorb the ASL and the cilia collapse onto the cell surface (Tarran, Button et al. 2005). This reflects the failure of the mutant CFTR to act as a

Cl^- channel or ENaC regulator, and occurs despite normal adenosine regulatory pathway in CF.

Unregulated absorption of ions and water across the CF airway epithelium causes shrinkage of the PCL in CF and dehydration of the overlying mucus layer (Figure 1.3). Shrinkage of the PCL impairs the action of the cilia and brings an increasingly adhesive mucus layer into contact with the epithelium. The disruption of the mucociliary escalator leads to sequestration of mucus in the lungs and immobilized mucus forms plaques. These plaques cause airflow obstruction and permit chronic infection with pathogenic bacteria. The exact process by which the infection is initiated is unclear, but may comprise inhaled bacteria that are not cleared efficiently, or the initial infection may follow an insult to the lung, e.g. viral infection or aspiration (Abman, Ogle et al. 1988). Whatever the initiating event, lungs of CF patients become colonised early in the course of the disease with bacteria such as Staphylococci or *H. influenza* (Rosenfeld, Gibson et al. 2001). These infections become persistent in the first few years of life and the inflammatory response leads to neutrophil recruitment and activation. This in turn stimulates hypersecretion of mucin. Bacterial products and cellular debris accumulate, along with polymeric DNA which makes the mucus more viscous and even harder to expel (Zahm, Girod de Bentzmann et al. 1995). The CF airways fill up with purulent secretions and a vicious cycle of infection, inflammation and progressive endobronchial destruction is established.

The clinical course is typically punctuated by periods of exacerbation, when bacterial infection gains the upper hand over host defence. At these times there is usually deterioration in lung function, and associated symptoms of infection. The involvement of different infecting organisms, particularly *Pseudomonas aeruginosa* and *Burkholderia cepacia*, further alters the delicate balance in favour of greater inflammation. The majority of bacteria in the CF airways are located within the mucus layer, and virtually none are intracellular or bound to the epithelium (Baltimore, Christie et al. 1989).

The formation of *Pseudomonas* biofilms appears to be encouraged by the thickened mucus. The mucus limits bacterial motility and diffusion of excreted proteins (Dawson, Wirtz et al. 2003). The resulting high bacterial densities are

detected by the bacteria's quorum-sensing mechanisms, which triggers a phenotypic change to biofilm forms (Davies, Parsek et al. 1998). In addition, the thick mucus layer and the increased oxygen consumption of the epithelial cells leads to an oxygen gradient across the mucus, with particularly hypoxic areas in mucus plugs (Worlitzsch, Tarran et al. 2002). This hypoxic environment favours the growth of Pseudomonas and the switching to biofilm formation.

It is acknowledged however that this may not represent the entire story. For example, primary ciliary dyskinesia results in a complete failure of mucociliary transport, but the patients do not usually suffer such severe generalised bronchiectasis, and it is consistent with a normal life expectancy (Schidlow 1994). It may be that defective CFTR leads to dysregulation of the host defences in other more complex ways. In vitro studies using CF epithelial cells have demonstrated an increased secretion of pro-inflammatory cytokines in unstimulated cells that is unrelated to the nature of the CFTR mutation and independent of effects on chloride transport (Weber, Soong et al. 2001). Other studies have looked at the response of CF and non-CF epilthelial cell cultures to stimulation with inflammatory cytokines and bacterial products (Aldallal, McNaughton et al. 2002; Becker, Sauer et al. 2004). Both of these studies showed considerable variability in response to stimulation, making it hard to demonstrate a clearly pro-inflammatory phenotype in CF cells in vitro. To overcome the problem of confounding genetic variability between different cell lines, Perez et al. applied a specific inhibitor of CFTR chloride conductance to well differentiated human tracheal cells grown in an air-liquid interface (Perez, Issler et al. 2007). Inhibition of CFTR was sufficient to cause an increase in IL8 in both unstimulated cells and in response to *Pseudomonas aeruginosa* products.

It is obviously much harder to investigate the development of inflammation *in vivo* since almost all patients with CF also have established chronic bacterial infection. A number of studies of infant bronchoalveolar lavage (BAL) fluid have found evidence of airway inflammation without concurrent lower airway infection (Balough, McCubbin et al. 1995; Khan, Wagener et al. 1995). Subsequent studies have since disputed this (Armstrong, Grimwood et al. 1997; Dakin, Numa et al. 2002), but it remains clear that inflammation is an early event in CF.

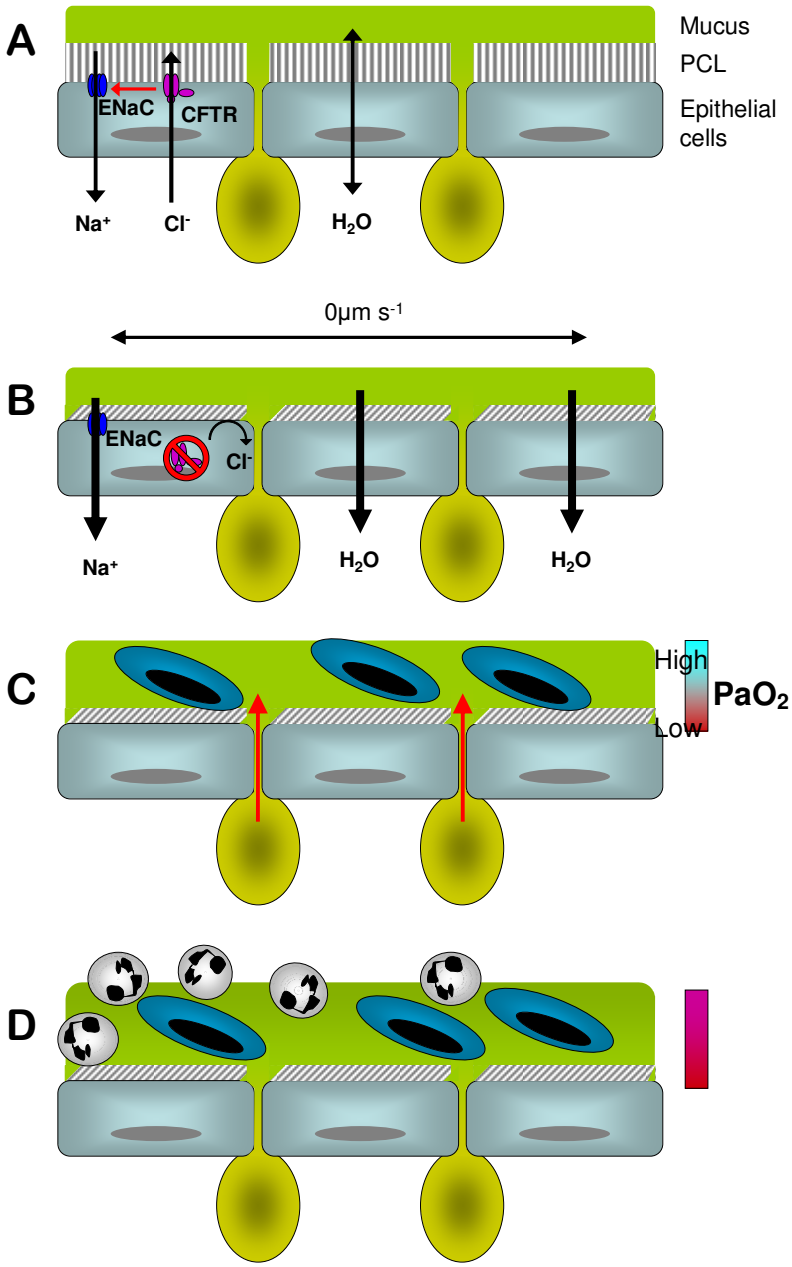


Figure 1.3: Proposed mechanism for the development of chronic airway infection in CF, after Boucher (Boucher 2007). In normal airways (A), hydration is controlled by Na+ absorption & Cl- secretion. In CF (B), the absence of CFTR leads to unregulated Na+ absorption and associated dehydration of the pericilliary layer (PCL) and mucus. Mucus becomes adherent in plaques which allow bacterial colonisation (C), encouraged by a hypoxic gradient across the plaque. The resulting inflammatory response (D) leads to a vicious cycle of inflammation, mucus secretion and retention, and ultimately airway destruction.

Effects in the airways

The harmful effects of CFTR dysfunction are particularly manifest in the small airways. Only relatively low levels of CFTR expression are found in the bronchial epithelium, but CFTR is expressed throughout the distal airway and is greatest in the respiratory bronchioles (Engelhardt, Zepeda et al. 1994). The pathological hallmarks of CF are infection and inflammation, and small airways are particularly susceptible to the effects of airway wall inflammation and obstruction.

High resolution computed tomography (HRCT) scans cannot image normal airways with a diameter of less than 2mm. However, abnormalities in the small airways can be visualised on CT, either indirectly in the case of mosaic attenuation due to gas trapping, or directly as mucus plugging or airway dilatation (Hansell 2001). CT studies have shown that there is progressive airway wall thickening, most marked in the peripheral airways, and that progression of this correlates with changes in mid expiratory flows (de Jong, Nakano et al. 2005). Unless there is associated airway dilatation, airway wall thickening will have a disproportionate effect on the luminal resistance of the smaller airways. Pathological studies have shown that this airway wall thickening is due to epithelial enlargement, smooth muscle thickening and epithelial metaplasia with goblet cell extension into the small airways (Bedrossian, Greenberg et al. 1976; Tiddens, Koopman et al. 2000).

Small airways are also particularly prone to mucus plugging, which has been described as affecting more than 50% of the total luminal volume in 64% of small airways in the severely affected lungs removed at lung transplantation (Burgel, Montani et al. 2007). Copious surface mucus has been shown in respiratory bronchioles at electron microscopy (Simel, Mastin et al. 1984). In addition to directly obstructing small airways, sequestration of mucus increases the surface tension, thereby further promoting airway closure and gas trapping.

There is a paucity of pathological data in young or healthy subjects. This means that, although we know newborns have histologically normal lungs (Chow, Landau et al. 1982), there is little information on how this progresses to the advanced changes seen in lungs removed at transplantation or autopsy. Specimens taken from patients who have undergone lung transplantation show a variety of airway

abnormalities, including bronchiectasis, fibrosis, inflammation, mucus impaction, pulmonary vascular changes and reduced small airway density (Hamutcu, Rowland et al. 2002). It is not clear whether the reduction in small airway density is due to progressive destruction or dilatation. It is also unclear how the different pathological processes interact to produce such variability in lung damage and destruction, both within and between individuals.

Other clinical features of CF

Although the most striking pathological effects of CF are manifest in the lung, deficiency of CFTR also leads to disease in other organs where the receptor is expressed. These include the gastrointestinal tract, liver, pancreas, sinuses, reproductive tract and sweat glands. In the majority patients, these conditions can be managed medically and are not fatal, but they do contribute significantly to morbidity and to the burden of disease.

Patients commonly present in the neonatal period with meconium ileus or in childhood with a failure to thrive. In addition to this, an increasing number of patients are now detected by neonatal screening, which presents opportunities for earlier identification and treatment of those with milder phenotypes.

Clinical management of CF

Over the last few decades there has been a striking and progressive improvement in life expectancy (Figure 1.4). A disease that was previously fatal in childhood now has a median life expectancy of mid to late 30's (Dodge, Lewis et al. 2007). This enormous improvement has been brought about by a better understanding of the pathology of CF, and more effective treatment. The treatment available however remains essentially symptomatic, and aimed at the consequences of the disease rather than correcting the underlying defect. Improved antibiotic therapy has been crucial to this improvement in life expectancy. Patients are now closely monitored for signs of infection and treated early and aggressively with at least two antibiotics. Other advances include the early eradication of *Pseudomonas*

(Wood and Smyth 2006), patient segregation to prevent cross-infection (Cheng, Smyth et al. 1996; Vonberg and Gastmeier 2005), nebulised DNase (Fuchs, Borowitz et al. 1994), and the use of nebulised and long term oral antibiotics to control infection (Ramsey, Pepe et al. 1999; Southern, Barker et al. 2004). The other major advance has been in the management of patients' nutritional status. Pancreatic enzyme supplements, specialist dietician services and the use of artificial feeds where necessary are all standard practice (Dodge and Turck 2006).

Recently, the use of nebulised hypertonic saline has been reported as showing a modest, but sustained, improvement in lung function and a more dramatic fall in the rate of pulmonary exacerbations and requirement for antibiotics (Elkins, Robinson et al. 2006). This is thought to occur as a result of improvements in mucociliary clearance caused by osmotically driven hydration of the mucus layer (Donaldson, Bennett et al. 2006).

However, all of this is not without cost. Quite apart from the financial cost of treatments, there is a considerable burden in terms of patient time and effort. Nebulised antibiotics and physiotherapy can each take up to 30 minutes to complete, and are usually prescribed twice daily. In addition to this, patients have numerous oral treatments to remember at different times of day, and may be on artificial feeds, inhalers and insulin injections.

In addition to the conventional therapies described above, there has been much interest in the possibility of designing therapies for specific genetic defects. Examples of such approaches include the use of drug screening programmes to look for compounds that will correct the abnormal ΔF508 processing or activate residual CFTR activity (Ma, Vetrivel et al. 2002; Verkman 2004). A number of compounds have been identified as being active in vitro against the G551D mutant (Pedemonte, Lukacs et al. 2005), but no such drugs have yet been used in large clinical trials. The use of aminoglycosides to rescue CFTR stop mutations has also failed to demonstrate consistent improvement in CFTR levels or function vivo (Clancy, Rowe et al. 2007).

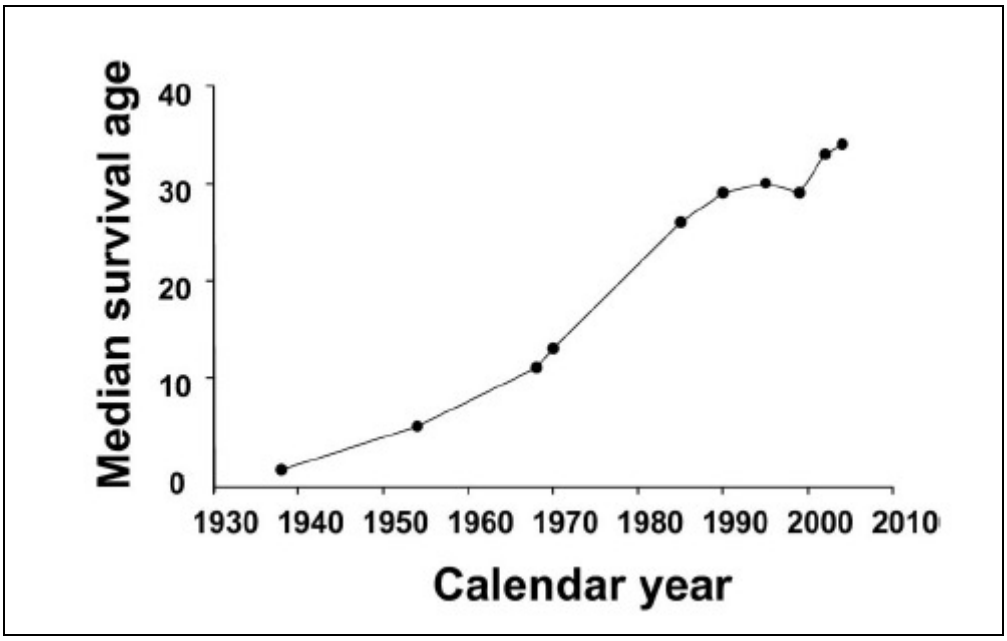


Figure 1.4: Median survival age for US patients with CF since first description of CF. Data from 1985 onwards are CF Foundation projections for median survival age for a patient born in that year with CF. From Davis (Davis 2006).

Gene therapy for CF

When the CFTR gene was first identified there was tremendous optimism that we would soon have a "cure". On the face of it, CF is an attractive target for gene therapy: it is a common single gene disorder with an abbreviated life expectancy; the site of major pathology (the lung) is easily accessible; and there is evidence to suggest that partial correction of the underlying defect would be able to achieve clinical benefit (Johnson, Olsen et al. 1992). The challenges however have been far greater than was first appreciated.

Viral vectors

The human immune system, with an evolutionary head start of several millions of years, has so far resisted attempts to circumvent its defences against the repeat administration of viral vectors. Typically adenovirus or adenovirus-associated viral (AAV) vectors have been used. Techniques that have been tried to enhance transfection efficiency include attenuation of the virus vector (Yeh and Perricaudet 1997), alteration of cell surface receptors to target vectors to the apical membrane (Kreda, Pickles et al. 2000), and masking of surface antigens by polyethylene glycol (O'Riordan, Lachapelle et al. 1999). For both these vectors many subjects have pre-existing neutralising antibodies, affecting even initial administration (Chirmule, Propert et al. 1999). Both the adenovirus and AAV are immunogenic and adenovirus elicits a T-cell mediated inflammatory response that can be fatal (Somia and Verma 2000).

Non-viral vectors

The most basic form of non-viral gene therapy is naked DNA. However, this is inefficient at gene transfer and subject to endonuclease degradation (Lechardeur, Sohn et al. 1999). In order to improve transfection efficiency, the DNA can be complexed with lipids to form lipoplexes, or poly-cation molecular complexes to form polyplexes (Montier, Delepine et al. 2004). These lack the specificity of viral

vectors, and have reduced transfection efficiency, but are not immunogenic and hence are suitable for repeat administration (Hyde, Southern et al. 2000).

Although there are a number of different formulations available for lipoplexes or polyplexes, one of the most promising so far has been Genzyme Corporation's Lipid 67 (GL67). In a recent preclinical study comparing different vector formulations using the same plasmid, the preparation using GL67 had greatest efficacy (McLachlan, Davidson et al. 2005). In addition to this, there are already clinical data on gene therapy using a GL67 vector (Alton, Stern et al. 1999), and toxicity data in normal subjects (Chadwick, Kingston et al. 1997).

The other key component of a gene therapy agent (GTA) is the plasmid. The first trial of nebulised gene therapy used a liposome consisting of GL67 and a synthetic plasmid with the CF gene and a cytomegalovirus promoter inserted. Seven of eight patients in the active arm reported flu-like symptoms within 6 hours of nebulisation. In addition, there was a statistically significant 7.5% reduction in gas transfer, and evidence of a systemic inflammatory response including raised C-reactive protein (CRP) and IL-6 in the active group. These features were not seen in the control (lipid alone) group, nor in the healthy volunteers previously dosed with lipid alone as part of a toxicity study.

It has since been appreciated that unmethylated CpG motifs in the bacterial DNA are recognised by the host innate immune system as foreign (Schwartz, Quinn et al. 1997; McLachlan, Stevenson et al. 2000). Removing these has been one of the major changes to the plasmid formulation since the earlier study. The other important improvements have been to source a promoter capable of early and prolonged gene expression.

The CF Gene Therapy Consortium

In the late 1990s, there were three groups in the UK working on non-viral gene transfer in CF, all of which published separate clinical trials of CFTR gene transfer to the nasal epithelium within a few years of each other (Caplen, Alton et al. 1995; Gill, Southern et al. 1997; Porteous, Dorin et al. 1997; Hyde, Southern et al. 2000). The only trial of nebulised gene therapy to the lung followed shortly after (Alton, Stern et

al. 1999). These three groups combined in 2001 to form the UK Cystic Fibrosis Gene Therapy Consortium, funded by a programme grant from the CF Trust. The advantages of amalgamation include the sharing of resources and experience, the removal of unnecessary project duplication, the formation of core facilities and improved access to patients (Alton 2004).

There is a highly focussed approach, concentrating on the development and delivery of a gene therapy agent that as near as possible fulfils the following criteria (Alton 2004):

1. Repeatedly administrable
2. Manufactured stably and to clinical standards
3. Deliverable by nebuliser
4. Acceptable safety profile
5. Able to reach clinical trial within 5 years

In order to realize these aims, the consortium has concentrated on the development of a GTA based on GL67 and a CpG-free plasmid, as discussed above. This has been tested in pre-clinical studies using sheep as a large animal model, since the anatomy and physiology of the sheep lung are much closer to that of man than the mouse lung (Harris 1997). The consortium has now reached a point where, pending the results of a pilot trial, the wave 1 gene therapy agent will be ready for large scale production and multi-centre clinical testing by the end of 2009.

Measuring response to gene therapy in CF

An important challenge of the gene therapy programme has been how to measure the response to treatment. Early gene therapy studies were limited in scope, and were designed as proof of principle rather than to effect clinical benefit (Caplen, Alton et al. 1995; Gill, Southern et al. 1997; Porteous, Dorin et al. 1997; Zabner, Cheng et al. 1997; Alton, Stern et al. 1999; Hyde, Southern et al. 2000; Noone, Hohneker et al. 2000). The endpoints used in these studies reflect this, and are summarised in Table 1.1.

Rather than a single endpoint, all of these studies relied upon a combination of markers of successful gene therapy, and the sophistication and number of these increased in the later studies. The endpoints were chosen on the basis of their ability to reflect presence of vector gene transfer, or presence of functioning CFTR. There was no attempt to demonstrate any clinically beneficial effect of gene therapy. Although all of these studies incorporated clinical assessments - including lung function in all cases, as well as measures of serum inflammation and immunology, radiological assessments in the form of chest x-ray or chest CT, symptom score, and nasal biopsy histology - these were used to assess safety of the gene therapy agent. In other words, they were looking for absence of deterioration in clinical state, rather than presence of an improvement.

Measures of biologic activity are acceptable for phase I or II studies, but ultimately it is necessary to demonstrate an improvement in clinical status (Ramsey and Boat 1994). The methods that can be used to demonstrate successful gene therapy are summarised in Table 1.2.

The first two of these methods, demonstration of successful gene delivery or expression, require invasive sampling of the lower airway at bronchoscopy. This is not without its' own risks, and is not suitable for frequent sampling. Even if samples can be obtained, the demonstration of mRNA or protein expression, particularly in the context of low levels of background expression, present their own challenges (Rowe, Accurso et al. 2007).

Assessment of CFTR activity is traditionally performed by measuring trans-epithelial nasal potential difference (Middleton and Alton 2006). This technology has been around for many years, and was instrumental in defining the function of the CFTR. However, the technique is operator-dependent and even in a single centre the reproducibility of repeat measurements on two separate occasions is poor (Yaakov, Kerem et al. 2007). Bioelectric measurements have also been performed in the bronchial tree (Knowles, Gatzy et al. 1981; Alton, Stern et al. 1999), but this requires the patient to have a bronchoscopy under a general anaesthetic and hence is not suitable for repeated measurements. In the trial by Alton et al., bronchial brushing samples were also taken to assess in-vitro cAMP-mediated chloride efflux (Alton, Stern et al. 1999).

Author, Year	Numr. subjects (active/control)	Endpoints used to assess efficacy of gene transfer				
		Nasal potential difference	Vector specific DNA & mRNA	Halide ion permeability	Bacterial adherence	Immunohisto-chemistry
Caplen NJ, 1995	9/6	✓	✓			
Gill DR, 1997	8/4	✓		✓		
Porteous DJ, 1997	8/8	✓	✓	✓		
Zabner J, 1997	12	✓	✓			
Alton EW, 1999	8/8	✓		✓	✓	
Noone PG, 2000	11	✓	✓			
Hyde SC, 2000	10/2	✓	✓	✓	✓	✓

Table 1.1: Endpoints used as measures of efficacy of gene transfer in previous (phase I) trials of non-viral gene therapy. In addition, all 7 trials included a number of measures of safety of gene therapy, including symptom scores, lung function assessments, chest X-ray or chest CT, serum biochemistry and immunology and histology of nasal biopsies.

Endpoint	Means of demonstrating endpoint
Gene transfer	• mRNA expression in target cells
CFTR expression	• Immunohistochemistry of airway epithelial cells
CFTR activity	• Trans-epithelial potential difference or chloride permeability • Mucociliary clearance • Airway surface liquid height
Clinical effect	• Improved lung function • Reduced lung inflammation • Structural improvement (e.g. as demonstrated on HRCT) • Improved quality of life • Improved longevity

Table 1.2: Methods of assessing response to gene therapy in clinical trials. Either gene transfer, expression or CFTR activity are essential endpoints in early, phase I, trials. In addition to these endpoints, phase III trials require evidence of clinical benefit.

Although assessment of CFTR expression and activity are important, ultimately, it is necessary to demonstrate clinical benefit. Ideally this would include improved survival or quality of life. These are difficult parameters to measure without large numbers of patients being treated and followed for a long period of time. Instead, there are a number of surrogate clinical endpoints that are used to measure improvement in lung function or clinical status. These were summarised in a consensus statement from the Cystic Fibrosis Foundation in 1994 (Ramsey and Boat 1994). The potentially useful measures of treatment efficacy in patients over 6 yrs of age are listed in Table 1.3. For Phase I and II trials, measures of CFTR function (e.g. mucociliary clearance and PD measurements) are sufficient. For phase III trials however, all the endpoints listed reflect changes in clinical status, either as improvement in lung function, reduction in inflammatory burden, or improved quality of life.

The development of more accurate and appropriate biomarkers of CF disease activity is not a problem limited to the development of CF gene therapy, and is an important challenge for all research into the effects of new therapies in CF.

Phase I and II clinical trials	Phase III clinical trials
• CT scanning of chest • Bronchoscopy with BAL, brushings and/or biopsy • PD measurements (upper and lower airways) • Mucociliary clearance • Complete lung function testing • Exercise testing • Aerosol deposition • Bronchial reactivity	• CXR score • Arterial blood gas or oximetry • Serum inflammatory markers • Bronchoscopy, BAL & brushings • Spirometry • Sputum for microbiology & DNA ***Measures of clinical efficacy*** • Anthropometry • Quality of life / illness severity scoring • Frequency of pulmonary exacerbations

Table 1.3: Proposed endpoints for trials of new therapies in cystic fibrosis, from the from the Cystic Fibrosis Foundation Consensus Conference, 1994 (Ramsey and Boat 1994).

Assessment of lung function in CF

Anatomy and physiology of the small airways

The human bronchial tree is a dichotomous asymmetrically branching structure of approximately 23 divisions. The large airways contain cartilage to maintain patency. Small airways are arbitrarily defined as non-alveolated and non-cartilaginous airways less than 2mm in diameter. With division, the airways become smaller and the histology gradually changes. In the proximal airway, there are abundant mucus-secreting goblet cells. These are absent from the distal airway in health, and the secretory cells of terminal bronchioles are the Clara cells. The epithelial cells are more cuboidal and ciliated cells are sparser, with fewer and shorter cilia. The airway walls are much thinner in the small airways and the proportion of smooth muscle increases towards the periphery. With successive divisions, the number of airways rises exponentially, together with the total airway cross sectional area. The contribution of each generation to total airway resistance therefore diminishes rapidly in more peripheral generations. The calibre of the small airways is maintained by a dynamic process that is dependent upon a variety of interacting factors and varies with the phase of respiration. Patency is maintained by the elastic pull of the surrounding connective tissue. The forces that act to collapse the small airways include smooth muscle tone and the surface tension of the airway surface liquid. During expiration, this is increased by the elastic recoil of airway tissue and the air pressure in the alveoli.

Small airways narrow during expiration even in normal subjects – this accounts for the differences in the inspiratory and expiratory flow-volume loops. In CF patients, there are additional factors that act to upset this dynamic balance in favour of airway collapse. There is airway wall thickening due to inflammation, smooth muscle hyperplasia, and destruction of the surrounding connective tissue (Hamutcu, Rowland et al. 2002). In addition to this, mucus is retained in the small airways due to failure of the mucociliary escalator. This can obstruct the airways directly (Hamutcu, Rowland et al. 2002), but will also increase the surface tension of the fluid in the airway, and hence favour collapse.

Spirometry

Whilst the forced expiratory volume in 1 second (FEV_1) is the currently accepted gold standard to monitor trials of new treatments for CF (Ramsey and Boat 1994), the rate of decline in this parameter has steadily reduced over the last decade (Que, Cullinan et al. 2006). Power calculations show that many hundreds of patients would need to be treated over a year, or more, to see any beneficial effect of a novel therapeutic agent aimed at the basic defect (Davis, Byard et al. 1997). However, the use of spirometry to measure FEV_1 and forced vital capacity (FVC) is well established in both clinical and research settings. It has the advantage that it is widely used, equipment requirements have been standardized, and normal ranges have been defined. FEV_1 and FVC can usually be reproducibly obtained, but the measurement is non-physiological, is dependent on effort and technique, and in particular is difficult to measure accurately in young children. More importantly, FEV_1 is insensitive to early disease. This is particularly true in children with CF, in whom spirometry now remains within the normal range for the majority until 18 yrs (Cystic-Fibrosis-Foundation 2005).

Recently a number of studies have looked at the structure of the lung using CT scanning (Demirkazik, Ariyurek et al. 2001; Brody, Klein et al. 2004; de Jong, Nakano et al. 2004). High resolution CT (HRCT) is a sensitive indicator of early CF lung disease and HRCT scores have also shown greater progression with time than spirometry (Helbich, Heinz-Peer et al. 1999; de Jong, Nakano et al. 2004). Evidence of bronchiectasis, peribronchial thickening, gas trapping and mucus plugging have all been demonstrated in those with spirometry within the normal range, including quite advanced changes in some cases (Brody, Klein et al. 2004; de Jong, Nakano et al. 2004). In addition, therapeutic responses have been shown on HRCT in the absence of any change in spirometry (Robinson, Goris et al. 2005).

Further evidence that spirometry is insensitive to early pulmonary disease has come from studies of gas mixing in the lung. In the presence of normal spirometry these have shown evidence of abnormal gas mixing on MRI ventilation scans of the lung (Mentore, Froh et al. 2005), or multiple breath washouts (Gustafsson, Aurora et

al. 2003; Aurora, Gustafsson et al. 2004). These will be discussed in more detail later in this chapter.

Another problem with spirometry is that normal absolute values change over time. In normal children, FEV_1 increases with age and in normal adults there is a gradual decline in FEV_1 with older age (Quanjer, Tammeling et al. 1993; Rosenthal, Bain et al. 1993). In CF patients this background annual decline is exaggerated, and is of the order of 100 ml/yr in those with moderate to advanced lung disease (Rosenberg, Howatt et al. 1992). Evolving normal ranges are a particular problem in adolescent CF patients. The delayed pubertal growth spurts frequently seen in CF (Haeusler, Frisch et al. 1994) make it difficult to define appropriate normal values in this population, just when robust treatment end points are most relevant. This causes difficulties in long term follow up studies, since continuous adjustment must be made for the subject's age and (in the case of children) height and developmental stage.

For this reason FEV_1 is usually quoted as a percentage of the predicted value. In the clinic, FEV_1 is considered normal if it lies within the range 80-120% of the predicted value. Thus, a patient could theoretically have lost 30% of their spirometric lung function (if their baseline was 120% predicted), before the FEV_1 was recognised as being outwith the normal range. This may help to explain the lack of sensitivity of FEV_1 to early lung disease on CT in the cross-sectional studies described above. Use of different prediction equations for "normality" can also have profound effects on the measured rate of decline in "percent predicted" values for spirometry (Merkus, Tiddens et al. 2002), particularly in adolescence.

The reasons for the insensitivity of FEV_1 to early airways disease are intrinsic to the measurement itself. FEV_1 is the volume of air expired within the first one second of a forced expiration, hence it measures the air flow over that period. Assuming that patient effort is unchanged with each attempt, in early lung disease it reflects a measure of total airways resistance. In more advanced lung disease the picture becomes more complicated by gas trapping and flow-dependent airway collapse due to reduced elasticity.

There is an exponential increase in total airways cross-sectional surface area with each division of the bronchial tree, illustrated in Figure 1.5. The small airways, of less than 2mm, are pathways of low flow and individually high resistance.

Because of the increase in total cross sectional area however, collectively they contribute only around 10% of total airways resistance in healthy adults (Macklem and Mead 1967). Hence, there can be quite considerable disturbance of the small airways, with relatively little effect on FEV_1. This was shown by Brown et al., who used beads of different sizes to obstruct small and large airways in explanted animal lungs and assessed the effect of this on pressure-volume curves (Brown, Woolcock et al. 1969). They demonstrated that in dogs, which have collateral channels like man, obstruction of 50% of the small airways caused only a 10% increase in total airway resistance, and had no measurable effect on vital capacity (VC) or pressure-volume curves.

Thus FEV_1 will be abnormal in conditions that cause obstruction of the larger airways or in conditions severely affecting the majority of peripheral airways. The insensitivity to mild disease in small airways has led to term the "silent zone". This refers to the deterioration in lung function that occurs between the start of a disease process and the ability to detect it on spirometry. The initial stages of the disease process in CF occur in the small airways (Brownlee 2006). In addition, it is these airways that are the target for nebulised gene therapy. In order to measure the response to therapy, and to identify patients with early (and potentially reversible) lung disease, there is thus a need for an assay of lung function that has the potential to reflect changes in the small airways.

As with any trial endpoint, the assay also has to fulfil the following criteria (Rosenfeld 2007):

- Minimally invasive and simple for the patient to perform
- Limited harmful or unpleasant side-effects
- Practical, with standardized equipment and interpretation
- Reproducible (with minimal error and variability)
- Sensitive
- Biologically relevant
- Stable, or behaves predictably, over time

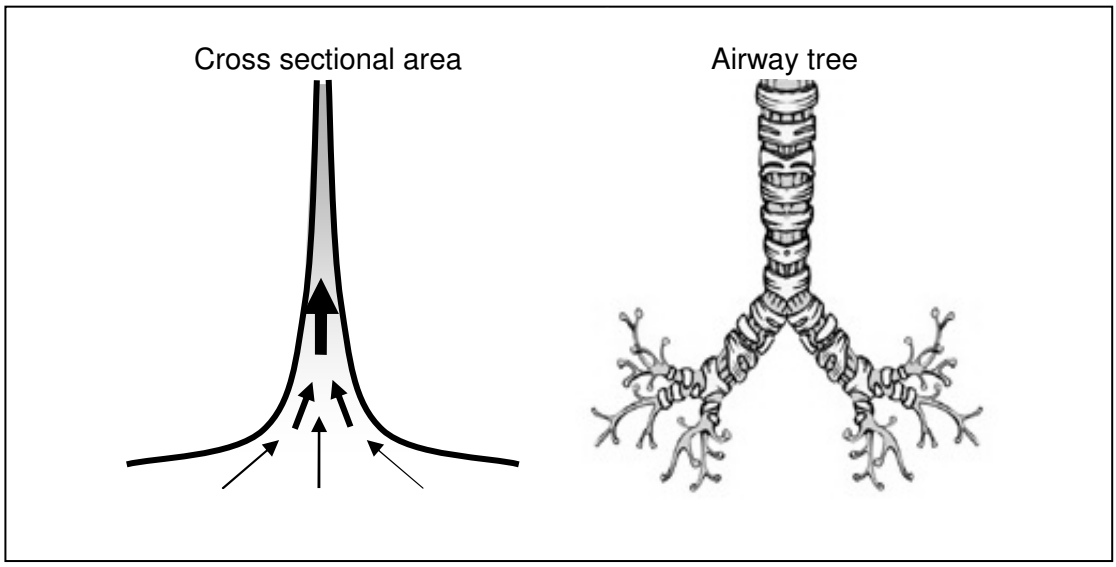


Figure 1.5: Illustration (not to scale) of the cross sectional area of the airways. With successive dichotomic divisions of the airway tree the cross sectional area at each level increases exponentially. Arrows on the left hand represent the flow of expired air in the airways: low flow (thin arrows) in multiple narrow peripheral airways and high flow (thicker arrows) in a smaller number of central airways.

Measurement of small airway function

Standard lung function measurements

Although FEV_1 is relatively insensitive to mild small airways disease, there are a number of other lung function assays that appear to be more sensitive. One of the best described of these is the forced expiratory flow between 25 and 75% of FVC (denoted as FEF_{25-75}). This is derived from the flow-volume loop generated during spirometry and calculated as the mean forced expiratory flow during the middle half of the FVC (that between 25% and 75% of the total FVC). A number of variations on this measurement also exist, but all have in common the measurement of the forced expiratory flow at low lung volumes, and are often referred to as measures of mid expiratory flow. Mid expiratory flow measurements are more sensitive to early airways dysfunction than FEV_1 (Kraemer, Blum et al. 2005), and are more sensitive to abnormalities on CT (Gustafsson, de Jong et al. 2007). Pathology studies have also shown that these measurements can be related to histological changes in the small airways (Petty, Silvers et al. 1982). A major barrier to the wider use of these tests however is that they are very dependent on patient effort, are poorly reproducible, both within and between visits, and have poorly defined normal ranges in different age groups (Timonen, Randell et al. 1997).

The forced oscillation technique (FOT) is an alternative method of assessing airway function that measures the impedance of the entire respiratory system. The forced oscillations are superimposed on the normal respiratory cycle, avoiding the need for any special respiratory manoeuvres (Oostveen, MacLeod et al. 2003). However, measurement can be affected by swallowing, integrity of mouthpiece or noseclip seal, irregular breathing or hyperventilation. In addition, the signal is attenuated by compliance of the upper airways, particularly at higher frequencies. A number of approaches have been developed to cope with this (Michaelson, Grassman et al. 1975; Peslin, Duvivier et al. 1985), but the standard practice is to minimise this upper airway shunt by compression of the cheeks (Oostveen, MacLeod et al. 2003). FOT is a promising technique that has been used in both adults and children, and reference data are available for both populations. However, the reference ranges are dependent on the exact measurement conditions, and frequency band assayed, for

which there is no universal standard. In addition, reproducibility is poor, with a day to day coefficient of variation of repeat measurements of around 10-11% in adults (Gimeno, van der Weele et al. 1993), and as high as 16% in children (Timonen, Randell et al. 1997). Finally, FOT is of uncertain value for measuring peripheral airway function, and cannot differentiate between intra and extra pulmonary disorders (Oostveen, MacLeod et al. 2003).

Inert gas washout tests

It has been appreciated for some time that there are regional differences in the distribution of ventilation even in normal lungs (Milic-Emili, Henderson et al. 1966). In early airways disease however, this heterogeneity of ventilation distribution is more pronounced (Mentore, Froh et al. 2005), possibly as a result of blockage or constriction of small airways by inflammation, remodelling or retained secretions. Even minimal heterogeneity in bronchoconstriction produces clusters of poorly ventilated airways through a positive feedback on surrounding smooth muscle, resulting in significant heterogeneity of ventilation distribution (Venegas, Winkler et al. 2005). This can be demonstrated on HRCT, where gas trapping is an early sign of small airways dysfunction (Hansell, Rubens et al. 1997). Ventilation heterogeneity can also be demonstrated on hyperpolarized helium MRI scanning, which permits direct visualisation of the distribution of a single inspiration, and which has been used to show changes in ventilation distribution in response to therapy (Samee, Altes et al. 2003; Mentore, Froh et al. 2005). Alterations in ventilation distribution impact upon the efficiency of gas mixing during breathing, and it is this that is measured by inert gas washout tests. These can either be performed on a single vital capacity breath, or during prolonged tidal breathing.

It is important to appreciate that these tests are not specific measures of small airways disease, but rather measures of overall ventilation heterogeneity, and that this can itself be affected by a number of different processes. Disease of large and medium airways will also produce regional differences in ventilation distribution, as will differences in the time constants of lung regions due to parenchymal or chest

wall disease. In patients with advanced CF lung disease, there may be extensive airway obstruction, with mucus plugging, bronchiectasis, and destruction of supporting tissue (Helbich, Heinz-Peer et al. 1999), and it is unlikely that differences in small airways alone are responsible for the ventilation heterogeneity seen in these patients. In well patients however, with little or no spirometric impairment (including children), it is likely that small airways changes are at least partially responsible for changes in gas mixing.

Evidence for this is, to a certain extent, circumstantial and it is not possible to know exactly what the MBW indices represent at a histological level since data on airway pathology in subjects with mild disease is not available and is not likely to be forthcoming. However, our understanding of CF is that the disease affects primarily the small airways, at least initially (Brownlee 2006). This leads us to believe that MBW indices are particularly sensitive to small airways dysfunction, since LCI appears to be particularly sensitive to early airways disease in CF (see below). Further support for this came from a paper by Gustafsson et al. looking at CT appearances, spirometry and LCI in 44 children (age 5-19 yrs) with CF (Gustafsson 2007). As had been previously reported, sensitivity of spirometry to structural abnormalities on CT was poor. LCI was the most sensitive to structural lung abnormalities, particularly to air trapping, for which it had a sensitivity of 94%. Normal LCI in a patient with CF almost excluded the presence of structural abnormalities on HRCT. In addition, LCI was also elevated in one third of those with a normal CT score, which may represent the presence of physiological abnormalities due to disease that is below the limit of resolution of the CT scanner.

1. Single breath washout

The classic single breath nitrogen washout test involves the administration of 100% oxygen to the subject during a slow inspiration from FRC to total lung capacity (TLC), followed by a slow expiration. The resulting trace of expired nitrogen against volume is conventionally divided into four distinct phases (Figure 1.6). The first phase represents the absolute dead space (e.g. of the apparatus, mouth

and upper airway). The second represents the rising nitrogen concentration as gas is expired from the bronchi. Together these two phases represent the anatomical dead space of the lungs. The third phase is the expiration of alveolar gas. In a "perfect" lung in which ventilation distribution was entirely even this would be a straight line. The final phase, a sharp increase in expired gas concentration seen in some subjects, represents expulsion of air beyond the point at which some airways have collapsed and is known as the closing volume (Forkert, Dhingra et al. 1979).

SBW tests can also be performed with exogenous tracer gas. In this case a gas mixture containing inert tracer, usually SF_6 or helium, is inhaled instead of 100% oxygen. The expired gas concentration profile is the inverse of that for nitrogen, since the concentration of tracer falls as gas is expired from the alveolar compartment.

It has been recognised since the 1940s that non-uniformity of gas mixing in the lung results in a sloping alveolar plateau (Fowler 1949), and ventilation heterogeneity can therefore be inferred from the steepness of this slope (also referred to as the phase III slope). Imperfect convective (bulk flow) gas mixing occurs between different regions of the lung as a result of differences in airway calibre and expansion of the lung. Sequencing between these regions contributes to the sloping alveolar phase, and this becomes steeper in disease. Interaction between convective and diffusive gas mixing in the periphery also contributes to variations in ventilation efficiency and a sloping alveolar plateau (Prisk, Lauzon et al. 1996; Dutrieue, Vanholsbeeck et al. 2000). Since diffusion is related to the molecular mass of the gas molecule, SBW tests can be refined by using helium and sulphur hexafluoride (SF_6) together. This allows inferences to be made about the ventilation of the most peripheral air spaces (Gronkvist, Emery et al. 2002). Greater heterogeneity of gas mixing results in a steeper phase III slope and an earlier onset of the phase IV (closing volume).

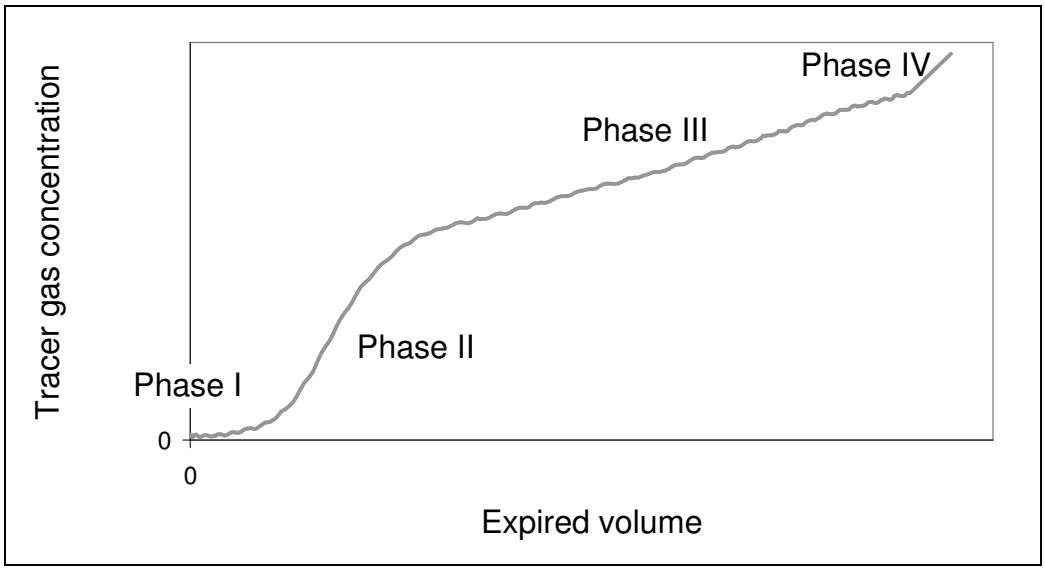


Figure 1.6: A typical plot of a single breath washout. Phase I corresponds to the equipment and upper airway deadspace, Phase II is the bronchial phase, Phase III corresponds to mixed alveolar gas and Phase IV to gas expired below the closing volume. This figure was derived from a multiple breath washout of a CF patient, and is also shown in more detail in Figure 5.2.

SBW tests have been used to demonstrate changes in phase III slope in patients with numerous different conditions, including CF. In addition, the slope has been shown to correlate with histological measures of small airways pathology, albeit in those with already well established disease (Cosio, Ghezzo et al. 1978). Although SBW tests have been used for many years in research, and have also been used in longitudinal clinical studies (Estenne, Van Muylem et al. 2000), they are not commonly used in clinical practice. Firstly the test requires a cooperative subject, though they have been performed successfully in children down to the age of 8 yrs (Ljungberg and Gustafsson 2003). Secondly there are differences in technique, including the nature of the tracer gas and the volume of gas inspired, and little standardisation between different units. Finally, there is no commercial apparatus available to conduct these tests, and the equipment required is very similar to that necessary for multiple breath washout studies which are easier to perform and more reproducible. Multiple breath washouts have the additional advantage that a number of different indices of ventilation maldistribution can be calculated from them since they contain data on the evolution of gas mixing over the course of several breaths.

2. Multiple breath washout

The basic principles behind multiple breath washouts (MBW) are relatively simple, and were first described more than 50 years ago (Becklake 1952). The test involves following the washout of an inert tracer gas from the lungs during relaxed tidal breathing (Figure 1.7). The tracer gas can either be resident nitrogen, washed out when the subject is switched to breathing 100% oxygen, or it can be an exogenous tracer gas that must first be washed into the lungs to equilibrium. Each approach has its own advantages and challenges, but the principle is the same: namely that the tracer gas should be inert and neither absorbed nor excreted by the body to any significant degree. As airways disease progresses, heterogeneity of ventilation distribution increases, gas mixing efficiency is reduced, and more tracer gas is retained after each breath. Washout of a defined fraction of the tracer therefore takes longer to complete and requires a greater cumulative expired volume. There are additional effects on the shape of the alveolar (phase III) slope of successive breaths

(Dutrieue, Vanholsbeeck et al. 2000). Indices of deranged ventilation calculated from washout curves include the lung clearance index (LCI) (Gustafsson, Aurora et al. 2003), moment ratios (Wall 1985) and normalised phase III slopes (Verbanck, Schuermans et al. 1997). These will be described in more detail in subsequent chapters.

Clinical use of MBW tests

Interest in MBW tests has experienced resurgence in recent years due to an increasing recognition of their usefulness at measuring gas mixing indices in children, combined with improvements in gas analyser technology. These tests were first described over 50 years ago, and originally involved nitrogen washouts (Becklake 1952). A major advance came about with the availability of personal computers to display and analyse the washout data. A number of studies, comparing small numbers of groups of subjects with different respiratory diseases, were performed in the 1970s-1980s (Saniie, Saidel et al. 1979; Fleming, Chester et al. 1980; Lutchen, Habib et al. 1990). These reported on moment ratios, a measure of overall ventilation heterogeneity derived from a plot of lung volume turnover versus normalized end tidal marker gas concentration. Moment ratios were found to be elevated in patients with CF, in asthmatics, in diffuse interstitial lung disease and in smokers. All of these studies were observational and involved small numbers with little appreciation of a clinical use of the measurements. Moment ratios, derived from MBNW, were also reported in 24 children with CF (aged 3.9 to 6.5 yrs) and 58 healthy controls (Couriel, Schier et al. 1985). Although they showed that moment ratios were higher in the children with CF, the authors reported considerable difficulty in collecting measurements in these young subjects. Wall et al. also reported moment ratios in 10 children with CF over a similar age range (3-6 yrs) and 36 controls, using distraction to improve the reproducibility of the results. They found elevated moment ratios in the subjects with CF and also reported a negative correlation between moment ratios and Shwachmann score, an index of CF disease severity (Shwachman and Kulczycki 1958). This time, there was an appreciation that these measurements had distinct advantages in young subjects over standard measures of lung function, which are difficult to perform in these young subjects.

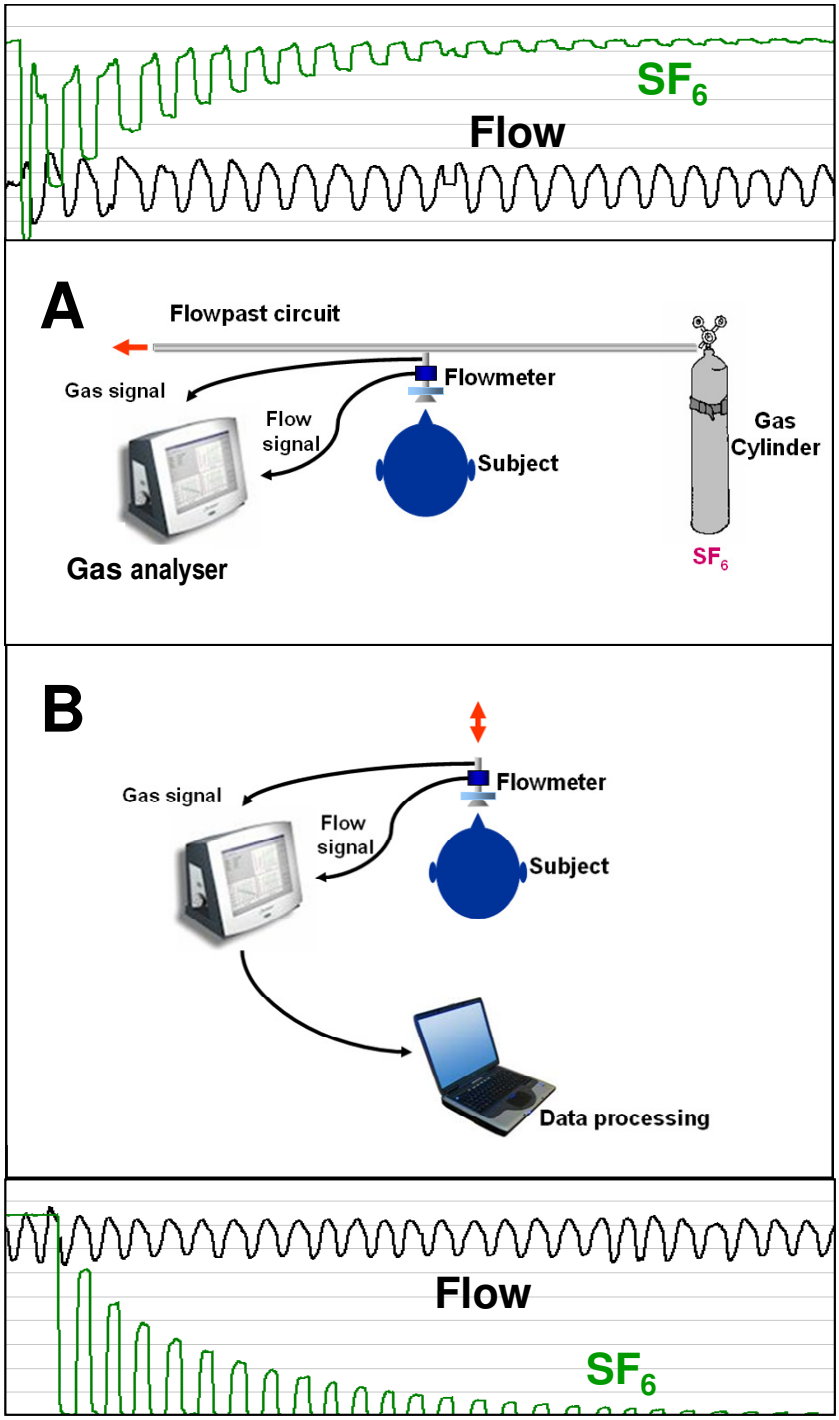


Figure 1.7: Principle of inert gas washout using an exogenous tracer gas. The subject first inhales inert tracer to equilibrium (A). With each successive breath the expired tracer gas concentration rises until it is equal to the inspired concentration (top tracing). The flowpast circuit is then removed, the subject breathes room air, and the washout of the gas measured (B). Tidal breathing is employed throughout. The bottom tracing shows a typical washout of 0.2% sulphur hexafluoride (SF_6).

The next major advance in this field occurred with the use of a mass spectrometer to measure 4% SF_6 as the inert tracer gas. This system was initially devised by Per Gustafsson and was used to demonstrate that lung clearance index (LCI) was elevated in 43 children with CF (aged 3-18 yrs) compared to 28 healthy controls (Gustafsson, Aurora et al. 2003). In addition, LCI was more sensitive than spirometry, being elevated in 22 of the 33 CF patients with normal spirometry. An identical system, using the same technique and analysis software, was subsequently established at Great Ormond Street children's hospital in London. Using this apparatus, Aurora et al. confirmed the findings in Sweden in school age children (Aurora, Gustafsson et al. 2004), and then went on to use the technique in pre-school children (Aurora, Bush et al. 2005). They also showed that in pre-school children LCI was higher in those patients infected with *Pseudomonas aeruginosa*. More recently, the same group have reported on LCI in infants as young as 10 weeks old. They reported that LCI was elevated in infants with CF (mean age 41weeks) compared to age matched healthy controls, and that these infants also had raised FRC and measurable impairments in spirometric indices generated using raised lung volume rapid thoraco-abdominal compression. LCI in normal subjects in all four of these studies was remarkably narrowly distributed (Figure 1.8). Although LCI was slightly higher in the infants, this may be due to differences in protocol (the test was performed supine) or due to the effects of serial deadspace (Schmalisch, Proquitte et al. 2006).

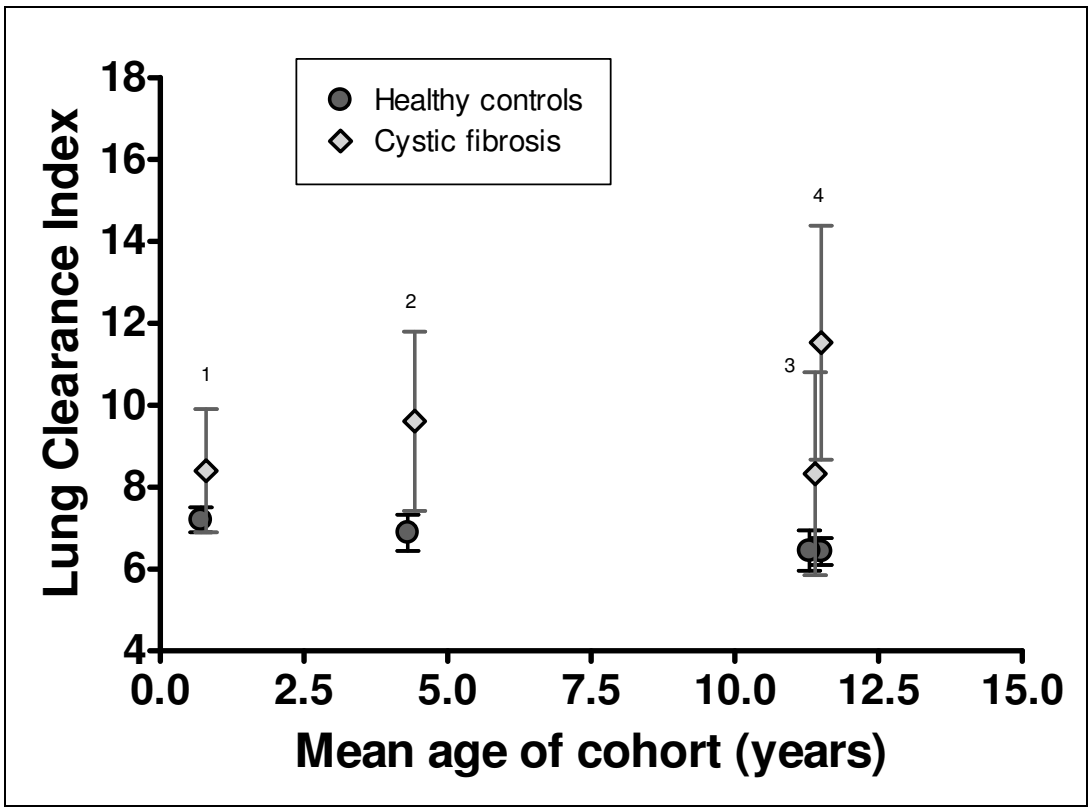


Figure 1.8: Mean and standard deviation of LCI obtained from CF patients and healthy controls in four separate paediatric studies. The two studies performed in school age children, #3 and #4 were performed on Swedish and UK populations respectively.

1. (Lum, Gustafsson et al. 2007)
2. (Aurora, Bush et al. 2005)
3. (Gustafsson, Aurora et al. 2003)
4. (Aurora, Gustafsson et al. 2004)

Longitudinal studies of gas mixing measurements are more complex to complete but a large Swiss cohort has been followed between the ages of 6 and 20 yrs by a group led by Richard Kraemer (Kraemer, Blum et al. 2005). 142 children with CF have had at least 4 serial annual evaluations of conventional lung function (spirometry, airways resistance and FRC at plethysmography), *Pseudomonas aeruginosa* infection status, and LCI (performed using a nitrogen washout apparatus). They found that LCI deteriorated first, followed by FEF_{50}, FVC and finally FEV_1. LCI was elevated in more than half of those with FEV_1 within the normal range. Furthermore LCI continued to increase, along with pulmonary hyperinflation and trapped gas, beyond the age of 12 yrs, whereas FEV_1 z-scores stabilised. In subsequent papers derived from the same dataset, they also showed that LCI was more elevated in those with allergic broncho-pulmonary aspergillosis (ABPA), but that the slope of longitudinal progression of LCI was greatest in those chronically infected with *Pseudomonas* (Kraemer, Delosea et al. 2006). Functional residual capacity (FRC, obtained from MBW) decreased with age, as gas trapping and LCI increased (Kraemer, Baldwin et al. 2006). LCI was the most sensitive discriminator between groups divided on the basis of chronic and intermittent *Pseudomonas aeruginosa* colonization of the lower airway, chronic *Staphylococcus aureus* infection, and those free from bacterial colonization.

Much of the recent work on MBW has focussed on measurements in children with CF. An exception to this is the work by the group headed by Sylvia Verbanck and Manuel Paiva. They have used a nitrogen washout apparatus to calculate progression of the phase III slope with sequential breaths of a washout and to generate measurements that are purported to reflect heterogeneity of gas mixing in the conducting airways (S_{cond}) and in the acinar (S_{acin}) compartment (discussed in detail in Chapter 5). In asthmatics, S_{cond} is a predictor of airways hyper-responsiveness and responds to treatment with bronchodilators, whilst in smokers S_{cond} shows a persistent improvement with smoking cessation (Verbanck, Schuermans et al. 1999; Verbanck, Schuermans et al. 2006; Downie, Salome et al. 2007).

Other factors which affect gas mixing efficiency

There are a number of technical and subject factors which can impact on gas mixing efficiency and affect measures of FRC or ventilation inhomogeneity. The fraction of a tidal breath that ventilates equipment dead space (i.e. the mouthpiece or mask, flowmeter and connections) is an additional component of overall ventilation heterogeneity. Dead space to tidal volume ratio (V_D/V_T) can affect measurement of LCI, with exponential increase in LCI as V_D/V_T increases above 0.5 (Schmalisch, Proquitte et al. 2006). This level of V_D/V_T however is well outwith the normal physiological range for adults, which lies between 0.05 to 0.2 (Habib and Lutchen 1991), and would require considerable equipment deadspace to raise it to a level at which it was likely to interfere with measurement. In children, and particularly in infants, V_D/V_T is greater, and, since tidal volumes are less, it is essential to maintain apparatus dead space as small as possible. This is currently recommended to be not more than 2ml per kg (Beydon, Davis et al. 2007).

Tidal volume can also affect LCI in the opposite direction if it is increased (Bouhuys, Lichtneckert et al. 1961). Increasing tidal volume voluntarily in healthy adults can result in an increase in FRC, but LCI remains unaffected except at extremes of tidal volume (Gronkvist, Bergsten et al. 2002). Increasing V_T /FRC ratio also reduces LCI in single compartment lung models (Bouhuys, Lichtneckert et al. 1961; Larsson, Jonmarker et al. 1988). On the basis of these findings, Larsson et al. proposed that measures of ventilation inhomogeneity that either took into account dead space, or were intrinsically independent of it, were superior to those such as LCI which appeared to be more susceptible to changes in V_D and V_T (Larsson, Jonmarker et al. 1988). The effects of changes in these variables in vivo, within the physiological range, are however far less than those demonstrated in simple lung models (Larsson, Jonmarker et al. 1988; Gronkvist, Bergsten et al. 2002). This may reflect the opposing effects of falling V_D/V_T and rising V_T /FRC, but increased V_T may also direct ventilation to previously less well ventilated lung regions with greater inhomogeneity.

Irregular breathing will result in an unstable end expiratory level, and changes in end tidal marker concentration will also generate changes in calculated FRC. In addition, apnoeas in infants have been shown to significantly decrease FRC and sighs

may lead to an elevation in FRC (Poets, Rau et al. 1997). In ventilated adults, slow insufflation times can increase some measures of ventilation inhomogeneity in those with impaired gas mixing, whilst end expiratory pauses reduce them (Larsson, Jonmarker et al. 1988). LCI however was relatively insensitive to changes in ventilatory pattern and has not been shown to change with increases in respiratory rate (Bouhuys, Lichtneckert et al. 1961). Finally, supine posture is known to reduce measures of FRC and to affect phase III slope analysis, but has no significant effect on LCI in adults (Gronkvist, Bergsten et al. 2002).

In summary, the documented effects on LCI of equipment dead space, and changes in respiratory pattern or tidal volume are small in healthy adults. In very young children, and particularly in infants, the effects are likely to be more pronounced and remain poorly understood (Beydon, Davis et al. 2007). In addition, it is possible that in diseased lungs breathing pattern and tidal volume may have greater effect on measures of ventilation inhomogeneity.

Practicalities of performing MBW tests

Although well established in a research setting, multiple breath nitrogen washout (MBNW) is a considerably more complex technique than it at first appears. Until recently, there has been no commercial equipment available, and all the studies so far have relied on apparatus developed in-house. The standard nitrogen gas analyser works by detecting the light produced by ionization of the gas sample in a high voltage ionization tube. The light intensity is proportional to the nitrogen concentration. This is filtered and measured by a photodiode and converted into an electronic output. The analyzer uses a vacuum pump to draw the sample into the ionization chamber and to maintain the pressure in the ionization tube. The output from the ionization chamber is very sensitive to the operation of this vacuum pump, and in particular to the setting of a small bore needle valve at the end of the gas sample line. Small alterations in the bore of the needle valve can cause large alterations in the measured concentration of nitrogen. The analyser and needle valve require frequent calibration. In addition, there can be quite pronounced drift of the gas analyser signal, which continues even after 1 hour of warming up.

During MBNW, the fractional nitrogen and oxygen concentrations alter during the course of both individual breaths and the washout as a whole. This alters the viscosity of the expirate, and hence the measured flow, by up to 12% (Saniie, Saidel et al. 1979). In order to accommodate this, continuous adjustment of flowmeter output is required according to the measured nitrogen concentration. With the availability of personal computers, this became possible electronically, but in the absence of an off-the-shelf commercial system, still requires individual programming by the user.

An alternative approach to this latter problem was developed by Verbanck et al. (Verbanck, Schuermans et al. 1997), and has subsequently been adopted by Downie et al. (Downie, Salome et al. 2007). Rather than measuring flow at the mouth, where the gas composition is changing, they have used a bag-in-box system. This involves the subject breathing into and out of separate inspiratory and expiratory bags contained with a sealed box. A flowmeter is fitted through the wall of the box so that changes in volume of the bags are registered as flow into or out of the box. This avoids the need for complex correction of the flow signal, and since the expired air is collected in a sealed bag also provides a method of verifying the calculated volume of expired nitrogen. However, the flow meter is distant from the mouth, and may be less effective at following small expiratory volumes or rapid respiratory rates, as are found in children. In addition the box is subject to thermal drift as the contents of the box warm up.

Finally, with all nitrogen washout systems, the contribution of additional body nitrogen excreted during the procedure may become significant with prolonged washouts, though is not considered to be significant in normal subjects (Crawford, Makowska et al. 1985). Also, sufficient time must be left between washouts for additional oxygen to be expired or absorbed, and the resting gas concentrations return to baseline. This is recommended to be at least 15 minutes (Wanger, Clausen et al. 2005), but in some cases, with severe obstructive or bullous disease, it has been suggested that this should be over 1 hour (Emmanuel, Briscoe et al. 1961).

An alternative approach is to use an exogenous inert marker gas that the subject must first breathe in, until inspiratory and expiratory marker gas concentrations are equal. The supply of gas is then disconnected and as the subject breathes room air the

marker gas is washed out from the lungs in the same way as nitrogen during the MBNW. This approach relies on the availability of an inert marker gas, and the two gases that have been used in previous studies are helium and SF_6. Helium is readily available in respiratory function labs, and is used in the determination of lung volumes in commercial closed rebreathing systems (Wanger, Clausen et al. 2005). The analyser used in these systems does not require a fast response time or high quality signal resolution and for multiple breath washout analysis a respiratory mass spectrometer is required. Both helium and SF_6 have been used in multiple breath washout studies, though only data from SF_6 washouts have been reported (Gustafsson, Aurora et al. 2003; Aurora, Gustafsson et al. 2004; Aurora, Bush et al. 2005; Aurora, Kozlowska et al. 2005).

The use of a respiratory mass spectrometer to measure SF_6 in multiple breath washouts has been a major advance in the understanding and use of this technique. The system was developed by Per Gustafsson in Sweden as a means to measure gas mixing in young children. The advantages of the mass spectrometer are that it offers a stable gas signal, with a rapid analyser response time (Aurora, Kozlowska et al. 2005). This has been crucial in the development of this technology in the assessment of very young subjects, including infants (Lum, Gustafsson et al. 2007). The mass spectrometer also offers the possibility of measuring more than one gas, so that simultaneous washouts of gas species with different diffusion coefficients (helium and SF_6) can be performed in order to explore the effects of diffusion on gas mixing (von Nieding, Lollgen et al. 1977; Gronkvist, Emery et al. 2002).

There are however a number of drawbacks to using a mass spectrometer in these measurements. Firstly, the cost of the mass spectrometer itself is considerable. Secondly, mass spectrometers are complex and temperamental devices, and spend a considerable portion of their time awaiting or being repaired. In addition, the equipment is bulky and static, and a separate supply of tracer gas is required (unlike the nitrogen washout system which can use the hospitals' piped oxygen).

For these reasons, MBW tests remain restricted to a small number of laboratories, where they are used primarily as research tools. Few units have reported on attempts to integrate these measurements into their annual assessments of CF lung function (Kraemer, Blum et al. 2005; Gustafsson, de Jong et al. 2007). The

technology has been developed by each group separately, and it has not been disseminated beyond close collaborations. Finally, no-one has previously attempted to use MBW tests in a multi-centre setting. The complexity of the equipment, and the individual experience required to conduct and analyse these tests, have made this an impractical assay for such studies.

Chapter 2 - Adaptation of Innocor gas exchange device to measure inert gas washout

Background

In order to measure inert gas multiple breath washout (MBW), a sensitive gas analyser and a flow meter are required. At time of preparation, no commercial system was available, and different techniques and gas analyser technologies were employed in different laboratories. It is important that any new apparatus be at least comparable in terms of performance to those used in previous studies, and any deficiencies of the apparatus be clearly recognised and assessed. To this end, technical recommendations are available for nitrogen washout apparatus in adults (Wanger, Clausen et al. 2005) and for using a mass spectrometer to perform MBW in children (Gustafsson 2005). Neither of these is exactly applicable to the technology and intended patient populations described here.

This chapter concerns the assessment of the Innocor gas analyser, in terms of gas analyser response time and signal quality. This performance is placed in context of previously described systems and guidelines. Modifications required to adapt Innocor for MBW measurements are described, and accuracy of the modified system assessed.

Introduction

Innocor gas analyser

The Innocor™ gas analyser (Innovision, Odense, Denmark) is designed to measure cardiac output at rest and during exercise by inert gas rebreathing (Agostoni, Cattadori et al. 2005). It does this by following the concentration of an absorbed gas (nitrous oxide, N_2O) and a non-absorbed gas (sulphur hexafluoride, SF_6) during an incremental exercise test using a photoacoustic multi-gas analyser. The difference in absorption of the two gases is proportional to pulmonary blood flow, and hence cardiac output. The device also measures oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) by following the concentration of O_2 and CO_2 in the expired air.

Innocor measures flow using a standard mesh-type flowmeter connected to an on-board differential pressure transducer. Oxygen concentration is measured using a laser diode absorption spectroscopy oxygen analyser. This is placed in series with the photoacoustic multi-gas analyser.

It is this photoacoustic gas analyser that represents the major advance of the device. This was originally developed for the space programme as a more robust and compact alternative to a respiratory mass spectrometer. Gas is drawn through the sample line and into an acoustically semi-closed measurement chamber by a pump. The chamber is pulsed with narrow-band infra red light at three different wavelengths. Different gases absorb the light energy at different wavelengths, proportional to their respective concentrations, and convert it to heat. The light energy is pulsed at three different frequencies using a rotating disk chopper. Thus gases heat and cool at different frequencies depending on the wavelength of light they absorb. These temperature fluctuations generate pressure waves as sound, which are detected by a highly sensitive microphone. The microphone signal is then filtered to separate the three modulation frequencies that correspond to the different gas concentrations. The amplitude of the sound wave is directly proportional to the gas concentration.

The chamber is insulated against external noise, with both upstream and downstream acoustic filters. The pulsations of the sampling pump are also attenuated. Temperature and pressure of the chamber are monitored and adjusted for.

The end result is a compact multi-gas analyser that is able to fit into a device no bigger than a computer monitor. The gas analyser can only measure gases consisting of two different atomic species. In the Innocor device, the selected light wavelengths are those absorbed by CO_2, SF_6 and N_2O. However, the machine could not be adapted to measure concentrations of monoatomic gases such as O_2 or Helium for instance. This is the reason for using a separate O_2 analyser placed in series with the photoacoustic gas analyser. The photoacoustic analyser is intrinsically stable and external calibration of the multi-gas analyser is only required every 12 months.

Comparison of Innocor with a respiratory mass spectrometer

Since the current standard method of performing MBW using SF_6 involves a mass spectrometer (MS), the performance of the modified Innocor gas analyser was compared to a MS used routinely for this purpose. The two devices operate at very different gas concentration ranges. Innocor is sensitive to very low concentrations of SF_6 and is linear only up to 0.4% SF_6. By contrast, the MS is normally used to perform washouts from 4% to 0.1% SF_6.

The respiratory MS used for this comparison was an Amis 2000 (Innovision, Odense, Denmark), used routinely for LCI measurements in children in the Children's Physiology Department at Queen Silvia Hospital, Gothenburg, Sweden. In order to make a simultaneous comparison between the MS and Innocor, a connector was prepared with two gas sample ports placed between the mouthpiece and a Fleisch no.1 flowmeter (Figure 2.1). This allowed a washout to be performed from 0.2% SF_6 while the two devices sampled the SF_6 concentration of the expired air simultaneously. An example of washout is shown in Figure 2.2, with the first and last breaths expanded to illustrate the differences in gas analyser response and signal quality. These measurements were made in Professor Gustafsson's laboratory in Gothenburg, Sweden, and his assistance with these is gratefully acknowledged.

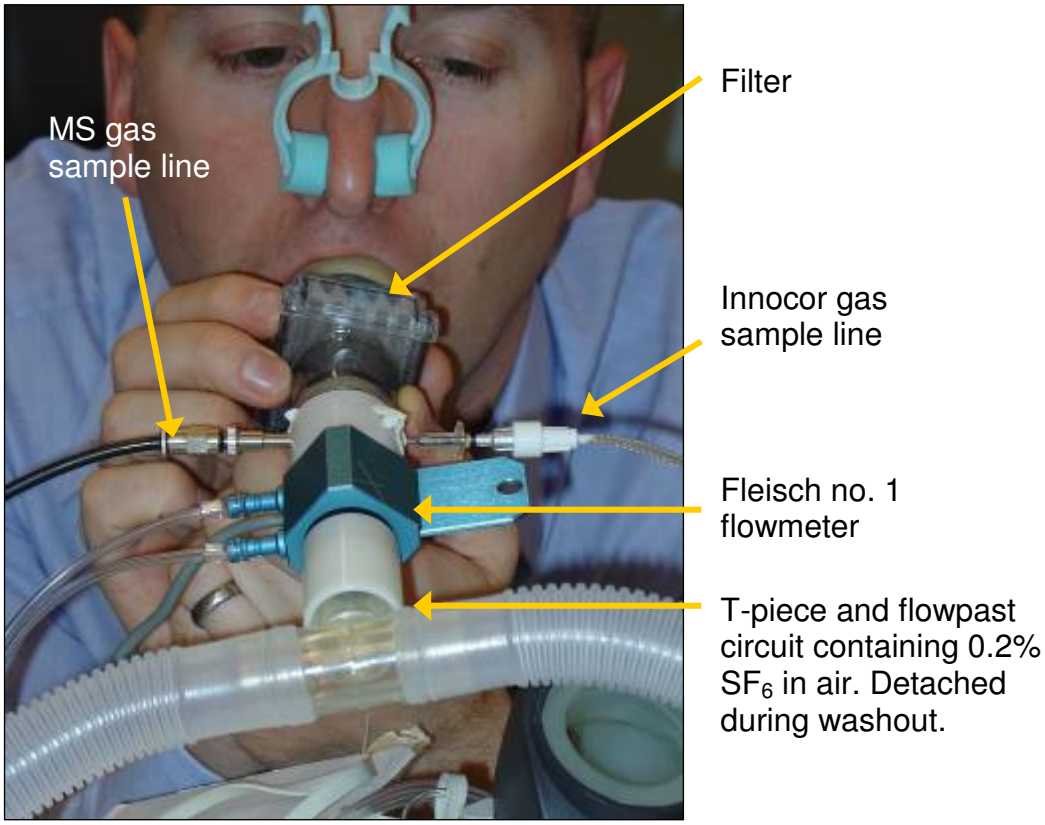


Figure 2.1: Apparatus used for simultaneous comparison of MS and Innocor multiple breath washout with key components identified.

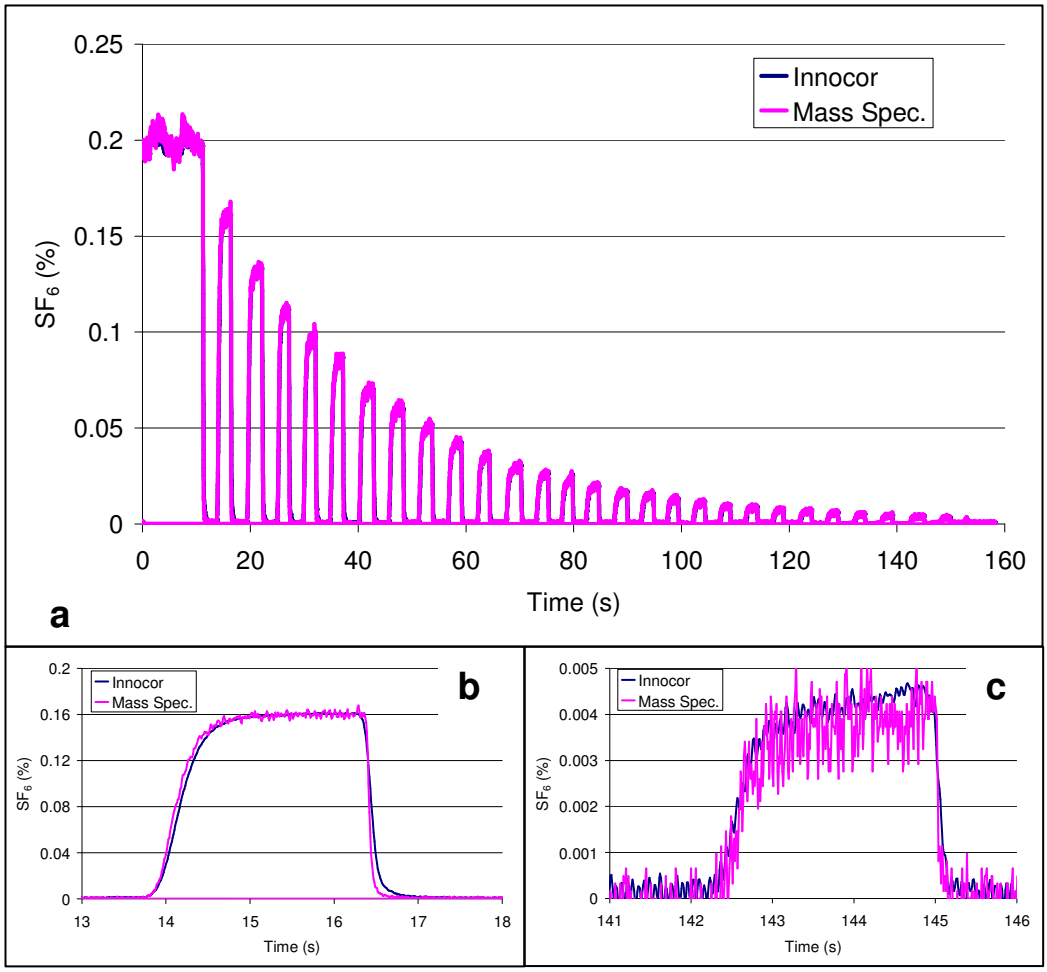


Figure 2.2a: Multiple breath washout from 0.2% SF_6 showing simultaneous mass spectrometer (purple) and Innocor (blue) gas signals. The two outputs have been aligned manually, but there is good overlay between the two outputs.

The first and last breaths of the washout are expanded in the lower panels, **2.2b** and **2.2c** respectively. **Figure 2.2c** illustrates the poor gas signal resolution of the mass spectrometer at the SF_6 concentrations encountered at the end of an Innocor multiple breath washout.

MS signal quality is insufficient for accurate washout analysis at the lower concentration range. Because of the limitations imposed by this difference in SF_6 concentration operating range, it has not yet been possible directly to compare washout calculations using the mass spectrometer and Innocor simultaneously *in vivo*. As an alternative to this, the first part of this chapter concerns non-synchronous comparison of Innocor gas analyser performance, in terms of signal quality and signal response time, to that of the same mass spectrometer. This also permits comparison of Innocor performance with published technical recommendations (Gustafsson 2005; Wanger, Clausen et al. 2005; Beydon, Davis et al. 2007). The second part of this chapter, describes the modifications to Innocor required to permit multiple breath washout measurements. The third part of this chapter concerns data analysis and adjustment of the raw gas signal data. In the final section, accuracy of the complete system is assessed.

2.1 - Technical validation of Innocor gas analyser

i) Signal:noise ratio of Innocor and Mass Spectrometer

Methods

Signal quality is expressed as the signal:noise (S:N) ratio. This is calculated as the ratio between the mean and standard deviation of a stable gas signal over 10 seconds (Beydon, Davis et al. 2007).

To assess the Innocor signal quality, a 3L gas bag was filled with air enriched with 0.2% SF_6 from a cylinder (BOC, Guildford, UK). The bag was then sealed except for the gas analyser sample port, and the contents mixed. Innocor was left to sample the gas within the bag for 5-6 minutes, in order to allow complete gas mixing. Signal quality was assessed on the final 10 seconds of this period. In order to assess signal quality at the lower SF_6 concentrations found at the end of a washout, the gas within the bag was partially emptied, diluted with oxygen from a cylinder, and mixed again. This was repeated until the Innocor analyser gave the SF_6 concentration at between 0.004 - 0.006%. Innocor was then left to sample the bag for a further 5-6

minutes and the final 10 seconds of this used for the signal quality assessment. Innocor samples at 100Hz. This was repeated 4 times, and an additional start of washout sample was also included.

The signal quality of the MS is determined by the number of channels open (i.e. the number of gases sampled) and the rate of sampling. The best quality signals (in terms of signal:noise) are achieved by sampling the fewest channels at the lowest rate. The MS was set to sample 4 channels at a rate of 33Hz (the same as the settings used during LCI measurement). MS signal:noise ratio was assessed in the physiology laboratory of Queen Silvia's Children's Hospital in Gothenburg. A stream of 4% or 0.1% SF_6 in air was generated by mixing 4% SF_6 with medical air, both supplied from separate cylinders. The MS sampled the gas stream for 10 secs.

Results

Since the Innocor device has a lower gas concentration operating range than the MS, signal quality is given at the starting and finishing concentrations of a washout, which are different for the two devices (Table 2.1). For both devices, there is a fall in signal:noise ratio as the gas concentration falls, but the Innocor signal quality remains superior throughout, despite much lower SF_6 concentrations and a faster sampling rate.

Conclusions

The Innocor signal quality is well above that recommended for apparatus used in MBW analyses (Beydon, Davis et al. 2007). This signal quality allows accurate rendition of expiratory volume vs concentration plots, and calculation of phase III slopes, even at the end of the washout when very low concentrations of SF_6 are being expired.

	SF_6 Concentration (%)		Mean (SD) Signal:Noise ratio	
	Start	**End**	**Start**	**End**
Mass spectrometer	4.0	0.1	200	13
Innocor	0.2051	0.0051	944 (46)	53 (6)

Table 2.1: Signal:noise ratios of Innocor and mass spectrometer at gas concentrations encountered at start and end of washout. The signal:noise is calculated as the ratio of mean to standard deviation of a stable gas signal over ten seconds. For the Innocor device, the figure represent the mean (SD) signal noise ratio of 5 repeats (start of washout) and 4 repeats (end of washout). The mass spectrometer data are the mean of three repeats, no SD data are available.

ii) Rise time of Innocor gas signal.

Methods

Step changes in gas concentration were generated using a specially constructed syringe, according to the method described by Brunner et al. (Brunner, Wolff et al. 1985) (Figure 2.3). The plunger end of the sealed syringe was filled with a test gas which was pumped into and out of a reservoir bag. A gas sample port half way down the syringe allowed rapid change of sampled gas from room air to test gas. The sample port was attached to the Innocor or MS gas sample line and a number of rapid changes in concentration performed. The response time is calculated as the 10-90% rise time of the signal, i.e. the time, in milliseconds, between 10 and 90% of the maximal gas signal deflection. The test gas used when assessing Innocor was a mixture of 0.2% SF_6 in air supplied from a cylinder, and expired air. The inclusion of expired air in the bag allowed the rise times of CO_2 and SF_6 to be calculated simultaneously and compared with each other. This is important because the CO_2 response is used to calculate the flow-gas delay daily, and is reported by the manufacturers as being the same as the SF_6 response. The plunger of the syringe used to assess Innocor gas rise times was sealed by a pair of o-rings. It has been assumed that leak between the o-rings is negligible, so that passage of the second o-ring over the gas sample port constitutes an instantaneous change in SF_6 concentration.

Raw data were exported to Excel for analysis. Resolution of the rise times was limited by the frequency of data sampling. For Innocor this is 10ms (100Hz).

Mass spectrometer SF_6 10-90% rise times were provided by Professor Gustafsson using his standard clinical system, a similar Brunner syringe, and 4% SF_6 in air as the test gas. Other MS settings were the same as in the previous section.

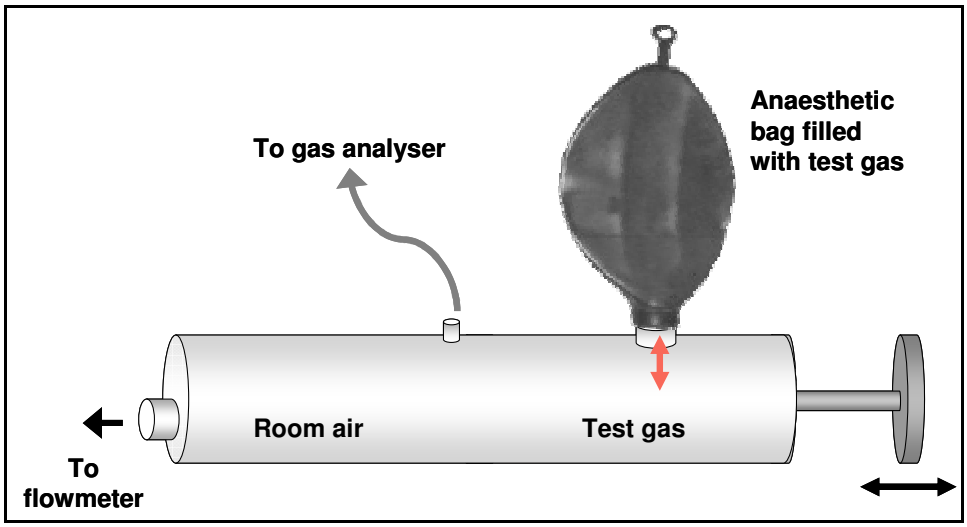


Figure 2.3: Diagram of syringe used to generate rapid changes in gas concentration between test gas and room air.
Test gas is contained within a sealed compartment at the plunger end of the syringe and moved in and out of a reservoir bag. A sample port half way down the syringe barrel allows rapid change of sampled gas from room air to test gas. If a flowmeter is attached to the syringe exhaust, near-simultaneous change in gas flow, and hence flow-gas delay, can also be calculated.

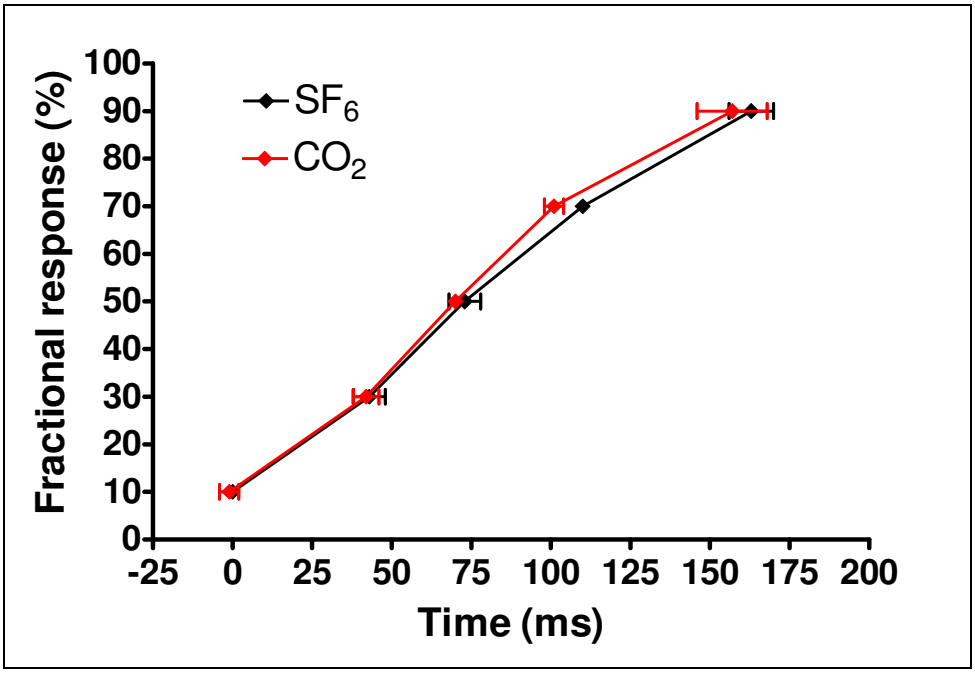


Figure 2.4: Comparison of SF_6 and CO_2 rise times. Each point represents the mean and SD of 10 repeat rise time manoeuvres. Step change in gas concentration was generated using a Brunner syringe, as described in the methods.

Results

The mean (SD) SF_6 10-90% rise time for Innocor was measured as 163 (7) ms, n=10. The mass spectrometer rise time was measured at 64 (5) ms, n=20, p<0.00001 compared to Innocor (unpaired T-test). The CO_2 rise time for Innocor was measured as 158 (10) ms, n=10, p=0.216 compared to that for SF_6 using Innocor (unpaired t-test). The CO_2 and SF_6 gas analyser responses are shown in Figure 2.4. There was no statistically significant difference in analyser response time at any of the fractional response points sampled with the exception of the 70% response. This was measured as 110 (0) ms for SF_6 and 101 (3) ms for CO_2, p=<0.0001 (unpaired T-test). It is possible that this represents an artefact, since the difference is less than the resolution (10ms), and further repeats may have reduced this. Importantly, the 50% rise times of the two gases were near-identical, and it is this that most closely approximates what Innocor uses to calculate the flow-gas delay.

Conclusions

CO_2 and SF_6 rise times are the same for the photoacoustic analyser, but are slower than the MS and also slower than those recommended for use in MBW tests in preschool children (Beydon, Davis et al. 2007). The implications of this rise time are considered in the discussion at the end of the chapter.

iii) Effect of expired air temperature and humidity on gas analyser accuracy

Expired air is warmed and humidified compared to inspired air. A particular criticism of ultrasonic gas analysers, which have also been used to perform multiple breath washouts, is that they are affected by the moisture and temperature of expired air. This must therefore be continuously monitored and adjusted for using complex empirically derived algorithms (Buess, Pietsch et al. 1986; Latzin, Sauteur et al. 2007). The manufacturers of Innocor claim that the photoacoustic gas analyser is stable during respiration. There are two features of the system that allow this stability.

Firstly, the temperature and pressure of the Innocor gas analyser chamber are sampled continuously at 2 KHz. The manufacturer has extensive knowledge of the effects of temperature and pressure on the detector and these data have been used to generate compensation algorithms.

Secondly, humidity of expired gas is controlled for by the use of a Nafion^{TM} gas sample line. Nafion is a synthetic polymer that selectively absorbs water vapour and releases it into a drier atmosphere. The humidity of the gas sample equilibrates with that of room air by the time the gas sample enters the machine itself. Since the temperature of the gas sample in the measurement chamber is already adjusted for, the analyser should be therefore unaffected by changes in humidity or temperature of the gas sample with phase of respiration.

This section concerns investigation of the stability of the SF_6 signal before and after humidification and warming.

Methods

0.2% SF_6 in air, supplied from a compressed gas cylinder, was passed through a humidification system at 10L/min. The humidification system used was a Fisher Paykel HC 100 (Fisher Paykel, New Zealand), designed for use with non-invasive ventilators. This device warms and humidifies a flow of gas by passing it over warmed water. Air exiting the device is warmed to 37^{o}C with 100% humidity, closely mimicking the conditions of expired air.

The exhaust port of the humidifier was connected to a length of 22mm diameter disposable plastic "elephant" tubing (Intersurgical, Berkshire, UK) The gas mix was sampled by inserting an 18G needle (Becton Dickinson, Oxford, UK), connected to the Innocor gas sample line, through the tubing into the stream of gas. The needle was inserted <1cm from the end of the tubing and temperature and humidity of the gas sample measured continuously using a digital thermohygrometr (Rotronic AG, Bassersdorf, Switzerland) (Figure 2.5). The thermometer and gas analyser were left in position for 2 minutes, and the average gas concentration measured over the final 30 seconds was calculated. After 2 minutes, the needle was removed, and a length of tubing cut from the end. The needle was then re-inserted 1cm from the end of the now shortened tube, with the thermometer once again placed over the end of the tube. This allowed the gas stream to be sampled at several points along the length of the tube, becoming increasingly warm with proximity to the humidification chamber. The tube was held vertically so that any condensation would run back into the chamber. A fan was positioned to blow the exhausted gas away, so that local temperature or humidity were not altered.

Controls included sampling the composition of the gas on the inlet side of the chamber and sampling the gas composition after passing it through the complete system but without water or heat added in the chamber.

Results

SF_6 concentration remained the same throughout the assessment, whether it was dry air at room temperature (25^{o}C), equivalent to inspired air conditions, or warmed and humidified air at 37^{o}C, equivalent to expired air (Table 2.2).

The difference between SF_6 concentration measured in the inlet and that at the exhaust mouth was 0.0004% absolute SF_6, or a 0.21% difference. The difference between air measured in the inlet and that at 35^{o}C was -0.0001% absolute SF_6, or 0.04% difference.

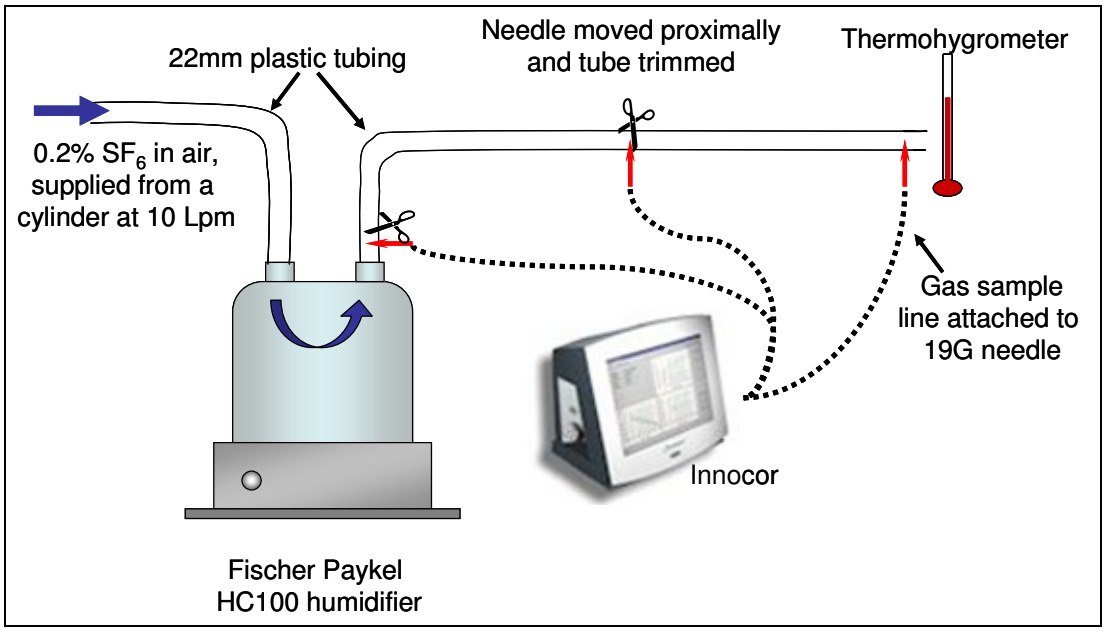


Figure 2.5: Diagram of apparatus used to assess effects of warming and humidifying gas stream on measured SF_6 concentration. The gas sample needle and thermohygrometer were placed at the end of a plastic tube attached to the exhaust of the humidifier. The tube was then cut and the gas sampled from the end of the shortened tube. The final sample is taken directly from the humidifier exhaust.
Although shown as horizontal for illustrative purposes, the exhaust tubing was held vertically to permit condensation to run back down into the humidifier.

Section	Distance from humdification chamber outlet (cm)	Temp ^{o}C	Relative humidity (%)	SF_6 concentration
3	76	31	100	0.205
2	37	33	100	0.205
1	0	37	100	0.205
Inlet	NA	25	0**	0.205
Dry control	20cm	25	0	0.205

Table 2.2: Mean SF_6 concentration and gas temperature recorded at different distances along exhaust tube from humidification chamber. Data are from a single experiment, but represent the mean SF_6 concentration over a period of 30 seconds of gas sampling.

* Recorded at humidifier exhaust

** Dry gas from cylinder

$^{++}$ No heat or water in humidification chamber

Conclusions

This assessment shows that the Innocor gas analyser [SF_6] signal is not affected by changes in the temperature or moisture content of the gas sample over the range encountered in the use to which the machine is put. The effects of cooling the gas stream were not assessed since performance below 20^{o}C is not relevant to the intended usage.

iv) Gas analyser drift.

Methods

A Douglas bag was filled with 0.2% SF_6 in air, and the gas in the bag mixed. The gas sample line was attached to an 18G needle inserted into a short length of plastic anaesthetic tubing, sealed at one end, and attached to the opening of the bag. The Innocor gas analyser was switched on, and allowed to sample the gas in the bag for 60 minutes. The difference between mean gas concentration over the final 1 minute was compared to that in the 2nd minute. Raw data were also plotted and inspected to ensure that the start and end concentrations were representative of the tracings over the entire sampling period.

Results

Mean SF_6 concentration in minute 2 was 0.2050%. Mean concentration in minute 60 was 0.2052%. This represents a drift of 0.0002% absolute change in SF_6 concentration, or 0.1% relative to baseline.

Conclusions

The Innocor gas analyser is stable, with no appreciable drift in gas concentration signal over prolonged sampling.

v) Long term analyser drift

Since the same cylinder of 0.2% SF_6 is used for many washout assessments over the course of several months, any drift in the analyser would be noticeable as a fall in the peak inspired SF_6 concentration. In addition to this, a small cylinder of 0.2% SF_6, retained as a spare, has been used to assess long term drift in the analyser over 7 months.

A stream of gas from the same cylinder was sampled by the Innocor gas analyser for at least 60 seconds. The mean (SD) of the SF_6 signal over a stable 30 second period was calculated. At time 0, 2 days after re-calibration, mean (SD) SF_6 concentration was 0.21629 (0.0022) % and 0.21650 (0.0021) % for two different repeats, giving an overall mean of 0.21639%. Two months later, the mean (SD) SF_6 concentration measured from the same cylinder was 0.21554 (0.0023) %. Five months after this, the overall mean SF_6 concentration was 0.21529 % (3 repeats). Over 7 months, a fall in the SF_6 concentration had occurred of 0.001107 % absolute, or 0.51% relative to the starting concentration. This represents a drift in SF_6 concentration of 0.073% per month.

The SF_6 analyser in the Innocor machine is re-calibrated annually by an engineer to an external standard.

Conclusions

There is minimal drift in the SF_6 analyser over an extended period. No short term drift has been observed between washouts or on consecutive days.

2.2 - Adaptation of Innocor device to permit MBW assessment

i) Modifications to Innocor patient interface

Because it is intended for measurements during an exercise test, the commercially supplied Innocor patient interface has been designed to be low resistance and consequently has a large deadspace of 136 ml. For accurate LCI measurement, deadspace must be at a minimum (Aurora, Kozlowska et al. 2005; Wanger, Clausen et al. 2005). The flowmeter was therefore replaced with a mesh type flowmeter (Hans Rudolph, Missouri, USA), equivalent to a Fleisch number 2 pneumotachograph (flow range 0-160 l/min). An alternative to the Innocor-supplied microbiological filter was also used (Barrierbac S; Tyco Healthcare, Hampshire, UK). This was selected for its low volume (deadspace) and for ease of connection with the flowmeter. Use of a filter permits the apparatus to be used with different patients without requiring full disassembly and cleaning. It also traps expired water vapour and prevents respiratory secretions from fouling the flowmeter mesh. A mouthpiece (Ferraris, Hertford, UK) was cut to fit over the filter end with minimal additional deadspace.

The gas sample needle was positioned on the supply rather than the mouthpiece side of the pneumotachograph. This is to avoid the possibility of the gas sample flow interfering with pneumotachograph function and to reduce re-inspired SF_6, as explained in the discussion. The gas sample line was attached to a shortened 20 Gauge needle (Becton Dickinson, Oxford, UK) in a hole drilled through the flowmeter housing. The modified patient interface is illustrated in Figure 2.6.

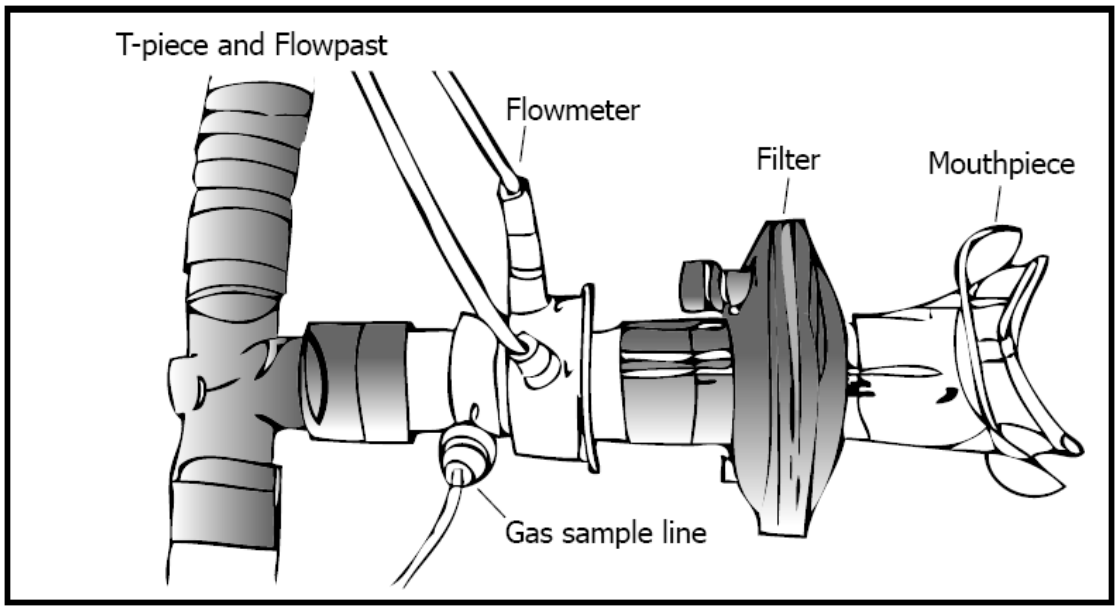


Figure 2.6: Modified patient interface used for inert gas washout measurements with Innocor gas analyser. The T-piece connects the patient to a bias flow circuit containing air enriched with 0.2% SF_6. This is removed at the start of the washout.

Pre-capillary dead space (that between needle and patient mouth) of the system, was calculated to be 46 ml for the complete apparatus. Calculations were based upon the known volumes of key components as described by the manufacturers, with volume of altered components (e.g. mouthpiece) calculated by filling with water. Post-capillary dead space (that between needle and end of flowmeter casing) was calculated mathematically to be less than 5ml, i.e. less than 1% of the typical minimum tidal volume. A fan was used to create a stream of air across the patient during washout so that additional expired SF_6 was not re-inspired.

The lines to the pressure transducer on the standard Innocor device are integrated into a large plastic cable that also contains lines supplying the rebreathing apparatus. This in turn is attached to a bulky unit which normally contains the flowmeter and valve assembly. The gas sample line runs separately. Since the only parts of this system required for MBW measurements are the gas sample line and pressure transducer line, a separate connector was constructed by the manufacturers which had all the ports occluded except those to the pressure transducer. This was connected to the flowmeter using equal lengths of tubing (Guttasyn, Hamburg, Germany), and permitted a lightweight patient interface consisting of the flowmeter and filter arrangement alone. This was then mounted on a swing arm, and attached using a retort clamp. The swing arm was clamped to the side of a wheeled table so that it could be brought to the subject and adjusted to ensure optimal comfort (Figure 2.7)

Details of the software settings required to perform a multiple breath washout using Innocor's operating system are found in a manual prepared for the modification and use of Innocor to measure inert gas washout. This is presented in Appendix A.

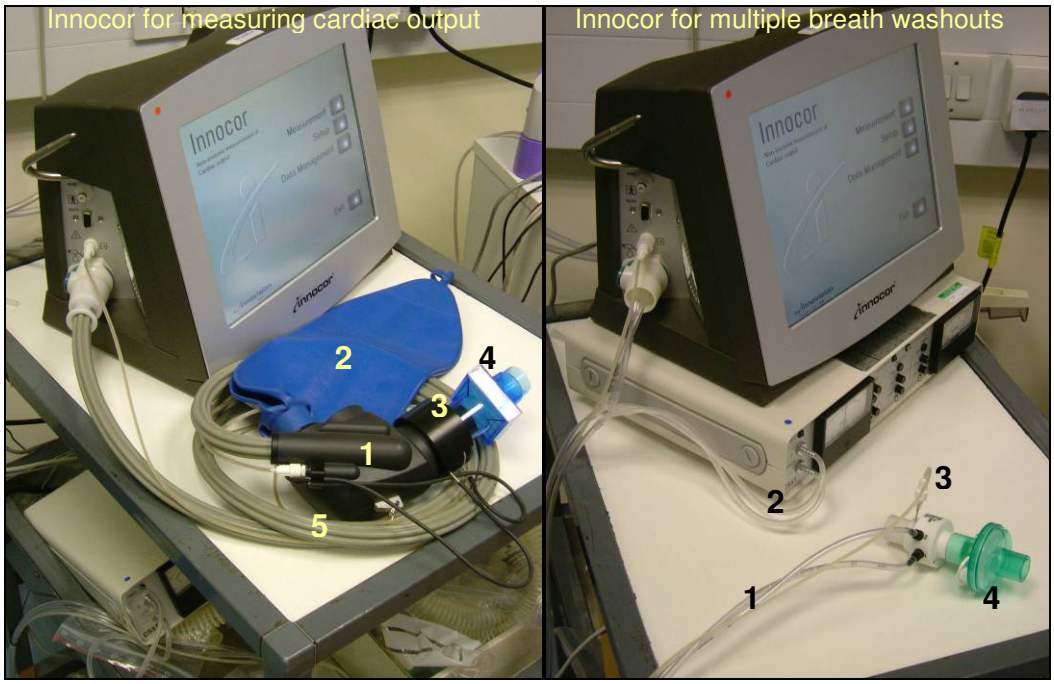


Figure 2.7: Standard Innocor set-up on left, with patient interface for cardiac output assessments. The interface consists of the main control unit (1), containing switching valves, a rebreathe bag (2), flow-meter (3) and filter (4), attached to Innocor analyzer by a cable (5) containing gas supply and pressure transducer lines.
The modified Innocor machine, for multiple breath washout assessments, is shown on the right. The cable has been replaced by two lines from the flow-meter (1), split to supply a separate pressure transducer for breath volume display (2), and a much smaller patient interface consisting of flowmeter (3) and filter (4) alone.

ii) Flowmeter accuracy

The flowmeter output was linearised using Innocor's onboard software according to the method of Yeh et al. (Yeh, Gardner et al. 1982). There is an additional daily calibration procedure of the flowmeter, in accordance with American Thoracic Society / European Respiratory Society guidelines on volume measurement (Wanger, Clausen et al. 2005). This involves emptying a 3L calibration syringe over 0.5 to 6s. Because the filter may alter airflow characteristics, a filter was also used during the calibration process. If the calculated gain was more than +/- 5%, or if the range of syringe volumes was greater than +/-3%, the system was checked for presence of leaks at connectors, and the calibration process repeated. If the error remained, a new flowmeter calibration was performed using Innocor's onboard software.

Because very low concentrations of SF_6 are used in the washout, it is not necessary to make adjustment for changing gas viscosity caused by changing gas composition of expirate. This is not the case with nitrogen washouts, for example, where the change in expired oxygen concentration (and hence expirate viscosity) can be considerable over the course of a single breath. During nitrogen washouts, this results in a difference in flow meter output of up to 12%, unless there is continuous adjustment of flowmeter response (Saniie, Saidel et al. 1979). In contrast, SF_6 has a viscosity similar to that of CO_2. Adding 0.2% SF_6 to dry inspired air reduces viscosity by less than 0.4%.

Since the apparatus was intended for use in multi-centre trials, it was important to ensure that the system was as simple as possible. For this reason, it was felt preferable to use a non-heated flowmeter. Previous studies have shown a fall in accuracy of such flowmeters due to formation of condensation on the mesh from exhaled breath water vapour, though this is more likely to be significant if a Fleisch-type of flowmeter is used (Miller and Sigsgaard 1994). The effects of a filter on condensation are however unknown. The following experiments were therefore designed to assess whether an unheated flowmeter and filter remained accurate after prolonged rebreathing.

Methods

In order to assess effects of prolonged breathing on flowmeter accuracy, a single subject breathed through the filter and flowmeter for a range of times, up to 20 minutes. The subject established tidal breathing and a noseclip was applied to ensure that all ventilation occurred through the flowmeter, as in a washout test. Flowmeter accuracy was assessed before and after rebreathing using a 3L calibration syringe at a range of different flow rates. Five fill-empty syringe manoeuvres were performed before rebreathing, and three afterwards. Continued fill-empty manoeuvres beyond this are likely to dry any condensation on the mesh and reduce the magnitude of any error caused by vapour condensation (Miller and Sigsgaard 1994). In addition, the flowmeter and mesh were visually inspected, after each rebreathing test, for the presence of condensation. Following testing, the data were exported and flow signals integrated to yield the calculated syringe volume. Pre-and post-rebreathing mean integrated volumes for syringe filling (inspiration) and emptying (expiration) were pooled to compare all data from each time-point.

Results

A total of seven trials were performed with different periods of rebreathing through the flowmeter; 3 x 5 minutes, 2 x 15 minutes and 2 x 20 minutes. No consistent changes were seen from the individual trials. Pooled data are summarised in Table 2.3 and illustrated in Figure 2.8. Mean (SD) measured syringe volume was 3.003 (0.021) L for syringe filling before and 3.023 (0.024) L for syringe filling after rebreathing. This difference was statistically significant, $p<0.0001$ (unpaired T-test), but represents an error of 20ml, or 0.67% of the delivered volume. For syringe emptying, measured volume was 3.005 (0.018) L before and 3.006 (0.016) L after rebreathing, $p=0.1752$.

A total of 110 manoeuvres were analysed, of which only a single one was outwith the range of accuracy ±3%, and only 9 (8%) were outwith the range ±1%. No moisture or debris were visible on the mesh when visually inspected.

Conclusions

The difference in volume signal observed after rebreathing is below the level of clinical significance. This supports the use of non-heated flowmeter and filter.

Time point	Measured volume range (L) (% delivered volume)	Mean	SEM	Coefficient of Variation (%)
Inspire Start (syringe fill pre-test)	2.955-3.056 (98.5-101.9)	3.003	0.003662	0.71%
Inspire End (syringe fill post-test)	2.987-3.095 (99.6-103.2)	3.023	0.005336	0.81%
Expire Start (syringe empty pre-test)	2.975-3.036 (99.2-101.2)	3.005	0.003031	0.59%
Expire End (syringe empty post-test)	2.973-3.040 (99.1-101.3)	3.006	0.003446	0.53%

Table 2.3: Measured volume of 3L calibration syringe using mesh flowmeter (equivalent to a Fleisch No.2) connected to Innocor pressure transducer pre and post prolonged (5-20 min) tidal breathing through flowmeter.
SEM – Standard error of the mean

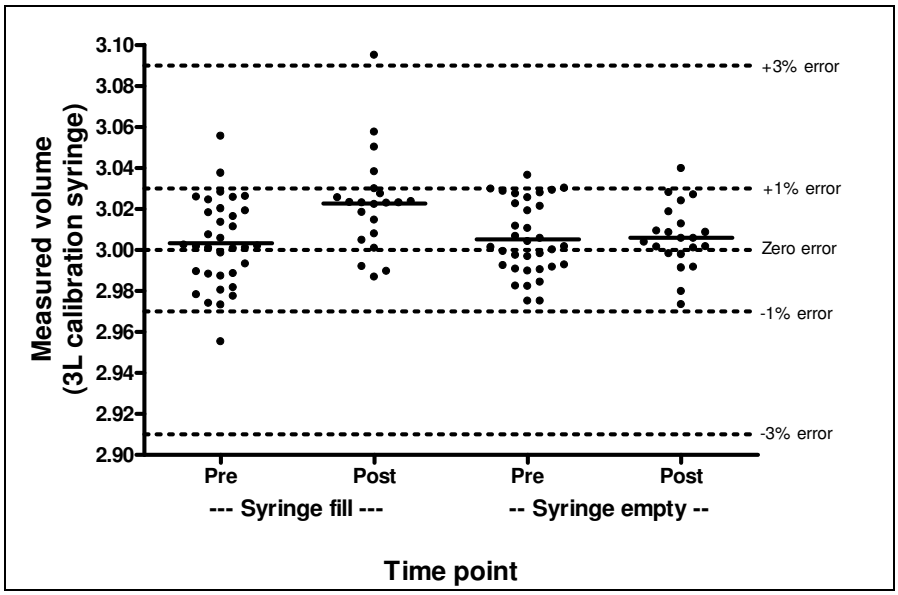


Figure 2.8: Flowmeter accuracy before and after prolonged rebreathing. Graph shows data pooled from 7 tests, representing different periods of rebreathing through the flowmeter (3 x 5 minutes, 2 x 15 minutes, 2 x 20 minutes). Five syringe fill and empty manoeuvres were performed before rebreathing and three afterwards. Horizontal solid lines in each group represent the group mean measured volumes. Horizontal dotted lines represent the limits of ±1% error and ±3% error.

iii) Flow-gas delay

Flow-gas delay is calculated on Innocor by the operator performing a series of slow expirations followed by fast inspirations. This manoeuvre generates a sudden change in flow at the same time as a sudden fall in CO_2 concentration. Flow-gas delay is calculated as the delay during this forced inspiration between change in flow (zero crossing) and change in CO_2 concentration (50% change in area under the curve, as described by Fowler (Fowler 1949)), taking into account the deadspace from the gas sampling point to the ambient air port. The flow-gas delay for SF_6 is claimed to be the same as that of CO_2. The recorded flow-gas delay is the mean of nine repeats, though outliers which differ by more than 25ms from the mean are excluded.

Methods

The assumptions behind Innocor's on-board assessment of gas signal delay were verified using the Brunner syringe (Figure 2.3) and by manual review of the flow-gas delay data.

CO_2 and SF_6 delay times were compared using the Brunner syringe. If the plunger is moved so that it is just obstructing the gas sample port, further sudden movement of the plunger will generate a near-simultaneous flow signal and change in SF_6 concentration. Attaching the flowmeter to the main syringe exhaust allows measurement of change in flow. To compare the delay of the two different gases, the reservoir bag of the syringe was filled with a mixture of 0.2% SF_6 and expired air (containing CO_2).

For comparison of the syringe-generated and breathing manoeuvre-generated calculations of gas delay, allowance must be made for the fact that the changes in flow and gas concentration signals are not perfectly simultaneous. This is because the depth of the plunger means that it must move 15mm before the gas sample port is exposed to the test gas. This displaces a volume of around 20ml, and takes an average time of around 70ms, dependent upon the speed of the plunger. For this

analysis, the zero time point for calculation of gas signal delay was after expulsion of 20ml from the syringe, derived from integration of the flow signal.

Finally, the delay obtained by Innocor's onboard software was compared to that derived manually from identical breathing manoeuvres.

All data were exported and analysed in Excel. Flow-gas delays derived from the Brunner syringe were calculated to the 50% maximal increment in gas concentration. Flow-gas delay derived from breathing manoeuvres was calculated as the delay between flow crossing zero on rapid inspiration, and 50% maximal fall in CO_2 concentration.

Repeatability of flow-gas delay calculations was assessed by repeat manoeuvres performed on the same day. A daily record of the flow-gas delay was also kept in a log book so that sudden changes, or trends across several days, could be detected.

Results

When assessed simultaneously using the Brunner syringe, mean (SD) SF_6 flow-gas delay was 1472 (10) ms and CO_2 flow-gas delay was 1469 (12) ms, n=12. This difference of 3ms was not statistically significant, p=0.56 by unpaired T-test.

At a different time point, mean (SD) of repeated flow-gas delay calculations performed by Innocor was measured at 1424 (4) ms. This was derived from 10 flow-gas delay calculations, each of which is the mean of 9 respiratory manoeuvres. Individual flow-gas delay repeats varied from 1391-1440 ms, but range of delivered mean delays varied by only 9 ms (range 1419-1428 ms). Mean (SD) SF_6 flow-gas delay on the same day, calculated from the time between expulsion of 20 ml from the Brunner syringe and 50% maximal increment in SF_6 concentration, was 1407 (8) ms (n=10).

Mean (SD) flow gas delay calculated manually from 50% fall in CO2 concentration during rapid inspiration, was 1440 (6) ms (n=11).

Conclusions

The delays calculated using the Brunner syringe verified that CO_2 and SF_6 have equivalent delay times, confirming the validity of this assumption used by Innocor to

determine flow-gas delay. Since concentrations of both gases are measured in the photoacoustic gas analyser identically, this is as expected.

Flow gas delay calculated using Innocor's on-board method appears to be highly reproducible, with a narrow range for assessments conducted on the same day (1419-1428ms). The assessments are themselves an average of 9 repeat manoeuvres however, and the individual repeats show a wider range of almost 50ms. Reproducibility can also be adversely affected if the manoeuvres are not identical. For instance if expiration is re-commenced as soon as inspiration is complete, the flow-gas delay is shortened, presumably since the final (slowest) part of the CO_2 fall time is attenuated. In order to obtain consistent results, it is necessary to pause for 1-2 seconds after each rapid inspiration before starting the next expiration.

Flow-gas delay calculated using the Brunner syringe was, on average, 17ms less than that calculated by Innocor. There are a number of reasons why this discrepancy may have occurred. The gas sample line attached directly to the syringe, whereas during a breathing manoeuvre the gas must first pass through the gas sample needle. Removing the needle will shorten the delay time by reducing resistance and increasing sample flow rate. Accuracy is also limited both by the resolution of the time delay (down to the nearest 10ms) and by the accuracy of the Brunner syringe. As discussed earlier, this does not generate a truly simultaneous flow and gas concentration change due to the distance the plunger must pass before the new gas mix is exposed to the sample port. The correction for this is based upon an estimated expired volume.

In contrast, the manually calculated CO_2 fall time delay, derived from breathing manoeuvres identical to those used by Innocor, was 1440ms – a mean increase of 16ms (1.1%) over that derived by Innocor's on-board software. Again, this analysis is limited by the 10ms resolution of the time signal.

Finally, the algorithm used in the manual analyses is slightly different to that used by Innocor. The measurement after 50% rise in SF_6 concentration for syringe manoeuvres, or 50% fall in CO_2 concentration for rapid inspirations, was taken as being the end point for the gas signal. Innocor however uses equality of areas above and below the gas concentration-time curve (as per calculation of Fowler dead space). Differences in method may explain some of the difference in results.

Despite the discrepancies, all assessments of gas delay were reproducible, and the Innocor method was between that derived from the syringe and that derived from rapid inspiration. More importantly, when gas and flow signals are aligned in the analysis software, the Innocor-derived flow-gas delay consistently provides accurate alignment.

iv) Expiratory volume feedback

In order to improve reproducibility of expired breath volumes, a system for dynamic inspiratory or expiratory volume feedback was developed. Innocor displays a three-breath sliding average tidal volume after a breath has completed, but does not provide real-time feedback. In addition, there are no outputs from the onboard differential pressure transducer, other than that to the on-board computer. In order to establish a separate flowmeter output, the lines to the pressure transducer were split using a Y-piece connector (Portex, Watford, UK), with one arm entering the Innocor device and the other a separate differential pressure sensor (GM instruments, Kilwinning, UK). This generates analogue outputs corresponding to flow, or integrates the signal itself to produce a volume output. The analogue volume output from this pneumotachograph was then converted to a digital signal using a 16-bit analogue-digital converter (Measurement Computing, Massachusetts, USA) and fed into a laptop via the USB port (Figure 2.9). The volume was displayed on a laptop screen using software written with Visual Studio (Microsoft, Washington, USA). As the subject expires a bar rises to represent the cumulative expired volume. This is reset to zero on inspiration by the software. An empirical adjustment factor to convert the voltage output to expired volume was derived using a 1L calibration syringe. The system was found to be sensitive to expiratory flow rate, with the total expired volume greater at high flow rates. However, the accuracy was found to be within +/-10%, which was satisfactory for the purpose of expiratory volume feedback. Subjects were first allowed to establish a relaxed breathing pattern. A paper arrow was then affixed to the computer screen at the expiratory volume level

they achieved. Subjects with very shallow or deep breathing patterns were encouraged, with the assistance of the visual feedback, to establish tidal volumes between 500-1000ml. Other than this, the system was used to aid reproducibility of tidal breath volumes rather than targeting to a specific expired volume.

The approach adopted in all the clinical studies contained within this thesis was to display the expired volume. It subsequently became apparent that this resulted in a tendency for some subjects to expire below their relaxed FRC. A cohort of nine healthy volunteers were reviewed, with washouts performed before and after the development and use of expiratory volume feedback. Mean (SD) V_T increased significantly with the use of expiratory feedback from 686 (112) ml to 880 (136) ml ($p=0.018$, paired t-test). This represents a mean increase of 32%. In contrast mean (SD) FRC fell significantly by 13% from 2.81 (0.76) to 2.43 (0.71) L ($p=0.036$). Mean LCI was unchanged: 6.85 (0.5) before using feedback to 6.89 (0.5) after its introduction ($p=0.78$). This is illustrated in Figure 2.10, which shows the change in mean V_T (averaged for three washout repeats) before and after expiratory volume targeting, against the change in FRC (red) and LCI (blue). Substantial changes in V_T and FRC have little effect on LCI.

As a refinement of this system, the tidal volume targeting has since been altered so that patients target their inspiratory volume and then expire to a relaxed FRC. In the studies presented in this thesis however, expiratory volume feedback has been employed as described above.

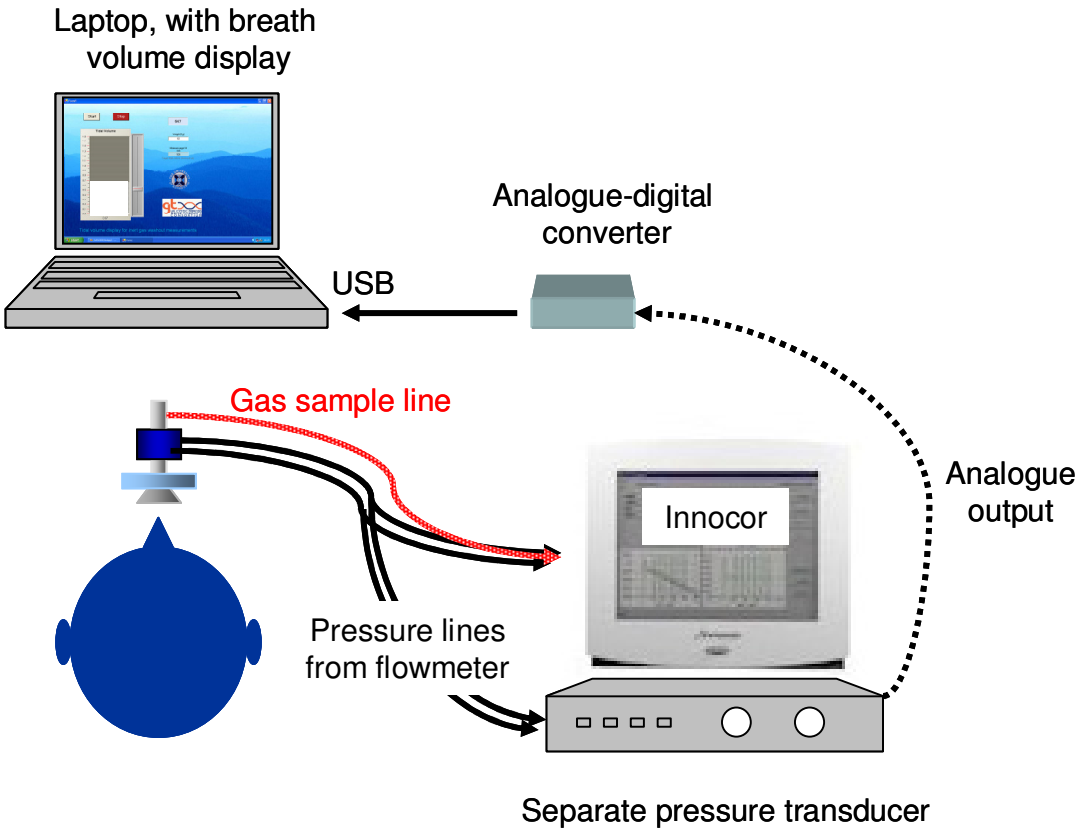


Figure 2.9: Diagram of tidal volume feedback system. The pressure lines from the flowmeter are split and fed into a separate pressure transducer, the output from which is interpreted and read by a laptop computer. As the breath volume increases, the bar on the left rises. Targeting of expiratory volume was achieved by affixing a paper arrow to the screen – this was found to be the most visually effective method of concentrating subject's attention on the expired volume.

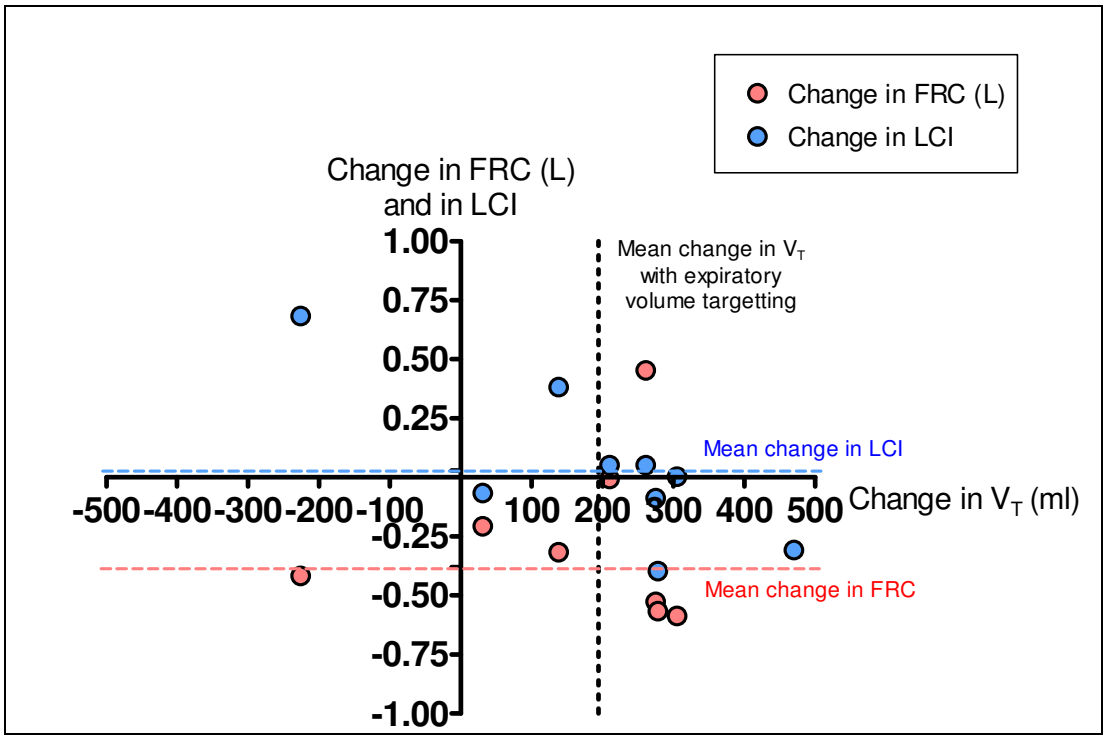


Figure 2.10: Effect of expiratory volume targeting on tidal volume (V_T), functional residual capacity (FRC) and lung clearance index (LCI) in nine healthy volunteers. The *x* axis represents the intra-subject absolute change in mean V_T between washouts performed before and after expiratory volume targeting. A positive value represents an increase in mean V_T with expiratory volume targeting. Change in FRC and change in LCI are plotted on the *y* axis and represented as red and blue circles respectively. A negative change in FRC or LCI represents a fall in that parameter with expiratory volume targeting. Overall mean change in V_T is represented by the vertical dotted black line, mean change in FRC by the dotted red horizontal line and mean change in LCI by the dotted blue horizontal line.

2.3 - Multiple breath washout analysis

Innocor generates raw flow and gas concentration data, sampled at 100Hz. These files are converted to text files and exported to custom-written software prepared using Testpoint (Capital Equipment, Massachusetts, USA). This software was adapted from the programme used in Professor Per Gustafsson's laboratory in Sweden, and has been used in a number of previous studies (Gustafsson, Aurora et al. 2003; Aurora, Gustafsson et al. 2004; Aurora, Bush et al. 2005). Modifications and revisions to the original analysis system were necessary – these were developed in collaboration with Per Gustafsson and his software engineer Eddie Bergsten. A screenshot of the analysis software is shown in Figure 2.11.

Zero flow is set by the Innocor device using a mechanical zeroing of the pressure transducer. This occurs every 60 seconds, and results in the loss of 1 second's worth of flow data whilst the pressure transducer is re-zeroed. A flow signal of 100L/s is generated for this second, easily visible on the flow vs time plot. For the purposes of washout analysis, it is important to maintain an un-interrupted flow signal. The flow-meter re-zeroing was therefore altered to occur after between 3-5 minutes, depending on the predicted length of wash-in of the subject. The wash-in was then continued beyond the point at which inspiratory and expiratory SF_6 concentrations had adequately equilibrated, until the re-zero had occurred. Wash-out was commenced immediately after this. Thus, at the start of a washout the flow-meter was re-zeroed. If wash-in was incomplete, it was continued until either the next re-zero (providing this was logistically possible), or until complete.

Inspiration is recorded as negative flow and expiration as positive. A change in phase of respiratory cycle was recorded as flow crossing zero. A filter is included to exclude very small variations around zero, as occur for instance at zero flow (e.g. pauses in respiration).

The full wash-in and wash-out were loaded and displayed, with the signals realigned according to the flow-gas delay, which was entered manually. The data were then cropped to focus on the wash-out only. New breaths were automatically identified as the flow crossing zero, but the start and end of breaths can be adjusted manually in case of error. Breath volume was derived by integration of flow, and

total SF_6 volume was derived from the integration of flow and gas concentration. The software also plots a graph of volume expired versus gas concentration in a separate window, and performs a linear regression of the phase III slope (Figure 2.11B). The default limits for calculating the phase III slope were between 65% and 95% of the expired breath volume, but these can be adjusted manually to achieve a best fit with the linear phase III segment. A washout is defined by convention to be complete when the end tidal SF_6 concentration has fallen to less than (or equal to) $1/40^{th}$ of the starting concentration (Gustafsson, Aurora et al. 2003). This convention is largely historical, being based upon the linear operating range of the nitrogen analysers originally used for multiple breath washout tests (2 – 80%). However, it has stood the test of time and represents a workable compromise between ending a washout too soon (and therefore losing sensitivity) and an excessively protracted procedure.

Functional residual capacity (FRC) is calculated from the starting fraction of SF_6 (C_{Init}), the final fraction of SF_6 (C_{End}), and the amount of exhaled SF_6 (V_{SF6}).

$$(C_{Init} \, x \, FRC) = (C_{End} \, x \, FRC) + V_{SF6}$$

So: $$FRC = V_{SF6} / (C_{Init} - C_{End})$$

LCI is defined as the number of lung turnovers required to washout the gas to $1/40^{th}$ of the starting concentration. In other words, the cumulative expired volume (CEV) divided by the FRC:

$$LCI = CEV / FRC$$

The other data outputs from the analysis software are:

1. Cumulative expired volume (L)
2. Number of breaths
3. Inspiratory and expiratory volume (L)
4. Raw and normalised phase III slope data for each breath of the washout (this is covered in more detail in Chapter 5)
5. 1^{st} and 2^{nd} order Moment ratios
6. Slope Index

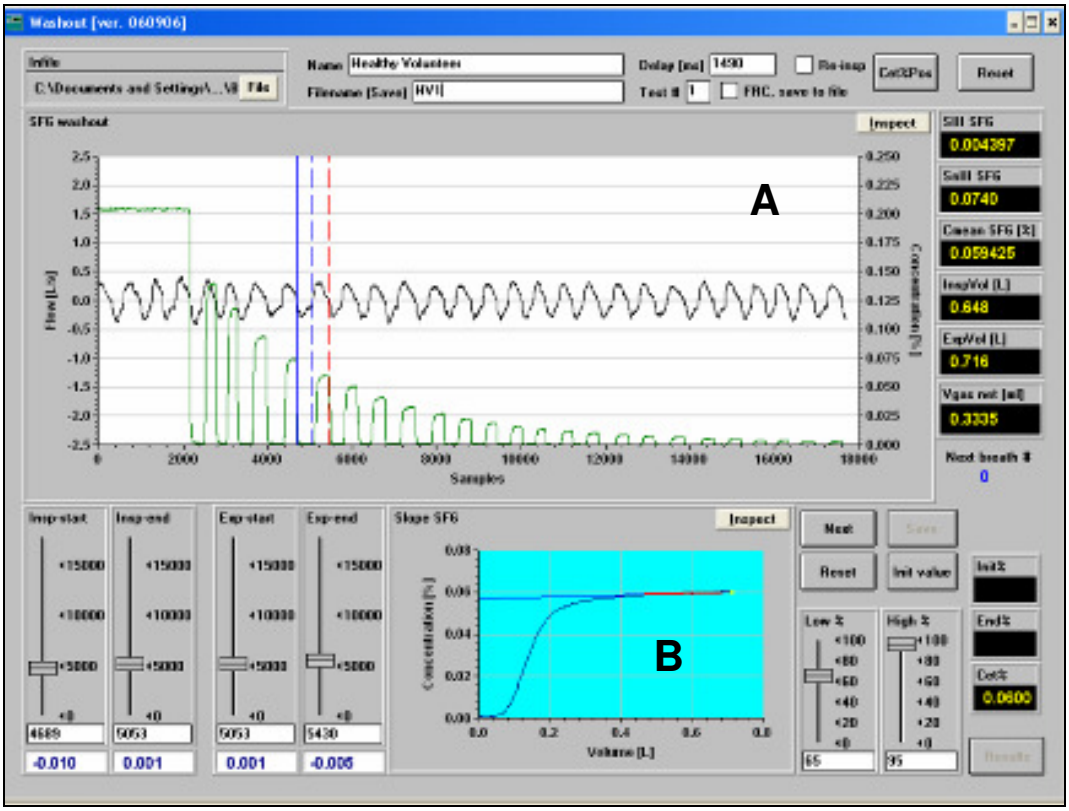


Figure 2.11: Screenshot of data analysis software. The upper window (A) shows the entire washout (flow signal in black, SF_6 signal in green). Expiratory SF_6 is integrated with flow to calculate the volume of expired SF_6, which is used in the calculation of functional residual capacity as described in the text. Each breath is identified and analysed in sequence and the lower window (B) shows a phase III slope analysis.

Moment ratios are derived from a plot of lung volume turnover (TO) against end tidal gas concentration (C_{ET}), normalised to 1.0 for C_{ET} at the start of the washout. The 0^{th} moment is the area under this curve, the 1^{st} moment the area under the curve of TO^2 versus C_{ET} and so on. The moment ratios are the ratios between these areas (Saniie, Saidel et al. 1979). Slope index is derived from the ratio between the slopes of C_{ET} versus TO at the start and end of the washout. Both of these indices emphasise the differences in the fall in C_{ET} between healthy subjects and those with lung disease. However, initial work identified problems with both of these indices, and they have therefore not been considered further in this thesis. Moment ratios showed poorer separation between health and disease than LCI, and were affected by the TO range analysed (Horsley, Macleod et al. 2007). Fleming et al. also showed greater sensitivity of LCI compared to moment ratios (measured to TO=10), but by contrast found LCI to be poorly reproducible (Fleming, Chester et al. 1980). Although they used a nitrogen rather than an SF_6 washout system, it is not clear why they found LCI so difficult to reproduce. The inclusion of more severely affected patients in the current studies may explain the poorer performance of moment ratios, since they are limited by the TO range over which they are analysed. Slope index appears to be highly variable. Neither index is currently reported by any other group working in this field.

Because of irregularities or pauses in breathing pattern, it is possible for errors in breath identification to occur if the automatic analysis is relied upon uncritically. For this reason, each breath was inspected in turn, and phase III slope and breath parameters adjusted if necessary. In collaboration with colleagues, guidelines have been prepared for the analysis of clinical washouts; these are presented in Appendix B.

BTPS correction

The raw data signals from Innocor are not BTPS corrected, nor is this correction applied in the analysis software. Cumulative expired volume, tidal volume and FRC were therefore exported into an Excel database and multiplied by an appropriate correction factor before entry into the results database. Since inspired

SF_6 concentrations were not being measured, it was unnecessary to apply any correction to inspiratory volumes. The temperature and humidity of the expired air was measured using an apparatus originally developed in our laboratory to monitor respiratory heat and moisture loss during tidal breathing (Noble, McCafferty et al. 2007). The sensor array was positioned distal to the mouthpiece and filter, and the temperature and humidity of the expirate measured over several breaths. This was found to be reliably stable at 35^{o}C and 98% relative humidity.

Environmental conditions in the test room were maintained in a steady state by the use of air conditioning (20^{o}C and 40% relative humidity). Temperature was recorded, and adjusted for if necessary. No dynamic adjustments were made for relative humidity or barometric pressure, which were not directly measured, and were taken 40% and 760 mmHg respectively.

FRC was also adjusted for the pre-capillary deadspace of the patient interface (46ml).

Signal Speeding

Background & Methods

Gas analyser response may limit the use of Innocor at faster respiratory rates, and in particular in pre-school children who typically have respiratory rates greater than 20-30 breaths per minute. The measured signal rise time of 163ms is greater than the 120ms quoted by the manufacturers, and is slower than the maximum of 100ms recommended for MBW tests in pre-school children (Gustafsson 2005; Beydon, Davis et al. 2007). The relatively slow signal rise time has at least two physical components that are amenable to improvement. Firstly, there is spreading of the gas concentration front within the gas sample line and in the in-line oxygen analyser. Spreading in the line could be reduced by shortening the gas sample line or altering its bore, but only at the expense of affecting the ability of the line to equilibrate gas sample humidity with room air. The second component relates to the intrinsic response time of the photoacoustic gas analyser itself, and is not alterable.

In Innocor, response time can be additionally altered by removing the separate oxygen analyser that is placed in series with the photoacoustic gas analyser. However this also disables the ability of Innocor to measure the flow-gas delay (since the on-board software will not perform the analysis in the absence of an oxygen signal). This renders the process of LCI assessment far more complex, and unsuitable at this stage for multi-centre trials. An alternative method of flow gas delay calculation would resolve this problem, but would require additional hardware and software. Further investigation of these restraints on gas analyser response ideally requires adjustment of Innocor's hardware and/or embedded software. This can only be done with the aid of the manufacturers, and is now being pursued by another researcher.

As an alternative, attempts were made to digitally enhance the signal response time according to the method of Arieli et al. (Arieli and Van Liew 1981). In this paper, the authors describe a mathematical correction that can be applied to a gas signal, which has the effect reducing the 10-90% response time of the gas analyser. First and second order corrections were calculated in Excel according to the following equations:

First order correction: $C_1=C_0 + \gamma_1.dC_0/dt$

Second order correction: $C_2=C_0 + (\gamma_1 + \gamma_2).dC_0/dt + \gamma_1.\gamma_2.d^2C_0/dt^2$

Where C_0 is the original concentration output, and C_1 and C_2 are the first and second order corrected concentrations respectively. γ_1 and γ_2 are first and second order time constants, calculated from the positive reciprocal of the slope of the linear regression of ln(1-response fraction) as a function of time. This was derived empirically from step changes in SF_6 concentration generated as described previously. In order to assess the usefulness of this correction, it was then applied to a sample washout to determine the effects on calculated FRC and on signal quality at both ends of the washout.

The same paper also describes the effect on calculated gas volume of realigning the flow gas delay to correspond to 80% of the maximum increment in gas concentration signal, rather than the standard 50% maximum increment (Figure

2.12). This has the effect of increasing the flow gas delay, and reducing error in determination of FRC. However, the misalignment of the signals will cause error on integration of falling gas concentration during inspiration. If re-inspired SF_6 is not being calculated, then adjustment of the flow-gas delay to correspond to the 75% response fraction should provide a simple alternative that will not have any detrimental effect on signal quality. In order to assess the effects of such an adjustment, the 50-75% response time was calculated from the same gas concentration curves used to calculate 10-90% response time. This additional time delay was then added to the flow-gas delay calculated during the standard Innocor calibration. A sample washout was analysed using the calculated flow-gas delay, and a range of adjustments to this. Finally, the effects of adjusting the flow-gas delay on a syringe model were assessed during a simulated washout.

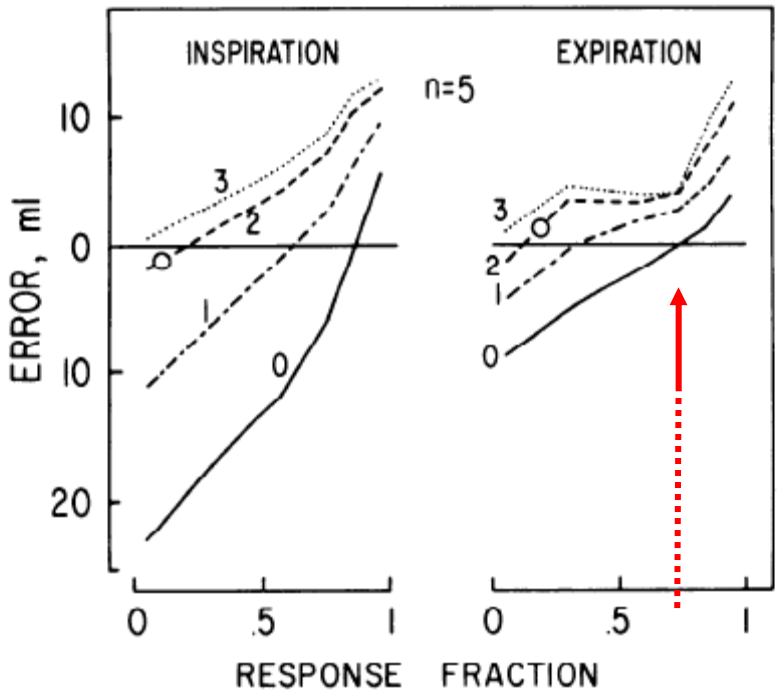


Figure 2.12: Taken from Arieli & Van Liew. J Appl Physiol **51** (6): 1417-22

Differences between calculated and measured amounts of Argon in a syringe, as a function of response fraction for which delay time is measured (i.e. a response fraction of 0.5 represents flow-gas delay measured to a 50% maximal rise in tracer concentration during a step-change in flow and gas concentration). The three curves in each graph (0-3) represent the effect of increasing correction orders (0=uncorrected, 1=first order correction etc.).

Left panel shows errors when concentration changed in inspiration only, as calculated at end inspiration, and *right panel* shows errors when concentration changed in expiration only, as calculated at end expiration. The circles represent the authors choice of correction order and response fraction, but the same accuracy can be achieved by using a response fraction of 0.8 and not applying any correction to the gas signal (red arrow). This has the advantage of being simpler to perform and does not have any deleterious effects on signal quality.

Results

First and second order Arieli corrections were applied to step changes in SF_6 concentration. The effects of these are illustrated in Figures 2.13 and 2.14. The first order correction reduced the 10-90% rise time of the SF_6 signal from 160ms to 135ms, a 16% reduction (Figure 2.14). However, the signal quality was adversely affected by these manipulations, with an overshoot in the peak SF_6 concentration and increased signal noise (Figure 2.13). Both of these effects were more pronounced in the second order corrections. In order to reduce these effects, values of γ_1 and γ_2 were adjusted to 2/3 of their calculated value, as suggested in the original paper (Arieli and Van Liew 1981). The adjusted corrections were then applied to a washout to determine the effects on signal quality and FRC. The first and last breaths of a washout performed by a normal volunteer (respiratory rate 11 breaths per min), and subjected to these corrections, is shown in Figure 2.15. Although the SF_6 concentration appears to rise faster after correction, the noise produced by the adjustment is excessive.

The effect of the 1st order correction on calculated FRC of the same washout is shown in Figure 2.16. This also illustrates the effect of adjusting the flow-gas delay to realign the signals. In this washout, the 1st order correction resulted in an increase in measured FRC of 1.8% (from 2.33 to 2.37 L) and the second order correction resulted in an increase in FRC of 3.7% (to 2.41 L). A similar effect was achieved by realigning the flow and gas signals by an adding 50ms to the flow gas delay. This resulted in a measured FRC of 2.39 L, an increase of 2.7%, and this method does not cause any deterioration in signal quality. Further adjustment of the flow-gas delay continues to increase the measured FRC. Accurate alignment of flow and gas signals is important for accurate calculation of FRC (and hence LCI), but variations of less than 25ms appear to have minimal effect on these measurements (up to 1.6%).

Because the subject's exact FRC cannot be known, and is variable with repeat washouts, this analysis was subsequently performed using a calibration syringe to deliver known volumes of gas through the system (see Part 4).

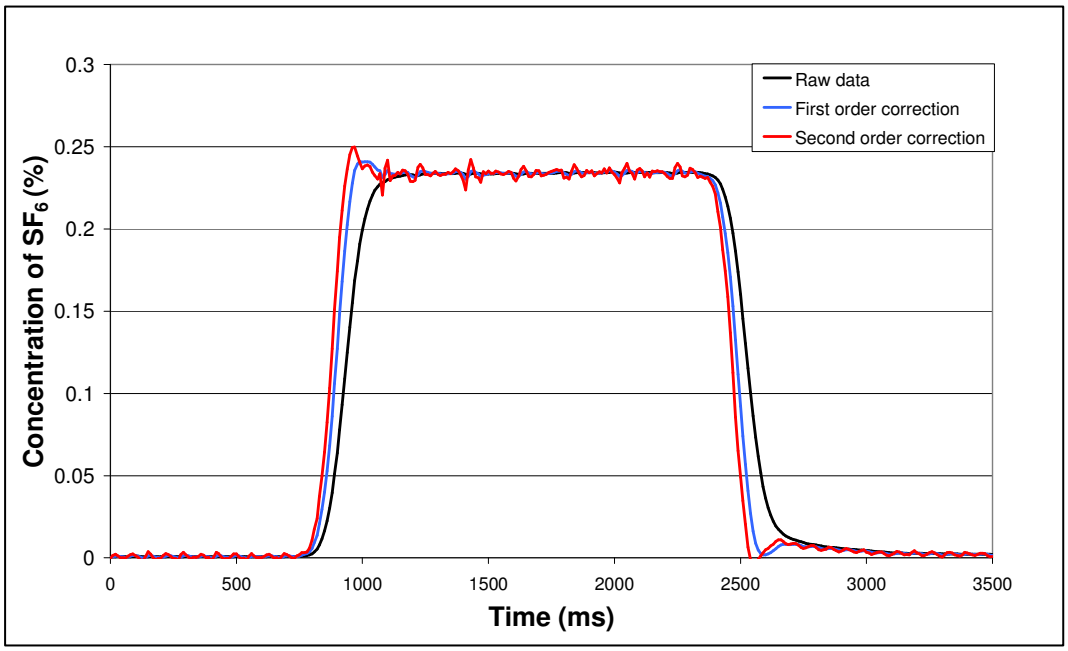


Figure 2.13: Effect of applying first and second order signal speeding corrections to a step change in SF_6 concentration, generated using a Brunner syringe. The raw SF_6 data are shown in black. The first order correction (blue) results in a steeper rise and fall in SF_6 concentration, but there is an overshoot of the gas signal and greater signal noise. These features are all exaggerated after a second signal speeding manipulation (red), with minimal additional gain in analyser response time.

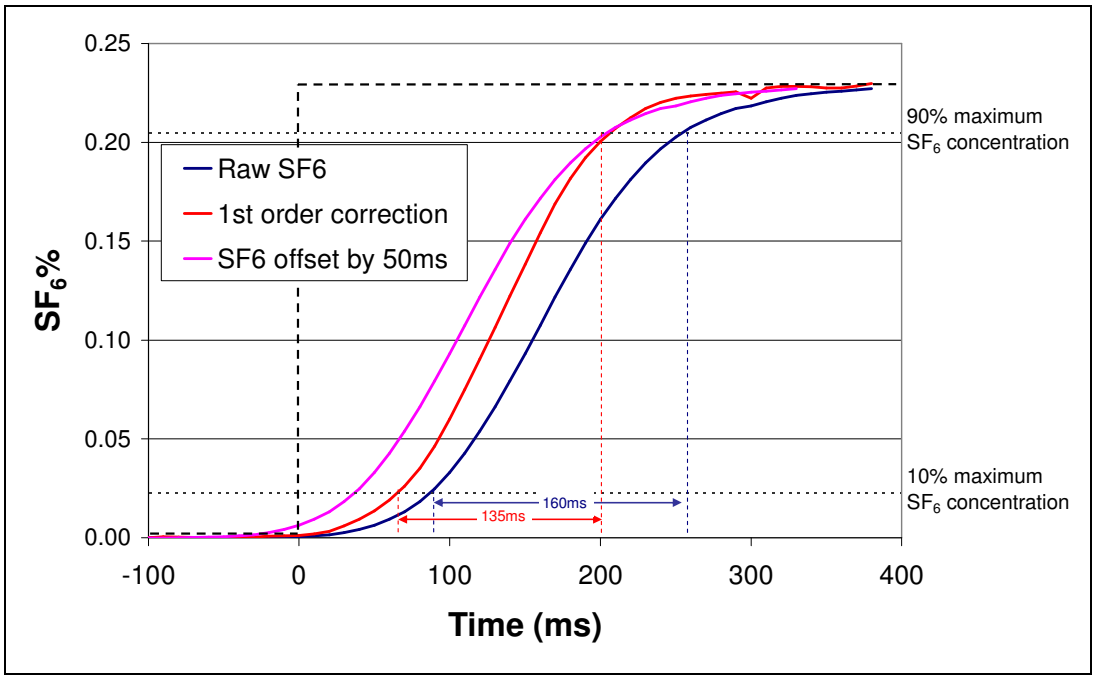


Figure 2.14: Effect of gas signal adjustments on analyser response. The simulated step change in gas concentration is shown by the black dotted line, and the Innocor gas analyser response by the solid blue line. The 10-90% rise time is also illustrated. The effect on rise time of a modified 1st order signal speeding correction is shown (red curve), as well as the effect of realigning to the 75% response fraction (purple curve). The 10-90% rise time after signal speeding correction is reduced to 135ms.

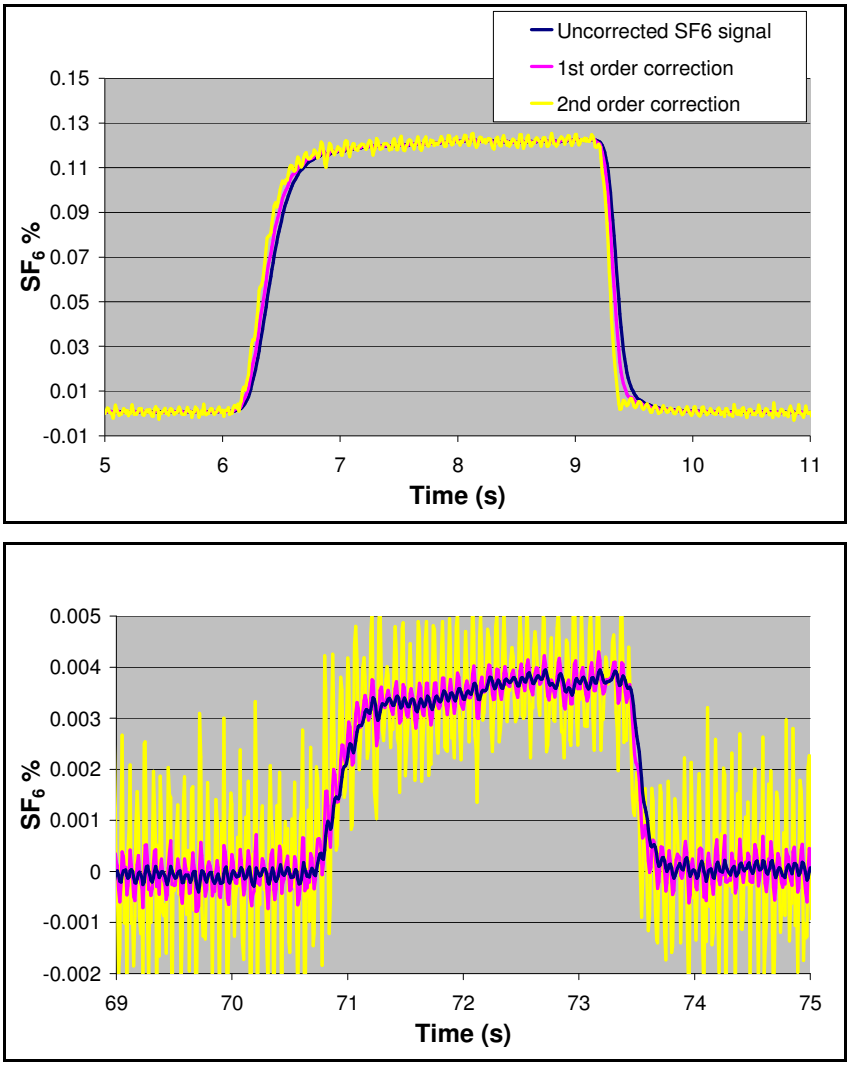


Figure 2.15: Effect of applying a gas signal speeding correction, as described by Arieli et al. (Arieli and Van Liew 1981), to a multiple breath washout of a healthy volunteer. The first and last breaths are shown in upper and lower panels respectively. The unaltered gas concentration signal is shown in blue. The pink and yellow traces represent the same gas concentration signal after first and second order signal speeding corrections respectively. The effects on signal quality are clearly shown in the lower panel, where the greater noise at low SF_6 concentrations is amplified by the correction.

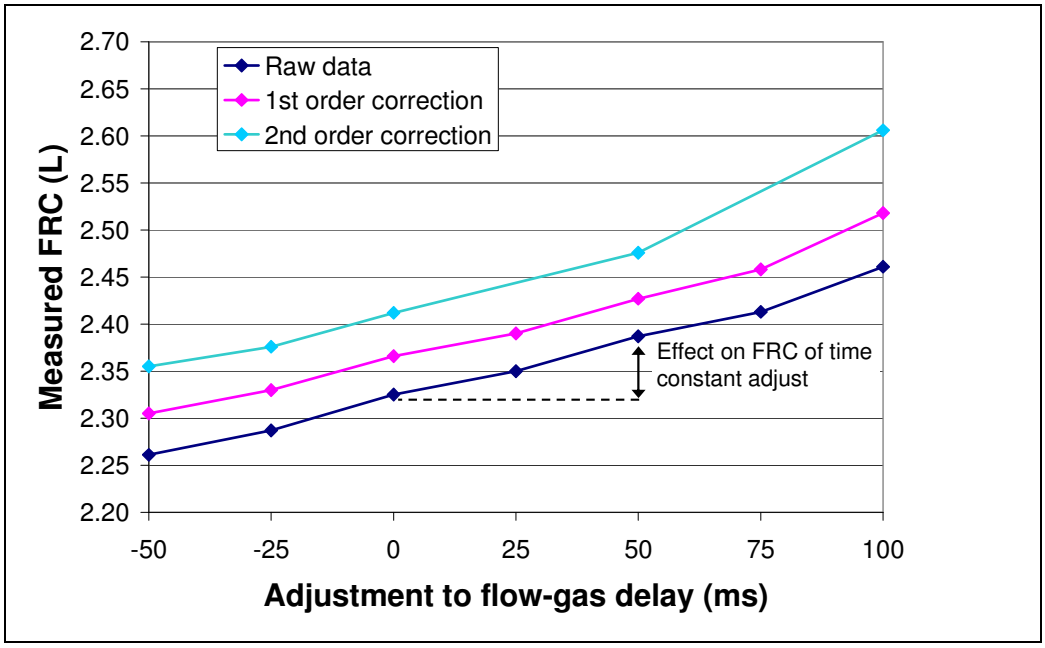


Figure 2.16: Effect of manipulating the gas concentration signal on measured FRC derived from the washout of a healthy volunteer. 1st and 2nd order corrections refer to the mathematical speeding of the SF_6 concentration signal according to the method of Arieli et al., as explained in the text. The effects of adjusting the flow-gas delay, as a simpler method of artificially speeding the gas concentration signal, are also shown.

Conclusions

Digitally enhancing Innocor gas analyser performance by applying signal speeding corrections to the gas signal results in a modest improvement in gas analyser response time, but only at the expense of signal quality. The effects on signal quality are tolerable at the start of the washout, where the signal:noise ratio is high, but are excessive by the end of the washout, and preclude the use of these corrections.

An alternative method of signal speeding has been described that does not affect signal quality. This involved the addition of a fixed time constant to the measured flow-gas delay in order to realign the flow signal with the 80% response fraction of the gas signal. Applying a similar time-shift correction to the Innocor derived washouts produces an effect on measured FRC similar to that of the digital speeding corrections. This was also seen by Bates et al., when they attempted to apply digital speeding to a MS signal using the Arieli's method. They showed that a time shift adjustment provided a ten-fold reduction in error of gas volume integration over an uncorrected signal, and was of similar accuracy to the more complex calculations required by Arieli (Bates, Prisk et al. 1983). The effects of this adjustment on accuracy of gas volume determination, and the modifying effects of respiratory rate, are explored in the next section.

2.4 - Accuracy of modified Innocor system.

Methods

The ability of the modified system to integrate flow and gas signals accurately and reproducibly was assessed using a gas calibration syringe which could be set to deliver different volumes (Hans Rudolph, Missouri, USA). This was filled with 0.2% SF_6 in air to starting volumes (FRCs) of 1 or 3L. Washouts were performed by incomplete filling and emptying of the syringe around this starting point, taking care to establish as regular and smooth a filling and emptying pattern as possible, at physiological rates. The volume of gas in the syringe was derived from the calculated "expired" volume of SF_6, using the clinical MBW analysis software, and was compared with the known starting volume of the syringe. The effects of different signal alignments on the accuracy of this integration were assessed by re-analysing the washouts with a series of adjustments to the measured flow-gas delay (-50, +25, +50 and +100ms). Because the syringe contains a substantial, but unknown, deadspace, it is not possible to know the volume of gas in the syringe at the start of the washout. Therefore the difference between mean volume in the syringe when set to deliver 1L, and mean volume when set to deliver 3L, was used to assess accuracy, since this should be measured accurately as 2L.

If the slow gas analyser response time has a deleterious effect on Innocor accuracy, it will be accentuated by fast respiratory rates. In order to investigate the effects of respiratory rate on accuracy of gas volume integration, the syringe washouts were repeated, using a different calibration syringe (Hans Rudolph, Missouri, USA) set to deliver 1L. Washouts were performed at slow and fast fill-empty rates, equivalent to slow and fast respiratory rates in a patient washout. These were analysed at the measured flow-gas delay, and after addition of a fixed time constant of 50ms.

Results

Six washouts were performed from a starting volume of 1L, and 4 from a starting volume of 3L, using a washout rate of between 15 to 30 fill-empty manoeuvres per minute. All washouts were analysed at the measured flow-gas delay

and 4 adjustments to this, corresponding to time constants of -50ms, +25ms, +50ms and +100ms. Accuracy was defined by the difference between the mean syringe volume when set to deliver 1L and when set to deliver 3L. The error +/- 95 confidence intervals, in millilitres, between these measurements is shown in Figure 2.17. A volume of 20ml represents a 1% error in gas volume integration.

The accuracy of the gas volume integration is reduced by 1.01% by the realignment of the flow and gas signals by +50ms, though the 95% confidence intervals of this error include zero.

Effect of increasing respiratory rate on accuracy

Figure 2.18 and Table 2.3 show the effect of increasing syringe fill-empty rate on volume determination. It is not possible to assess "accuracy" of volume determination per se, since the exact volume in the syringe (including the deadspace) is not known. However, it can be seen that there is little difference in the measured syringe volume at slow fill-empty rates (mirroring the conditions during adult tidal respiratory rates) after addition of 50ms to the measured flow-gas delay. Addition of the 50ms time constant causes an increase of 18ml (1.5%) in the measured gas volume. At higher rates (average 67 per min, range 54-78per min), using the uncorrected flow-gas delay, there is a much greater change in the measured syringe volume of -36ml (3.0 %). This has been presumed to be due to error in accuracy of integration of flow and gas signals and higher fill-empty rates. Use of the additional time delay method of signal speeding however corrects the measured syringe volume to that measured at slower rates. Because of the small numbers of repeats, none of these differences were statistically significant.

It can also be seen that the error caused by rapid rates is modest. Without the additional time constant, there is only a 3% fall in measured volume at rates of up to 78 per minute. Above a rate of 60 "breaths" per minute however, the software is inaccurate at identifying breaths, and this process must be carried out manually.

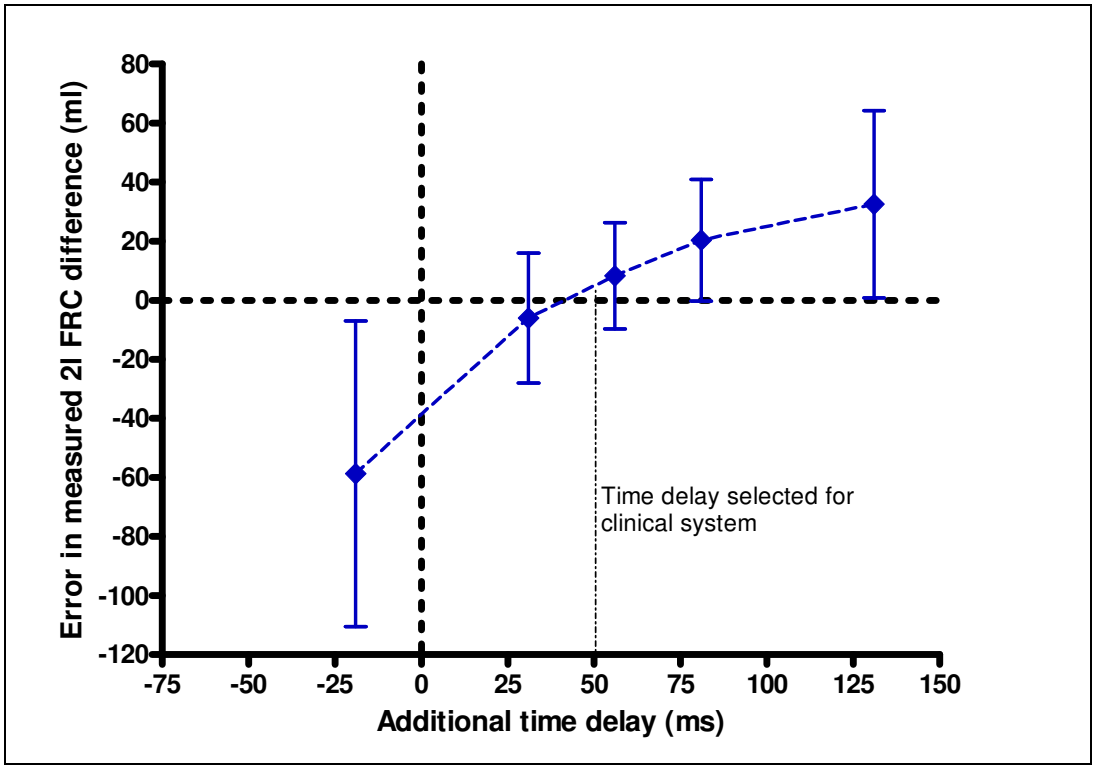


Figure 2.17: Mean and 95% confidence interval of effect of altering the measured flow-gas delay on accuracy of determination of 2000ml volume difference between syringe set at 1L and at 3L. Six washouts were performed from a starting volume of 1L and four from a starting volume of 3L.

	Additional time delay added to measured flow-gas delay	
	0ms	**50ms**
Slow rate Mean (SD) measured syringe volume (L) *Mean (range) fill-empty rate: 10 (9-12)* *Mean (SD) tidal volume: 721 (189) ml*	1.17 (0.02)	1.19 (0.02)
Fast rate Mean (SD) measured syringe volume at (L) *Mean (range) fill-empty rate: 67 (54-78)* *Mean (SD) tidal volume: 716 (111) ml*	1.13 (0.02)	1.18 (0.03)

Table 2.4: Effect of increasing empty rate and adding to measure flow-gas delay on volume determination of a calibration syringe set to 1L. The measured FRC includes 46ml of apparatus deadspace and an unknown dead space volume in the end of the syringe. This deadspace is constant and does not alter if the syringe is set at a greater starting volume.

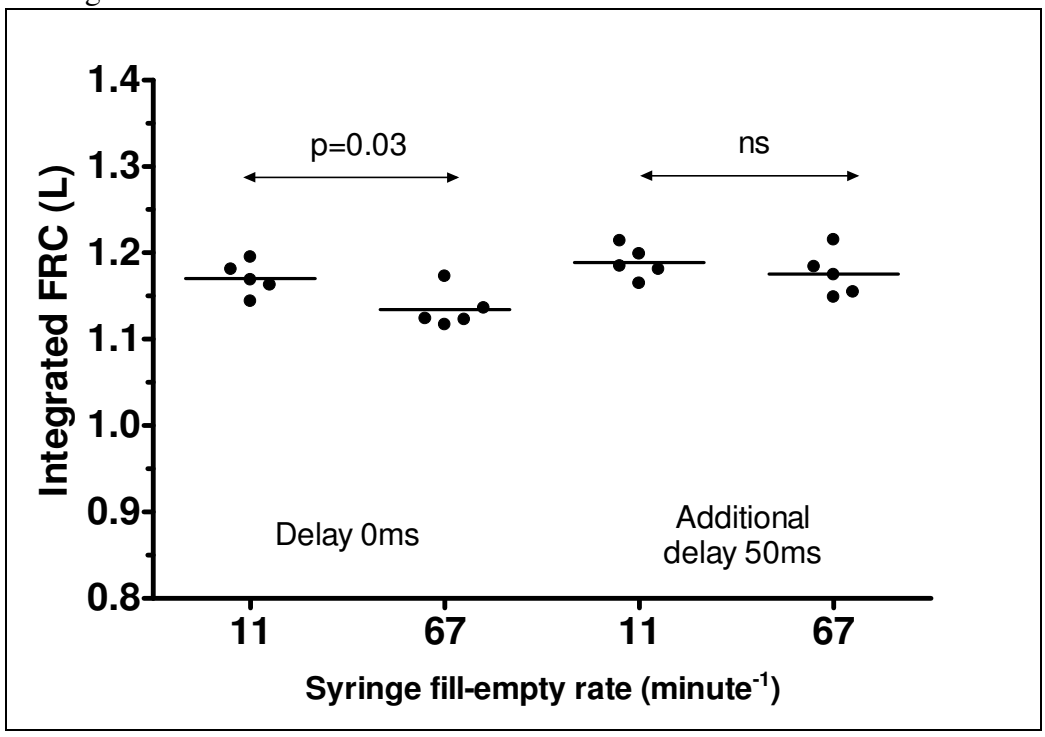


Figure 2.18: Measured syringe volume at fast and slow washout fill-empty rates, and two different flow gas delays: that measured by Innocor, and after addition of 50ms. Each point represents a separate assessment, mean is indicated by the horizontal line, significance assessed by paired t-tests.

Conclusions

The system is able to integrate expired SF_6 accurately and changes in the flow-gas delay of 10-20ms have little effect on the accuracy of volume determination at physiological "respiratory" (fill-empty) rates. The addition of a 50ms time constant increases the measured volume of the gas in the syringe, and reduces accuracy, by 1%. At faster rates however, the additional time constant increases accuracy. An additional delay of 50ms corresponds to the 50-75% gas signal rise time, and would be expected to improve accuracy as per the findings of Arieli et al. (Arieli and Van Liew 1981). However, even without any adjustment to the flow-gas delay, the effects of faster breath rates are modest, and within the 3% error of volume determination generally permitted in physiological apparatus.

The software has a default setting to correct for re-inspired SF_6, i.e. integrating the inspiratory flow and SF_6 concentrations in order to measure and account for tracer gas residing in the post-capillary deadspace at the start of inspiration. This setting was in response to the problems faced by Professor Gustafsson when performing washouts on children. In the apparatus described here, post capillary deadspace is only 5ml, and tidal volume is greater, so the possible contribution of re-inspired SF_6 is much less. The analysis software was modified to remove the automatic adjustment for re-inspired SF_6. When the re-inspired SF_6 adjustment was included, accuracy was reduced at all time points and fill-empty rates (data not shown). This adjustment has therefore been omitted from calculations in the studies presented in this thesis.

Discussion

The modifications described in this chapter are designed to permit the use of an Innocor gas analyser in assessments of multiple breath washout tests. The device lends itself well to this purpose, since it contains both a differential pressure transducer for a flowmeter, and a highly sensitive gas analyser. In order to adapt the device, a new patient interface has been constructed with a low dead space. A separate pressure transducer has also been added in order to allow dynamic feedback of expired volume. Finally, in collaboration with Professor Gustafsson, the analysis software has been reformatted and adjusted to analyse raw data generated by Innocor.

The ideal comparison would be to compare performance of both the mass spectrometer and Innocor simultaneously, as has been done for other gas analysers (Fuchs, Buess et al. 2006). However washouts would have to be performed at the operating range of the Innocor gas analyser, since the response is not linear above 0.4% SF_6. Without altering the sampling protocol, the signal resolution of the mass spectrometer shows excessive noise at this level, making such a comparison invalid; this is an unavoidable limitation of the current comparison. Since a simultaneous comparison of the two devices has not been possible, the gas analyser has instead been evaluated in terms of signal quality and response time and these features have been compared to those of a mass spectrometer used to measure LCI in children. Finally, the accuracy of the system has been confirmed, and methods explored of improving this using signal speeding manipulations.

The performance of the Innocor and MS apparatuses are compared in Table 2.5. Also included in this table are a summary of the equipment recommendations. These come from the ATS/ERS guidelines on multiple breath nitrogen washouts in adults (Wanger, Clausen et al. 2005), and from the recent ATS/ERS guidelines on pulmonary function testing in pre-school children - this also contains a section on multiple breath washout analysis (Beydon, Davis et al. 2007). Although neither of these scenarios exactly reflects the application for which the Innocor has been adapted, they do provide a useful guide to the necessary system specifications.

Characteristic	ATS/ERS guidelines for MBW in children[1]	ATS/ERS guidelines for MBNW in adults[2]	Mass Spectrometer[3]	Adapted Innocor
Deadspace	1-2ml/kg	<100ml	12.5ml*	46/36ml**
Gas sample flow	<100ml/min	N/A	20ml/min	120ml/min
10-90% response time	100 ms	<60 ms	64 ms	160 ms
Sample rate	50Hz	>40Hz	Up to 100Hz^{+}	100 Hz
Signal:noise ratio (start of washout)	>100	N/A	200	944

Table 2.5: Technical comparison of mass spectrometer and Innocor with ATS/ERS guidelines for lung clearance measurement in children and for multiple breath nitrogen washout (MBNW) in adults.

N/A – this parameter not covered in the guidelines.

* This system does not include a filter, which contributes to the majority of the deadspace in the adapted Innocor system.

** Deadspace can be reduced for children by the use of a smaller filter

$^{+}$ Sample rate can be varied depending on number of channels open

1: Beydon, Davis et al. 2007

2: Wanger, Clausen et al. 2005

3: Aurora, Kozlowska et al. 2005

Despite operating at a much lower SF_6 concentration range, Innocor has a superior signal resolution throughout the range of concentrations experienced in a washout. The resolution of the MS signal can be improved, but only at the expense of the analyzer sample rate. Accurate signal resolution is particularly important when performing analysis of phase III alveolar slopes (Verbanck, Schuermans et al. 1997) (see Chapter 5).

However, when the modified Innocor is compared to the nitrogen washout or the paediatric washout recommendations, the gas sample flow rate and the rise time of the gas signal fall short of the recommendations. Gas sample flow is particularly important with paediatric subjects, since a sample rate of 120ml/minute may represent a significant proportion of the minute ventilation and there is an additional risk of back sampling ambient air at low expiratory flows. The guidelines therefore specifically recommend that gas sample flows of greater than 100 ml/min are undesirable and that compensation for this is required.

In adults however, this is far less likely to be relevant. Adult minute ventilation is usually greater than 7 L/min, and a gas sample flow rate of 120 ml/min therefore represents a far smaller proportion of minute ventilation. Another problem of the sample rate is that, if the sample port was placed between the patient and the flowmeter, it might be sufficiently high to cause a bias-flow across the flowmeter. In order to resolve these problems, the gas sample port of the patient interface has been placed distal to the flowmeter (Figure 2.6). Air drawn in by the sample needle will follow the path of least resistance. With the distal placement of the gas sample needle described above, this will be ambient air drawn 5cm into the tube rather than air drawn through the apparatus across the flowmeter. With the sample needle placed between mouth and flowmeter, air may be drawn across the flowmeter. Observations of measured flow with the needle connected and disconnected did not identify any excess flow meter inaccuracy attributable to gas sampling.

This positioning of the gas sample line is important when using Innocor for another reason. If there were significant dead space distal to the gas sample line (post-capillary dead space), then this would be re-inspired when the patient breathed in. On expiration, this volume of gas would be counted towards the total, leading to a falsely elevated FRC. The volume of gas re-inspired can be calculated, and indeed

the analysis software has the ability to do this. However, a slow gas signal fall time leads to additional error in this process. By positioning the gas sample line at the distal end of the patient interface, with a post capillary dead space volume of less than 5ml, the volume of re-inspired SF_6 can be ignored. This simplifies the calculations, and removes the error due to the slow signal fall time.

The adverse effect of this gas sample line positioning is to increase the pre-capillary dead space to 46ml (this includes the mouthpiece, filter and flowmeter). This volume is subtracted from the derived FRC at the end of the washout, but is included in the CEV and FRC for calculation of LCI. This is an important distinction, and there has been some discussion amongst those involved in measuring gas mixing about whether to include pre-capillary deadspace in these calculations. However, they form a part of the FRC involved in gas mixing, and larger pre-capillary deadspace will alter measures of heterogeneity. In this thesis therefore all measurements of gas mixing, including Sn_{III} analysis, include the pre-capillary deadspace as part of the measurement.

It has been assumed that, with the typical tidal volumes and respiratory rates encountered in adult subjects, the positioning does not adversely affect response of the system. This assumption may not be correct in very young children, or in subjects breathing very rapidly with small breath volumes. In order to achieve this small deadspace, low volume components have been used which means relatively high resistance to flow. At tidal breathing flow rates, this is not a problem for subjects. Resistance becomes more noticeable at higher flow rates and the system would be unsuitable for use in exercise testing.

The majority of the deadspace (20ml) is due to the presence of the bacterial filter. This is not used in the mass spectrometer-based apparatus, but is necessary for the current application. By doing this, the need to dismantle and clean the flowmeter after every patient is removed, which greatly simplifies the procedure and permits increased patient throughput, an essential feature for use in clinical trials outside of the laboratory. For adult subjects, the total dead space remains well within the limits set by both sets of recommendations.

The only important compromise for Innocor compared to the MS is the gas analyser response time, which is slower than that recommended for use in pre-Air

drthat gas analyser response times slower than 100ms will introduce error into the monitoring of gas concentration changes at fast respiratory rates. Support for this comes from a paper by Tang et al. (Tang, Turner et al. 2005). In this study, the authors looked at the effects on the derivation of Fowler dead space (DS) (Fowler 1948) of varying the rise time, and flow-gas signal alignment, of a computer generated CO_2 expirogram. Since the expirogram was generated by a computer model of the lung, they were also able to investigate the effects of expiratory time constant. The computer modelling was supplemented by real data from ventilated patients. The authors showed that there is a linear relationship between 10-90% rise time and error in determination of Fowler DS. This is affected by the expiratory time constant, and increases exponentially when this falls below about 0.8 (equivalent to a respiratory rate of 25 per minute). It was also shown however that mis-alignment of the flow-gas delay has a greater effect on accuracy, and that this too rises exponentially with a time constant of less than 0.8.

This paper provides comprehensive support both for a fast rise time when integrating flow and gas signals, and for accurate flow-gas delay correction. However, in the scenarios described, rise time was a theoretical 0ms when assessing signal alignment, and it is likely that the effects of signal alignment will be less profound when the rise time is slower. What the authors also demonstrated was that a re-alignment of the flow-gas signals, as described in this chapter, corrects for the error of the rise time.

An earlier study by Schena et al. (Schena, Thompson et al. 1984) also considered the effects of rise time on capnogram. They altered the rise time in a mechanical lung model by varying the gas sample tubing length and gas sample flow rate. They found that increasing rise time shifted the capnogram to the right, and that predicted end-tidal CO_2 values could not be achieved if the rise time was excessive compared to the respiratory cycle time. However, in the example they quote, the rise time is 0.94 seconds, and the respiratory rate is 60 per minute, both of which are considerably in excess of the circumstances in which Innocor has been employed in the present studies.

Neither study presents any evidence for the use of 100ms as a definitive cut off value for signal rise time in all circumstances. Both demonstrate that errors caused

by rise time are increased at fast respiratory rates, and the selection of 100ms as a cut-off by the guidelines' authors appears to have been a pragmatic choice of upper limit in order to reduce error in subjects with known fast respiratory rates (i.e. infants) (Beydon, Davis et al. 2007). In adults however, with respiratory rates of typically 10-20 per minute, the effects of rise time are likely to be far less significant, and a 10-90% rise time of 160 ms should not by itself be a contra-indication to the use of Innocor for this purpose.

In order to investigate this, the accuracy of the system was assessed using a calibration syringe as a single compartment lung model. Different volumes of gas were then washed out at different fill-empty ("respiratory") rates to assess the accuracy of the system at faster breathing rates. The system can accurately and reproducibly determine the volume of gas in the syringe at washout fill-empty rates similar to those experienced during adult tidal breathing. At faster fill-empty rates, error is introduced into determination of gas volume, presumably as a result of lag in the gas signal. This error is modest (3%), but confirms the assertion that analyser response time will impact on accuracy at faster respiratory rates (Tang, Turner et al. 2005; Beydon, Davis et al. 2007).

In order to improve this, methods of digitally speeding the gas signal have also been explored, according to the method described by Arieli et al. (Arieli and Van Liew 1981). Although this results in a modest improvement in rise time, the signal quality suffered excessively and that the signal:noise ratio at the end of the washout was unacceptable. As an alternative to this, the flow signal has been realigned with the 75% response fraction of the gas signal by the addition of a fixed time constant to the calculated flow-gas delay, a device that has previously been shown to provide similar accuracy to more complex mathematical manipulations (Bates, Prisk et al. 1983). This approach is facilitated by omitting calculation of the re-inspired SF_6 volume, as discussed above. If re-inspired SF_6 was included, different time constants for inspiratory and expiratory flow would be needed in order to avoid excessive mal-alignment and additional slowing of analyser response during inspiration. This approach improves the accuracy of integration of expired SF_6 volumes at higher respiratory rates but has little effect at the lower rates normally encountered in adult washouts. Indeed, the major limitation at rates above 60 breaths/min is that the

ability of the analysis software to automatically identify respiratory cycle change is diminished. The current set-up falls short of the recommendations for MBW apparatus in pre-school children, but in school age children (>5 yrs) and adults Innocor should be capable of accurately calculating FRC.

There are alternative methods of improving analyser response time, including adjustment of gas sample flow rate and removing the oxygen analyser from the system. Initial investigation of these methods has established that they cannot be effected without the assistance of the manufacturers - work that is now ongoing and will be the subject of a separate thesis.

The final system has been constructed with the intent that it be suitable for multi-centre use and relatively high patient throughput. In these regards it differs from apparatus previously used in single centre research studies. The use of a MS to measure LCI by following changes in exogenous SF_6 is now well described in children (Aurora, Kozlowska et al. 2005; Gustafsson 2005), and is probably the accepted gold standard technique in this population. The MS offers the additional advantage that it can measure a wider range of different gases, which is a useful option when measuring vital capacity single breath washouts (Gronkvist, Emery et al. 2002). However, the MS is an expensive, temperamental and bulky piece of equipment that cannot readily be taken out of the laboratory. In contrast, Innocor contains both the gas analyzer and the pneumotachograph in a single portable unit. Indeed, Innocor has already been used (in its original capacity to measure cardiac output) at Everest base-camp (Ghofrani, Reichenberger et al. 2004). This advantage should not be underestimated: if the measurement of LCI is ever to achieve widespread usage outside of specialised units, then the size, robustness and cost of the apparatus are critical. Without substantial technical support, mass spectrometers are likely to prove more difficult to use in multi-centre studies. A supply of SF_6 is required for both systems, but the concentration required for Innocor is $1/20^{th}$ of that used in the MS washouts, which reduces both gas wastage and costs.

The disadvantages of Innocor are the high gas sample flow rate and slow analyser response time. As discussed above, the system has been modified to

account for the effects of both these aspects and accurate of gas volume determination has been demonstrated.

There are a number of assumptions inherent in the final clinical system:

1. Flow gas delay is accurate, and does not change appreciably during the washout.
2. Pre-capillary deadspace (46ml) does not have any significant effects on gas mixing.
3. Because the post-capillary deadspace is minimal (5ml), the amount of SF_6 re-inspired is negligible and does not need to be accounted for in the calculation of total volume of expired SF_6.
4. Resistance to tidal breathing is insufficient to have any significant effect on FRC or gas mixing.
5. The effects of changing SF_6 concentration on expirate viscosity are negligible, and no dynamic adjustment is required.

These assumptions are reasonable for use of the apparatus in adult subjects and older children. Because of the small breath volumes and rapid respiratory rates encountered in pre-school children, they may not be valid in this population.

The clinical use of the system described here is the subject of subsequent chapters.

Chapter 3 - Measurement of LCI in CF patients and healthy controls

Introduction

This chapter concerns the measurement of lung clearance index (LCI) using the apparatus described in Chapter 2. Previous studies have established the feasibility and utility of multiple breath washout (MBW) for measuring CF lung disease in exclusively paediatric populations (Gustafsson, Aurora et al. 2003; Aurora, Gustafsson et al. 2004; Aurora, Bush et al. 2005; Kraemer, Blum et al. 2005). The sensitivity to early airways disease, and ability to perform the manoeuvre in children too young to co-operate with spirometry, have driven the development of LCI measurements in children. There is less obvious need for a sensitive measure of early CF lung disease in adults. However, in the specific context of CF gene therapy, LCI offers the possibility of monitoring lung disease and subtle responses to treatment in milder CF patients, and also of being a more specific marker of small airways disease than conventional lung function assessments. The hypothesis behind this chapter was that LCI would be more sensitive in adults than FEV_1 to lung disease in CF.

The aims of this chapter are to:

1. Establish the feasibility of LCI measurements using the modified Innocor apparatus.
2. Establish a normal range in healthy adults.
3. Compare LCI in CF patients with healthy adults.
4. Investigate the associations between FEV_1, FEF_{25-75} and LCI.
5. Investigate the association of LCI with other markers of clinical severity.
6. Assess the inter and intra-visit reproducibility of the technique

Methods

Subjects

Cystic fibrosis patients were recruited from those attending the Scottish Adult CF Service, based at the Western General Hospital in Edinburgh. In order to maximise the numbers and quality of the data, the results presented here are an amalgamation of data from three separate studies. The majority of subjects were recruited for measurement of LCI whilst attending routine clinic appointments. A number of these patients were recruited at the time of their annual review, which also includes an assessment of full lung function. These additional data are also described in more detail in Chapter 5. Nine patients are drawn from those recruited to a study looking at the effects of physiotherapy on LCI (Chapter 6). This involved two assessments of LCI – the first (baseline) measurement is equivalent to the one-off assessments in the other study. Finally, 10 patients are drawn from those recruited to the UK CFGT consortium Tracking study. This involves three longitudinal assessments of LCI over the course of treatment for a pulmonary exacerbation of CF (described in detail in Chapter 4). Data taken from this study are from the final visit that patients made. Ideally this represents an assessment of LCI at full recovery, though in practice not all patients attended for all visits.

There is bound to be some variability in the clinical state of patients, as in any cross section of CF patients. Unstable or acutely unwell patients were not recruited and a summary of clinical condition was noted for each patient visit. However, since the aim of the chapter is to explore cross-sectional relationships between different lung function measurements, no additional attempt has been made to control for clinical condition

A number of subjects were recruited to more than one study and underwent assessment of LCI on more than one occasion. Wherever possible the most recent measurement of LCI has been selected. The exceptions to this are if there was poor reproducibility of LCI within-day repeats at the later visit or if there were missing data (e.g. mid-expiratory flow data are not available for all subjects).

It should be noted that a deliberate attempt was made to recruit subjects from the least affected end of the severity spectrum; in other words those with the least impairment of FEV_1 were targeted specifically. Furthermore, from initial observations it was clear that those with the most severe lung disease, and most impairment in FEV_1, were also least suitable for this test. This is because both wash-in and wash-out phases of the test were prolonged, making the test more burdensome for the patient. The subjects recruited do not therefore represent an un-biased cross section of the patients attending the Scottish Adult CF Service.

Healthy volunteers were recruited from amongst colleagues at the Molecular Medicine Centre and Department of Respiratory Medicine. Healthy volunteers were defined as non-smokers with less than 10 pack years smoking history, with no active lung disease, and taking no regular respiratory medications.

All three of these studies were approved by the Lothian Research and Ethics committee and all patients and controls provided informed written consent.

The majority of the healthy volunteer data, and a large proportion of the CF patient data, were included in a paper describing the development and use of Innocor to measure LCI (Horsley, Gustafsson et al. 2008).

Washouts in subjects under 16 yrs

In order to explore the change in LCI with age, data are included on LCI in healthy children, aged 5-16 yrs. These washouts were performed at the Royal Hospital for Sick Children in Edinburgh by Dr Kenny Macleod. Although the washouts were performed by Dr Macleod, the apparatus was configured to be compatible with the foregoing validation work (see Chapter 2), and the analysis used was that developed above for use in adults. The only additional modification for use in children was that a smaller filter was used (9070/01, Air Safety Ltd, Morecambe, UK), which reduced the pre-capillary deadspace to 36ml. Data analysis was also performed by Dr Macleod, using the same analysis protocol as used in adults, (described in Appendix B), and are presented here with his permission.

Healthy paediatric controls were recruited at the Royal Hospital for Sick Children from those attending for follow-up of stable upper-limb fractures as well as children of hospital staff. Subjects had no history of recurrent wheeze, pertussis or

tuberculosis; no previous diagnosis of asthma or previous use of asthma medication; no prematurity (<34 completed weeks gestation); no neuromuscular weakness or bone disease likely to affect respiration; and no congenital cardiac disease requiring medication. This study was approved by the Lothian Research and Ethics Committee. Parents of children provided informed, written consent and children provided assent or written consent, depending on age and understanding.

Multiple breath washout

Washout tests were performed with the subject seated and suitably distracted by watching television. A noseclip was applied and tidal breathing established whilst the subject breathed through a mouthpiece attached to a filter and flowmeter. This was connected via a T-piece connector (Intersurgical, Berkshire, UK) to a flowpast circuit consisting of 22mm disposable plastic anaesthetic tubing (Intersurgical, Berkshire, UK) with a reservoir bag on the upstream (gas supply side) of the circuit.

The first part of the test is the wash-in, during which the subject breathes 0.2% SF_6 in air from the flow-past circuit. This is supplied from a compressed gas cylinder, with the flow rate adjusted to ensure that rebreathing does not occur, confirmed by visual inspection of the gas concentration tracing - usually greater than 12-14 L/min. During the wash-in, SF_6 concentration and flow signals are displayed on the Innocor screen by selecting the option “Show online data” from the “Show results” menu (see Appendix A for full details). A 1 second average of SF_6 concentration is displayed alongside the graph of changing gas concentration versus time. In addition, an average expired tidal volume is calculated and displayed by Innocor, though there is no real-time tidal volume display. A separate display of expired volume is obtained on a separate laptop screen, as described in Chapter 2 (Section 2.iv).

During the first part of the wash-in, subjects were allowed to establish a comfortable breathing pattern. They were closely observed for evidence of air leak around the mouthpiece. Subjects unused to respiratory testing occasionally found

the mouthpiece and / or resistance of the system unusual at first. It commonly took up to 1 minute for some subjects to establish a comfortable breathing pattern. Once this had occurred, a paper arrow was affixed to the laptop screen at a position corresponding to the subject's own tidal volume in order to aid expiratory volume reproducibility. Occasionally subjects continued to find the test difficult after the first minute, and breathed either with excessively shallow or deep breaths. If this occured, visual feedback of expired volume was used, along with verbal encouragement, to aid the subject in achieving a comfortable breathing pattern with a tidal volume of between 500 ml and 1 litre. During the test, subjects were encouraged to relax and to watch TV. Visual feedback was provided on an additional screen, and used to aid a regular relaxed breathing pattern, particularly in those who initially found this difficult. Both screens were visible to the patient in the same field of vision.

The wash-in phase was continued for at least 4 minutes in adults, and in all cases until inspiratory and expiratory SF_6 concentrations differed by less than 0.004% (absolute difference in SF_6 concentration). In normal adults, wash-in was usually complete after 3-4 minutes. In patients with mild-moderate CF, wash-in typically required 5-7 minutes of tidal breathing. In one early assessment in a subject with severe CF lung disease, wash-in was incomplete after 12 minutes. These data are not included here. Aside from this, wash-in was not continued beyond 10 minutes in any subjects.

Once wash-in was deemed complete, based upon observation of the gas concentration versus time plot and the average SF_6 concentration displayed on the graph, the flowpast circuit was manually detached during expiration by removing the T-piece from the flowmeter. The subject was warned that this was going to happen beforehand, and the process was demonstrated to the subject before the start of the test to familiarise them with the manoeuvre. Just before removal of the T-piece, a stream of air was directed over the end of the flowmeter using a fan, to ensure that expired SF_6 was blown away and not re-inspired.

During the washout the subject breathes room air until the end tidal SF_6 concentration had fallen to less than 0.005% (1/40th of the SF_6 concentration during wash-in). Because of the imprecision of the Innocor SF_6 concentration display, it is

difficult to identify this point precisely. Washouts were therefore continued for several breaths beyond this point to ensure that this has been achieved successfully.

Washout analysis

Each subject completed three wash-outs. Data were extracted from Innocor's hard disc and analysed after all three manoeuvres had been completed. If there was a clear problem with a washout test during the procedure (e.g. air leak, interruptions to breathing pattern), then the subject was asked to repeat it. Occasionally problems were only apparent during detailed analysis. Since these washouts could not be repeated at this stage, this necessitated excluding them from the analysis. As an additional quality control measure, washouts where the FRC differed by >10% from both of the other two repeats were also excluded. LCI is quoted as the mean of at least two reproducible repeats from washouts of satisfactory quality. Criteria for a satisfactory washout test include:

1. Adequate wash-in, i.e. inspiratory and expiratory SF_6 concentrations were within 2% of each other.
2. No obvious air leak.
3. Acceptably regular breathing pattern. Considerable variation in breath volume or frequency can still be analysed, but if excessive this makes the analysis very difficult and poorly reproducible.
4. FRC within 10% of the other repeats.

Occasional pauses in respiration, swallows and coughs were not a reason for exclusion of a washout on their own, but if excessively frequent could impair accuracy of FRC and LCI determination.

A standardised operating procedure for the conduct of a washout test is given in Appendix A. Detailed instructions on data analysis and interpretation are presented in Appendix B.

Spirometry

Spirometry was measured according to American Thoracic Society / European Respiratory Society guidelines (Miller, Hankinson et al. 2005) using either an Easyone spirometer (ndd Medizintechnik AG, Zurich, Switzerland) or a Vitalograph 2120 (Vitalograph, Buckingham, UK). Predicted values for FEV_1 and mid-expiratory flows are those provided by the European Community for Coal and Steel (adults≥17 yrs) (Quanjer, Tammeling et al. 1993) and Rosenthal et al. (children≤16 yrs) (Rosenthal, Bain et al. 1993). Three reproducible measures were required for a satisfactory result. The best of the three manoeuvres, defined as the result with the greatest sum of FEV_1 and FVC, was recorded. Measurements were performed without a noseclip.

Statistical analysis

Data were analysed using Prism (GraphPad Software Inc, CA, USA). Results are quoted as mean (SD) unless otherwise stated. Within test repeatability for LCI was determined by calculating the coefficient of variation (CV) as 100 x SD x $mean^{-1}$. Inter-visit reproducibility was assessed using the Bland-Altman technique (Bland and Altman 1986). Numerical values for different measures of lung function were assessed for normality using the D'Agostino and Pearson Omnibus normality test. If both parameters were normally distributed, means were compared using unpaired t-test. If either parameter was not normally distributed then means were compared using a Mann-Whitney U test. For comparison of parameters across three or more groups, ANOVA or Kruskal-Wallis ANOVA was used, depending on whether data were normally distributed. The 95% limits of normality for LCI were calculated as mean +/- 1.96 x residual standard deviations. A *p* value of below 0.05 was considered as statistically significant.

Results

53 healthy adult volunteers were enrolled for measurement of LCI. One of these was subsequently excluded because he was unable to establish a regular and relaxed breathing pattern. Washout data were collected from 50 adult CF patients. An additional patient with CF was enrolled but was unable to perform the test satisfactorily and declined to complete three washouts. Demographics and main outcome data for the two groups of subjects are given in Table 3.1.

The proportion of male CF subjects was greater than that of adult male healthy volunteers (56% vs. 40%), though this was not statistically significant (Pearson Chi Squared test, p=0.115). The median age of the healthy volunteers was significantly higher than that of CF patients; 31.5 vs 24 yrs, p=0.0026 (Mann-Whitney U test). This reflects both the reduced age of the CF population generally, and also a deliberate attempt to recruit healthy volunteers over a wide age range.

	Healthy Volunteers (age≥17yrs)	CF patients	Difference (95% CI)
Number	52	50	
Male / Female	21 / 31	28 / 22	
Median Age yrs [Range]	31.5 [19-58]	24 [17-49]	7.5*
Mean (SD) FEV_1 % predicted [Range]	102 (12) [73-133]	66 (21) [21-111]	-35** (-42 to -29)
Mean (SD) FEV_1/FVC (%) [Range]	84 (6) [71-95]	65 (12) [35-87]	-18** (-22 to -15)
Mean (SD) FEF_{25-75} % predicted [Range]	117 (26) [83-174]	34 (21) [6-94]	-83** (-95 to -71)
Mean LCI (SD) [Range]	6.7 (0.4) [6.0-7.8]	13.0 (3.5) [6.3-20.4]	6.3** (5.4 to 6.3)
Mean CV% LCI (SD)	3.6 (2.1)	4.6 (2.5)	1.0^{+} (0.1 to 1.9)

Table 3.1: Demographics, spirometry and lung clearance index of adult healthy volunteers and cystic fibrosis patients.

LCI = lung clearance index

CV% = Coefficient of variation (%) for intra-visit repeats.

* p=0.0026 compared to healthy controls (Mann-Whitney U test)

** p<0.0001 compared to healthy controls (unpaired T-test)

$^{+}$ p=0.047 compared to healthy controls (Mann-Whitney U test)

Spirometry

The mean (SD) FEV_1 was significantly different between the two groups, being 102 (12) % predicted in healthy controls versus 68 (23) % predicted in CF patients, $p<0.0001$.

Subjects with CF also had significantly lower FEF_{25-75} % predicted than healthy controls (34 versus 117 % predicted, $p<0.0001$). This is traditionally taken as a more sensitive measure of small airways disease than FEV_1 alone.

A single normal volunteer had an FEV_1 of 73% predicted. This subject was a 29 yr old female, with no respiratory history or symptoms, who struggled to complete the spirometry assessment adequately on the occasion quoted. On a previous assessment she had achieved an FEV_1 within the normal range.

LCI in normal subjects

There was a narrow normal range of LCI in adults of 5.95 – 7.43. This was independent of age, gender or height of the subjects.

Change in LCI with age

Data on LCI in subjects under 16 yrs, derived using identical apparatus and protocols, has been provided by Dr Kenny Macleod for this analysis. A summary of the additional subjects included in this analysis is given in Table 3.2.

Figure 3.1 shows the relationship between LCI and age for the combined paediatric and adult cohorts (min 5 yrs, max 58 yrs). In those subjects over the age of 18 yrs there was no relationship between LCI and age. When the cohort of subjects under the age of 17 yrs was combined with these data, there was a weak but statistically significant correlation with age (Pearson $r^2=0.11$, $p=0.0026$). The small dependence of LCI on age is best summarised by a normal range (95% limits of normality) in adults of 5.95-7.43 and in children (≤16 yrs) of 5.36-7.37. A weak relationship between height and LCI in the combined cohorts disappeared on multiple regression analysis. By contrast, FEV_1 varied between 64 and 133% predicted in the same group of 82 healthy adults and children.

Child healthy volunteers *(age≤16 yrs)*	
Number	30
Male / Female	18 / 12
Median Age yrs [Range]	12 [5-16]
Mean (SD) FEV_1 % predicted [Range]	91 (12) [64-117]
Mean LCI (SD) [Range]	6.4 (0.5) [5.4 – 7.4]

Table 3.2: Demographics, spirometry and LCI of healthy child volunteers (age 5-16 yrs).

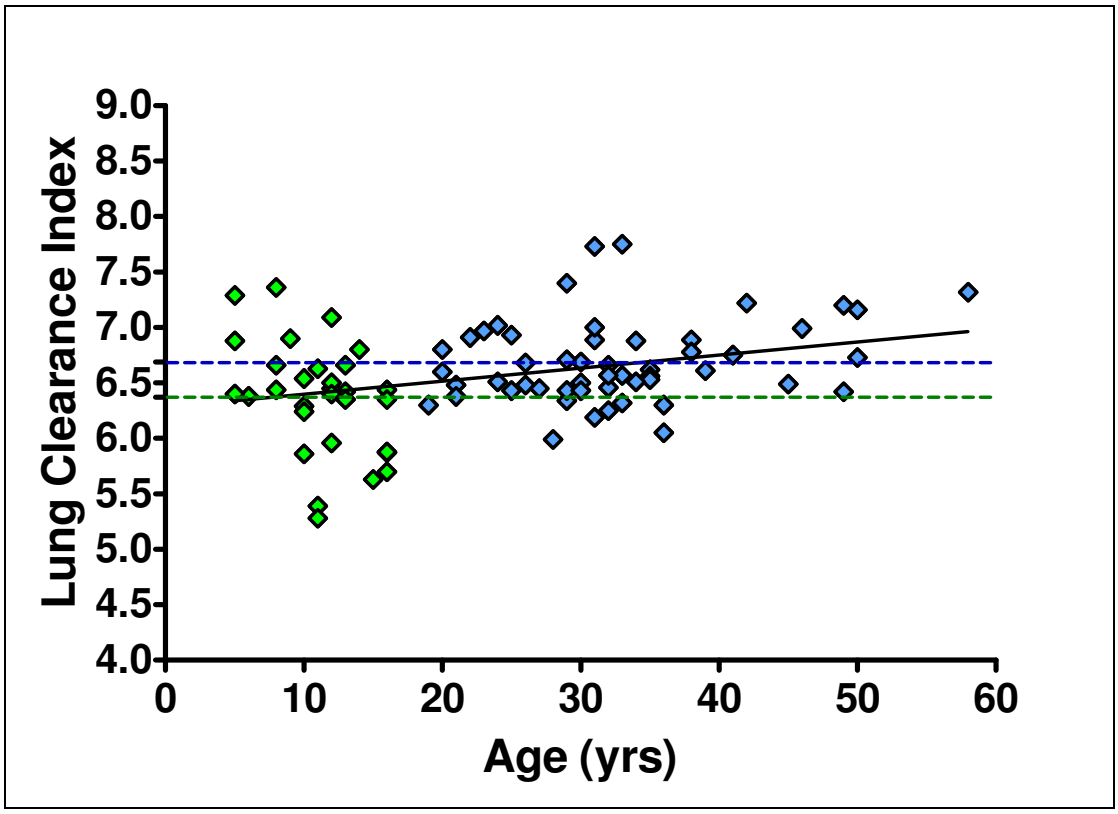


Figure 3.1: Effect of age on LCI. In adults (blue) there is no significant correlation between age and LCI, which remains stable over the age range of 17-58 yrs. The normal range is wider, and lower in children ≤16 yrs (green), which results in a weak but statistically significant correlation of LCI with age over the range 5 to 58 yrs, shown by the solid black line (r^2=0.11, p=0.0026). The means of the two populations (>17 yrs and ≤16 yrs) are represented by the blue and green horizontal dotted lines respectively.

LCI in patients with cystic fibrosis

The group mean (SD) [range] for CF patients was 13.0 (3.5) [6.3-20.4], $p<0.0001$ compared to 6.7 (0.4) in healthy controls. Figure 3.2 shows the relationship between FEV_1 % predicted and LCI for healthy control and CF adults. Unlike in healthy controls, where LCI was restricted to a narrow range, in CF patients LCI increased with reducing FEV_1 % predicted (Pearson $r^2=0.64$, $p<0.0001$).

There were 12 CF patients with $FEV_1 \geq 80\%$ predicted; all but one had LCI greater than the upper limit of normal. These subjects are represented in Figure 3.2 by those CF subjects (red symbols) plotted on the right of the vertical dotted line. A summary of these subjects is presented in Table 3.3. Although for two of these subjects the LCI was only just above the upper limit of normal, it can be seen that even in those with FEV_1 % predicted well within the normal range (>90% predicted), this does not exclude the presence of substantial gas mixing abnormalities. By contrast LCI was marginally elevated in only two healthy adults (measuring 7.7 and 7.8), consistent with the use of 95% confidence limits to define the normal range. The sensitivity of LCI for detecting CF was 98%, versus 76% for FEV_1.

LCI was also compared to FEF_{25-75} % predicted (Figure 3.3). There were fewer data points available for this assay (n=37 CF subjects and 25 healthy volunteers). There was only a single CF subject with FEF_{25-75} greater than 80% predicted and this subject had an elevated LCI (8.62), and an FEV_1 of 98% predicted. There was a significant correlation between FEF_{25-75} percent predicted and LCI: Pearson $r^2=0.71$, $p<0.0001$. Based on these data, the sensitivity of FEF_{25-75} for detecting CF was 96%, versus 100% for LCI in the same population. In healthy volunteers the range of FEF_{25-75} percent predicted varied from 83 to 174 %.

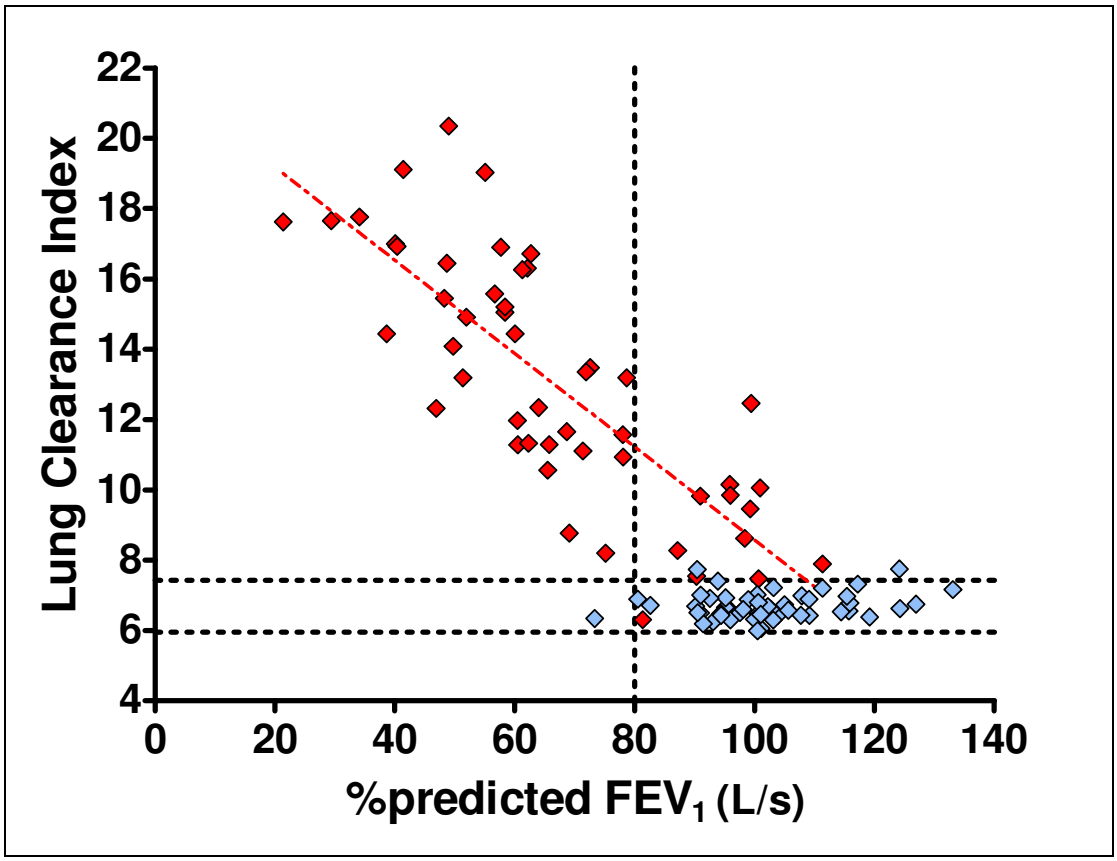


Figure 3.2: Lung clearance index (LCI) v FEV_1 % predicted for adult healthy volunteers (blue) and patients with cystic fibrosis (red). The horizontal dotted lines represent the 95% limits of normality of LCI, calculated from the healthy adult population The vertical dotted line represents the lower limit of normal for % predicted FEV_1. Linear regression of FEV_1 % predicted versus LCI is shown for CF patients by the red dotted line; Pearson r^2=0.64, p<0.0001.

Age	Gender	Genotype	Genotype severity class	FEV_1 % predicted	LCI
20	M	ΔF508 / 4096-7A→G	IV	81.4	6.3
26	F	ΔF508 / 711+3A→G	IV	87.2	8.3
39	F	ΔF508 / D1152H	IV	90.4	7.5
30	M	ΔF508 / G551D	III	91.0	9.8
41	F	ΔF508 / A455E	V	95.9	10.2
23	M	ΔF508 / ?	?	96.0	9.9
18	F	ΔF508 / ?	?	98.4	8.6
27	F	1585-1G→A / P67L	IV	99.3	9.5
20	M	ΔF508 / ΔF508	II	99.4	12.5
22	M	G542X / A455E	V	100.7	7.5
33	M	ΔF508 / ?	?	100.9	10.1
49	M	ΔF508 / 5T	V	111.3	7.9

Table 3.3: Summary of the characteristics of the 12 cystic fibrosis patients with FEV_1 % predicted within the normal range (≥80% predicted). Only a single subject had an LCI within the normal range (less than 7.4).

Severity class of CF mutation is based upon the class of the non-ΔF508 mutation, as described by Welsh et al. (Welsh and Smith 1993), and described in more detail in Chapter 1.

"?" = mutation unidentified after extended testing. Diagnosis based upon a combination of clinical features and sweat testing.

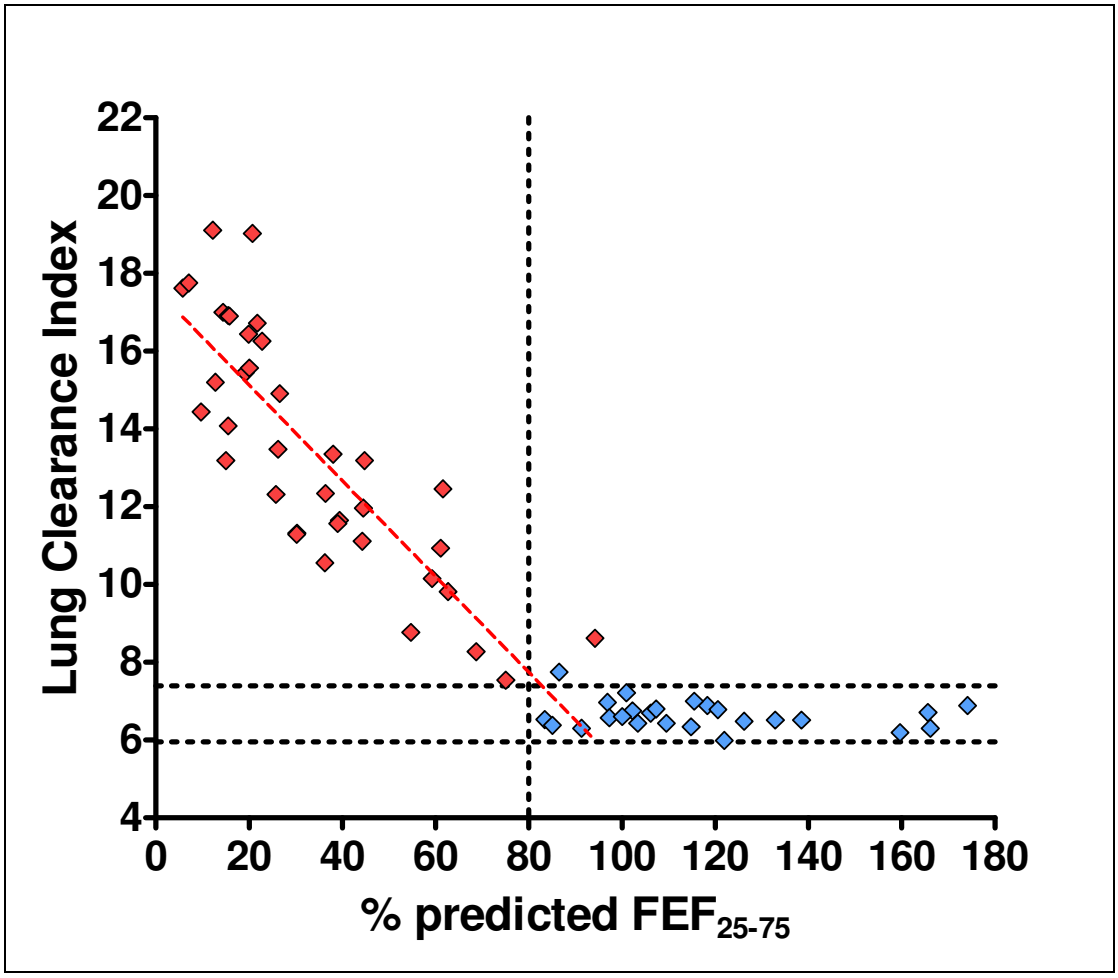


Figure 3.3: Lung clearance index (LCI) v FEF_{25-75} % predicted for adult healthy volunteers (blue) and patients with cystic fibrosis (red). The horizontal dotted lines represent the 95% limits of normality of LCI, calculated from the healthy adult population. The vertical dotted line represents the lower limit of normal for % predicted FEF_{25-75}. Linear regression of % predicted FEF_{25-75} and LCI is shown by the red dotted line; Pearson r^2=0.71, p<0.0001.

Applicability of LCI technique in normal subjects and patients

A washout test was excluded if the measured FRC differed by ≥10% from both of the other two washouts. This is in line with current recommendations on measurement of FRC by washout (Wanger, Clausen et al. 2005), and provides a built-in quality control of washout performance and analysis. In adult subjects, this resulted in the exclusion of a total of 6 tests (2 from controls, 4 from patients), representing less than 2% of the total number of repeats from both healthy volunteers and CF patients. All three washout repeats from an additional single adult healthy volunteer could not be analysed because the subject was unable to achieve a regular and reproducible breathing pattern. Two additional washout repeats, one from a healthy volunteer and one from a CF patient, were also excluded because of evidence of air-leak during wash-in that had only become apparent on formal data analysis. Finally, a single washout repeat from a CF patient was excluded because irregularities in breathing pattern made it impossible to analyse accurately.

Repeatability of washout at same visit

After exclusion of washout repeats that failed to meet quality control, the mean (SD) intra-subject coefficient of variation (CV) for FRC derived from repeat washout manoeuvres on the same visit was 3.2 (2.0) % for adult healthy volunteers, and 3.6 (2.3) % for CF patients. The mean (SD) CV for LCI was 3.6 (2.1) % for healthy adults, and 4.6 (2.5) % for CF patients. There was no significant correlation between the LCI CV and FEV_1 % predicted.

If the 6 washout repeats excluded on the grounds of reproducibility were included, mean CV for FRC increased to 3.5% for healthy volunteers and 4.1% for CF patients. Mean CV for LCI increased to 3.7% for healthy volunteers and increased to 5.1% for CF.

Since three washout repeats were performed, it is not possible to perform a Bland-Altman analysis that contains all three repeats in a single analysis (Bland and Altman 1986). If a learning effect was significant however, this would be most pronounced between the first and final washouts. Bland-Altman analysis was

performed on all pairs of washouts for healthy controls and CF patients (i.e. washout 1 vs 2, 2 vs 3 and 1 vs 3), but only the graphs of washout 1 vs 3 are presented here (Figure 3.4). Repeatability of washouts in healthy controls was acceptable, with no evidence of a learning effect. Inter-test reproducibility of LCI for individual washouts on the same day was between 0.68 and 0.88 (mean of 0.77), or around 10%.

Repeatability in CF patients was considerably poorer (mean inter-test reproducibility of 1.99), but this was skewed by some big differences between repeats in those with higher LCI (Figure 3.4b). Although there was no evidence of a learning effect, it is clear that LCI was less reproducible in subjects in whom it was very abnormal. This feature was consistent regardless of the pair of washout repeats selected for comparison. These are also the subjects in whom the test is most difficult to perform, since protracted wash-out and wash-in are required, which can become uncomfortable.

Inter-visit reproducibility of washout in healthy adults

Repeat measurements of LCI were performed in triplicate on 16 healthy volunteers after an average (SD) of 36 (40) days. A Bland-Altman plot of the difference between repeat measures versus the mean of the measurements is shown for FRC in Figure 3.5 and for LCI in Figure 3.6.

For FRC, the 95% limits of agreement between the two measurements were -0.43 to 0.45 L. For LCI, the 95% limits of agreement for the two measurements were -0.78 to 0.46. Thus the inter-visit reproducibility of the FRC measurement was around 400 ml and that of the LCI measurement was 0.6. This is similar to, but slightly better than, the mean intra-visit reproducibility.

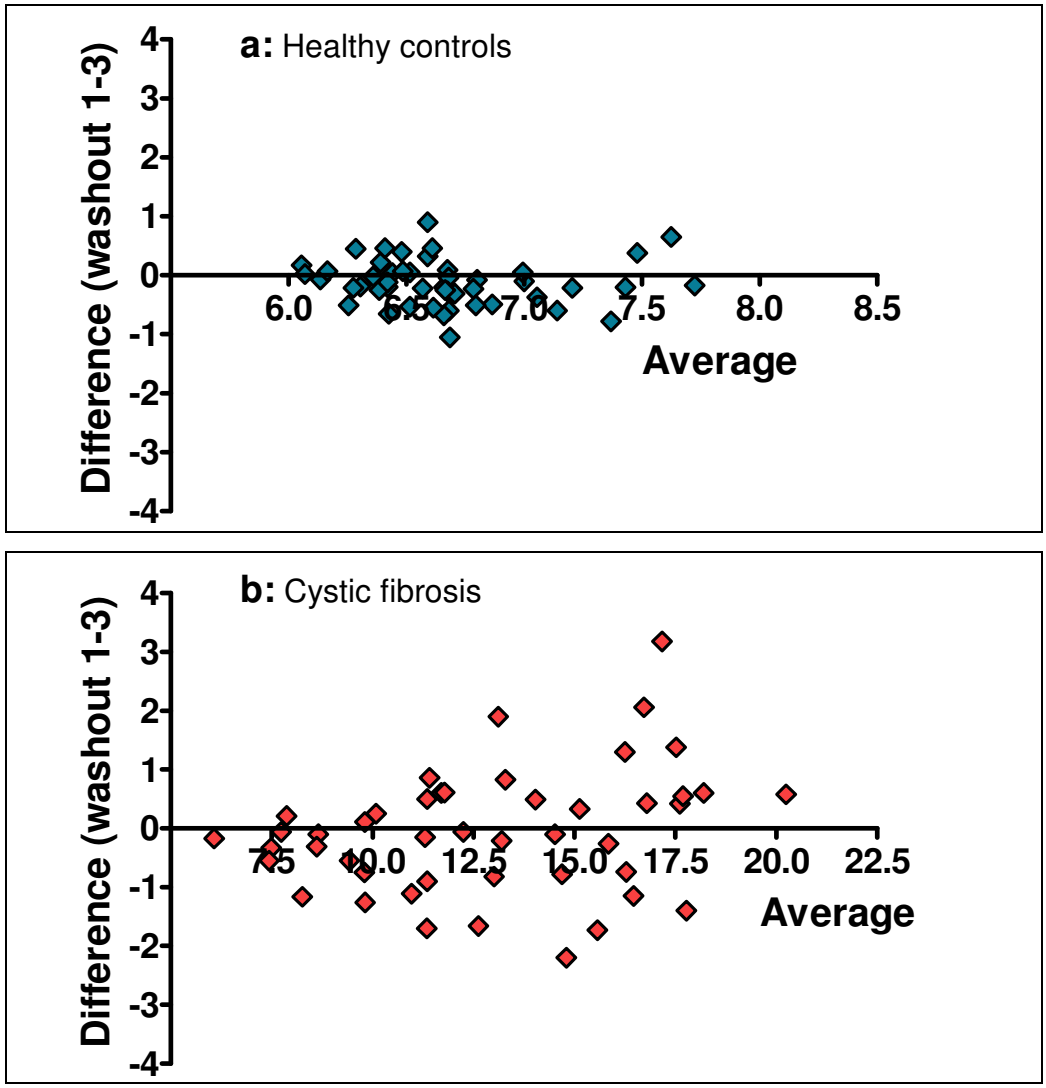


Figure 3.4: Bland-Altman plots of lung clearance index for first and third washout repeats for (a) healthy controls and (b) patients with cystic fibrosis. There is no evidence of a learning effect, and repeatability for healthy controls is both acceptable and consistent. However, repeatability for CF patients deteriorates in those with most severely abnormal gas mixing, and highest LCI.

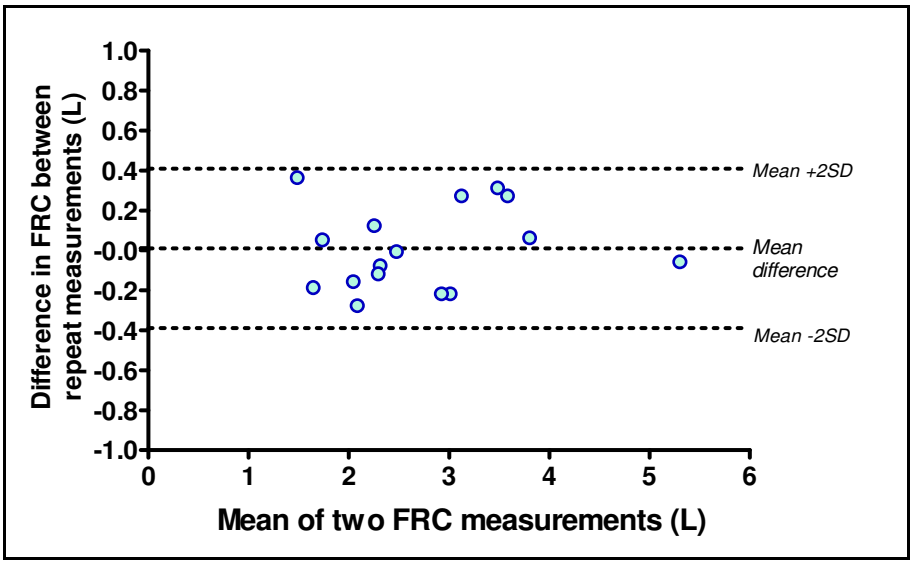


Figure 3.5: Bland-Altman plot of difference between functional residual capacity (FRC) measured on two separate occasions (quoted as mean of triplicate repeats), and mean of the two measurements of FRC. Horizontal dotted lines represent the mean and 95% limits of agreement of the difference between measurements on the two occasions.

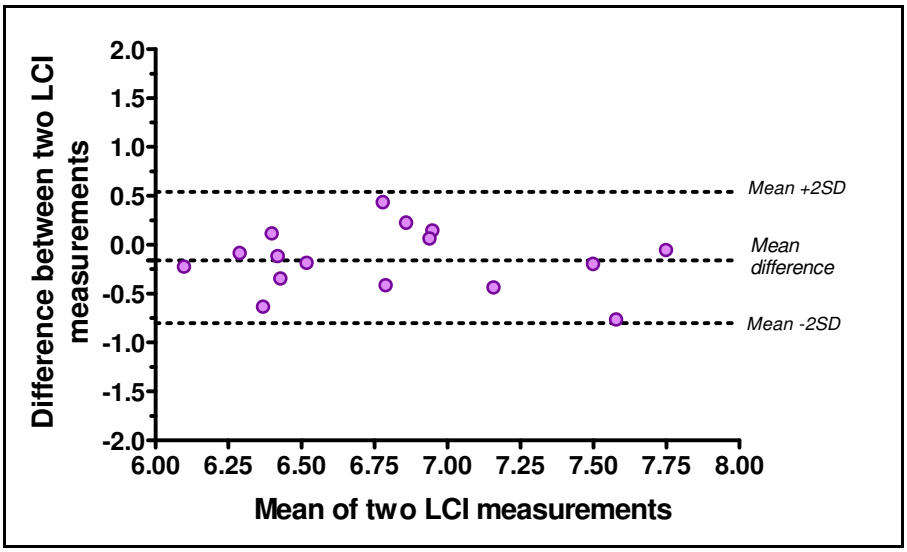


Figure 3.6: Bland-Altman plot of difference between lung clearance index (LCI) measured on two separate occasions (quoted as mean of triplicate repeats), and mean of the two measurements of LCI. Horizontal dotted lines represent the mean and 95% limits of agreement of the difference between measurements on the two occasions.

Discussion

It has been demonstrated in this chapter that clinical measurement of inert gas washout is practical using the modified Innocor device. It has also been shown for the first time that, in adults with CF, a simple measure of ventilation heterogeneity is more sensitive than spirometry in detecting lung function abnormalities. Finally, it has been shown that measurement of LCI is reproducible both within and between visits, and that there is little change over a wide range of subject age.

Ventilation heterogeneity is thought to be altered by small airways dysfunction (Cosio, Ghezzo et al. 1978; Verbanck, Schuermans et al. 2004). Measurements of ventilation heterogeneity should therefore reflect the earliest pathology in CF - as has already been shown in children (Gustafsson, Aurora et al. 2003; Aurora, Gustafsson et al. 2004; Aurora, Bush et al. 2005). This is also the region of the lungs which is likely to be a key target for gene therapy. LCI therefore offers the ability to measure dysfunction in the airways of interest, and also to extend the range of patients suitable for these assessments.

The preceding chapter concerned the technical characteristics of the Innocor device and a method of modifying it so that it was able to perform inert gas multiple breath washouts. In this chapter, the clinical application of the device has been demonstrated. In addition, a second modified Innocor device has been established at the Royal Hospital for Sick Children in Edinburgh, in order to perform MBW tests in children. Standardised procedures for the conduct of washout tests and for data analysis have also been developed (Appendix A & B). The data on LCI in subjects under 16 yrs were collected using this second device, and standardised protocols. In doing so, the feasibility of using this technique and equipment in a multi-centre setting has also been demonstrated. This is the first time that MBW measurements have been performed using the Innocor gas analyser, and the first time that LCI data have been collected simultaneously at two different sites. Although Paul Aurora and Per Gustafsson have been involved in paediatric studies in Gothenburg and London using identical mass spectrometer-based equipment, these studies were performed and published sequentially (Gustafsson, Aurora et al. 2003; Aurora, Gustafsson et al. 2004).

Furthermore, these are the first data on LCI in CF adults; previous studies have only reported measurements in subjects only up to 19 yrs. Even in adult patients with normal spirometry, the LCI may be markedly elevated, indicating significant "silent" lung damage. Some of the patients with normal FEV_1 gave no symptoms and were on no treatment, the diagnosis of CF having been made incidentally. Yet despite this, there was abnormal gas mixing in almost all cases. There is a risk that the extent of lung disease in such patients may be underestimated and hence under-treated.

LCI has been reported in small numbers of adult subjects in several studies, but only a single study has been identified where large numbers of healthy non-smokers have been assessed (Orzalesi, Hart et al. 1965). This study employed nitrogen washouts and reported a mean (SD) LCI in 26 adults (>15 yrs) of 7.1 (1.3). The mean LCI is very similar to that reported here (6.7) but the wider range of normal LCI in the earlier study may reflect both the smaller numbers and the presence of some smokers within the cohort. This study also found LCI to be unaffected by gender or age of subjects. An earlier study reported a more elevated LCI in 80 adult males, ranging from 9.0 to 10.0 depending on age (24-65 yrs) (Bouhuys 1963). This study did not control for smoking however and also reported a positive association between smoking history and LCI in older subjects.

The mean (SD) LCI in normal subjects determined here is also very similar to that reported in the literature in children and adolescents (Table 3.5). In preschool children (mean age 4) this has been reported as 6.9 (0.4) (Aurora, Bush et al. 2005), and in school age children (mean age 11 yrs) as 6.5 (0.5) (Aurora, Gustafsson et al. 2004) and 6.3 (0.4) (Gustafsson, Aurora et al. 2003) in two different populations from UK and Sweden respectively (Figure 1.8). The range for LCI reported here is almost identical to those reported previously in children of the same age. It can also be seen that LCI is remarkably consistent across different populations and technology.

There was a weak, albeit statistically significant, rise in LCI with age. The clinical significance of this is unclear, since the magnitude of the difference (over a 53 year age range) remains very small and is less than inter-visit reproducibility. This is in contrast to the paper by Orzalesi et al., who did not find any significant

Reference	Age range (yrs)	Number of subjects	Mean LCI (SD)
Current study, adult volunteers	19-58	52	6.69 (0.38)
(Orzalesi, Hart et al. 1965)	15-45	26	7.1 (1.3)
	8-15	20	7.0 (1.0)
Current study, paediatric controls	5-16	30	6.37 (0.51)
(Gustafsson, Aurora et al. 2003)	4-18	28	6.33 (0.43)
(Aurora, Gustafsson et al. 2004)	6-16	33	6.45 (0.49)
(Aurora, Bush et al. 2005)	2-6	37	6.89 (0.44)

Table 3.5: Summary of previously published mean and standard deviation of lung clearance index (LCI) in healthy control populations. Data from this chapter are included for comparison.

difference between mean LCI in children (8-15 yrs) and adults (Orzalesi, Hart et al. 1965). Serial deadspace is known to affect LCI in infants and neonates (Schmalisch, Proquitte et al. 2006). However, the change in normal values of LCI was in the opposite direction to that which would be caused by a greater dead space/tidal volume ratio (as found in infants). It is possible therefore that this represents a true effect of age on lung elasticity and hence gas mixing. This is not unexpected, since it is known that mid-expiratory flows can be diminished in those over the age of 60 yrs, even in healthy non-smokers (Fowler, Pluck et al. 1987), and it would not be unreasonable to expect some impairment of airways function prior to this. The age related change in LCI is small however, and this supports the use of LCI in long term follow-up studies. By contrast, there is a wide range of "normal" for FEV_1 and FEF_{25-75} % predicted, which are influenced by the choice and accuracy of the normal range selected (Roca, Burgos et al. 1998). It has been shown that LCI may be affected by large changes in tidal volume, respiratory rate or FRC (Bouhuys, Lichtneckert et al. 1961; Gronkvist, Bergsten et al. 2002). In the present studies, tidal volume feedback was used to control tidal volume and respiratory rate within a range which should not affect the result. Since LCI is a ratio of cumulative expired volume and FRC, it is independent of small changes in FRC over the physiological range. This is supported by the reproducibility of LCI and the narrow range of LCI in healthy controls found in the current study. Furthermore because it is normalised for FRC, differences due to physical size are already accounted for. The normal range of LCI is therefore unaffected by age, height or gender of subject, leaving only the effects of gas mixing.

This is especially important for longitudinal studies, particularly in children. Since spirometry changes with age, height and gender, it is normally expressed as a percent predicted. But this means accepting a wide range of FEV_1 which would be considered "normal" for any individual, and the equations most commonly used to determine normal range change in late teens (Quanjer, Tammeling et al. 1993; Rosenthal, Bain et al. 1993). Use of different prediction equations for "normality" can have profound effects on the measured rate of decline in "percent predicted" values for spirometry (Merkus, Tiddens et al. 2002). Normal LCI however remains unchanged, allowing any deviation to be easily identified. This is a major advantage

of LCI over spirometry, particularly in adolescents. It is a significant challenge to accurately predict the "normal" FEV_1 in CF patients during the adolescent growth spurt. This is often delayed in CF, and it is insufficient to base the choice of prediction equations on age alone.

The entire assessment, comprising three washout repeats, typically took around 20-30 minutes for healthy volunteers, but 30-45 minutes for CF patients. This is considerably more burdensome than spirometry alone, and likely to place some limitation on the routine use of washout tests. Assessment time could be shortened by performing only two repeats, but this would require fast and accurate online data analysis to inspect the washout tracings and confirm acceptable reproducibility and quality. This is not possible with the current apparatus.

Over 90% of adult subjects were able to complete all three washout manoeuvres without difficulty and generate reproducible measurements of FRC and LCI. Even for CF patients, the whole process (wash-in and wash-out) usually took little more than 10 minutes, and considerably less in children. Despite the relatively uncontrolled conditions, the mean CV for repeat FRC is similar to that described in the literature, which varies from 3.5-6.7% for plethysmography and 4.9-10.4% for helium dilution (Hankinson, Stocks et al. 1998). The mean CV for LCI is also better than that described in children (Aurora, Gustafsson et al. 2004). To a certain extent, this level of reproducibility represents a "best case scenario", since washouts were excluded if they were poorly reproducible. In addition, some subjects attended on more than one occasion, and would therefore be more experienced with the technique than patients not previously exposed to it. There is a tendency for those with more severely affected lungs to tolerate the prolonged wash-in less well and generate less reproducible data, and this can be seen in Figure 3.4b. Reproducibility of LCI is affected by the magnitude of the abnormality. This may be intrinsic to the measurement error of the system, but it may also be a feature of the patients in whom LCI is highest. Dry air from the cylinder can cause discomfort and cough in some subjects with plentiful mucus. Those with the most severely affected lungs, and most elevated LCI, are more likely to shift mucus within and between washouts, which may change the distribution of ventilation (Mentore, Froh et al. 2005). The variability in repeat LCI measurements in these subjects could thus be a real effect

of changing ventilation distribution in severely damaged mucus-filled lungs, rather than an artefact of the measurement. In these patients, LCI is not an ideal measure of lung physiology, and its' main advantage is in those with far less severely affected lungs. Nonetheless, the technique was found to be well tolerated and reproducible in the majority of subjects.

Repeat measurements of LCI at a separate time point, in a cohort of the adult healthy volunteers, also demonstrate good inter-visit reproducibility of 0.6. This equates to less than 9% of the mean LCI, and is similar to the intra-visit reproducibility. Data on reproducibility of LCI in healthy volunteers have not been presented previously. Because of the nature of CF lung disease, and the difficulty in defining stability, it is beyond the scope of this thesis to assess long term reproducibility in patients with CF. Some data on short term reproducibility have been collected as part of a study looking at the short term effects of physiotherapy, and these are presented in Chapter 6. When assessing the significance of a change in LCI it is reasonable to take 0.6 as the level of a significant difference between two different measurements when LCI is within or close to the normal range. It is however likely that this will overestimate the significance of changes in those with more elevated LCI.

From Figure 3.3, it would appear that FEF_{25-75} is almost as good a measure of airways dysfunction as LCI, with a similar sensitivity (96% vs. 100%). FEF_{25-75} is undoubtedly a quicker manoeuvre to perform than LCI, and can be performed on most standard spirometry equipment. However, it is known that these tests are dependent upon patient effort, and are poorly reproducible both within and between visits (Timonen, Randell et al. 1997). Data on the repeat measures of FEF_{25-75} were not collected in this study, but LCI was shown to have good within and between visit reproducibility. In addition, there is a narrow range of normal LCI, across a wide age range. The normal range of FEF_{25-75} in the same control subjects ranged from 83 to 174% predicted, and the normal ranges are poorly defined in different age groups (Timonen, Randell et al. 1997). LCI thus appears to be a potentially more sensitive measure than FEF_{25-75} with the added advantages of better reproducibility and more well defined normal range. Further work is required to more clearly define the advantages of each measurement in comparison to the other.

In summary, it has been demonstrated that a robust and compact gas analyser can be used to measure LCI and that this can be applied in a multi-centre setting. In adults with mild CF lung disease, LCI is a more sensitive indicator of lung pathology. This is likely to be particularly important as improved genotyping permits the identification of subjects with milder mutations. It also offers the possibility of measuring abnormality, and hence improvement, in subjects with milder CF lung disease in clinical trials of gene therapy.

The following chapters include the longitudinal assessment of LCI over the course of an exacerbation in CF patients, and the assessment of LCI before and after physiotherapy.

Chapter 4 - Change in physiological, functional and structural assessments of CF lung function over the course of an exacerbation.

Introduction

As has been discussed in Chapter 1, a major challenge of the cystic fibrosis (CF) gene therapy programme has been how to measure the clinical response to treatment. Ideally this would involve demonstrating an improvement in either lung function, survival or another clinically relevant outcome (e.g. exacerbation rate). With improvements in the standard medical management of CF have come great advances in patient wellbeing. The falling rate of decline in lung function and exacerbation rate, and improvements in survival, mean that these parameters are no longer appropriate end points for the majority of studies (Davis, Byard et al. 1997). This problem is exacerbated in the context of gene therapy studies, where there is a particular interest in delivering gene therapy to those with reasonably well preserved lung function in order to maximise transfection efficiency. Almost by definition, this subgroup of the CF population tends to have least in terms of symptoms and annual lung function decline.

Surrogate clinical endpoints can be used to measure improvement in clinical status in trials, and the important features of any new trial endpoints are summarised in Table 4.1. The preceding chapters have dealt with the practicalities of performing MBW measurements, and with the sensitivity and reproducibility of LCI. There are at least two further aspects of LCI measurements however that have not yet been addressed, and which are essential if the measurement is to find a role in the assessment of lung function after gene therapy. Firstly, LCI must respond in some way to an intervention known to improve the underlying pathology. For gene therapy, this ideally means that LCI would respond to an intervention, in a stable population, that is known to improve survival or quality of life. In CF, there are no such "gold standard" interventions that are not already widely adopted, and it would be unethical to withhold them for these

Feature
Non-invasive and simple for the patient to perform
Devoid of harmful or unpleasant side-effects
Practical, with standardized equipment and interpretation
Reproducible (with minimal error and variability)
Sensitive
Biologically relevant
Stable, or behaves predictably, over time
Changes with disease status

Table 4.1: Features of biomarkers for use as endpoints in therapeutic trials in cystic fibrosis, after Rosenfeld (Rosenfeld 2007).

purposes. An alternative to this is to investigate how LCI, and other biomarkers, change in unwell patients as they are treated for an exacerbation. This is clearly not entirely analogous to the situation in which gene therapy is used, but provides an opportunity to investigate how biomarkers improve with therapy, how different assays correlate with other, and also provides data that can help to inform power calculations for the use of assays as trial endpoints. Biomarkers with relevance to disease status in CF would be expected to improve in the majority of patients. On the other hand, biomarkers that are poorly reproducible or do not change with treatment of an exacerbation, are less likely to be appropriate gene therapy trial endpoints.

The second important aspect of this investigation is that it allows the UK CF Gene Therapy Consortium (UKCFGT) to assess the feasibility of performing MBW measurements in multiple trial centres. These are all important issues that have not been addressed previously for MBW tests.

In this study a number of assays, including the best understood biomarkers from the literature, as well as novel markers developed in-house, have been brought together. The study was initiated by the UKCFGT consortium and involved recruitment and assessment of patients at three sites: the Western General Hospital and the Royal Hospital for Sick Children in Edinburgh, and the Royal Brompton Hospital in London. This chapter only includes the assessments performed in Edinburgh, and only includes established markers of function or inflammation. Although more samples were collected than those presented here, they are the subject of other researchers' investigations. The assays that will be discussed in this chapter are summarised in Table 4.2.

The aims of this chapter are to:

1. Assess the change in LCI with treatment of an exacerbation
2. Explore relevant correlations between the different markers of disease activity, including LCI, spirometry, symptoms, inflammation and HRCT appearances of the lung.

Although not addressed specifically in this analysis, this study has also permitted assessment of the practicality of performing these assays in a multi-centre study.

Assay	Advantages	Disadvantages
Lung Clearance Index[1,2]	• Sensitive • Reproducible	• Requires specialised, custom-built apparatus
HRCT chest[3,4]	• Sensitive • Provides information about lung structure	• Radiation exposure • Cost
Haematology and biochemistry, incl. CRP	• Simple • Widely available	• Less relevant to pulmonary disease
Sputum IL-8[5,6]	• Easy for patient if expectorating • Biological relevance (airway inflammation)	• One of many sputum cytokines, with complex interactions • Reproducibility • Not all patients produce sputum easily • Previously showed no change with treatment[7]
Sputum differential and total cell counts	• As above	• Previously showed no change with treatment[7]

Table 4.2: Assays selected for inclusion.

1: Aurora, Gustafsson et al. 2004, Horsley, Gustafsson et al. 2008 3: Nasr, Gordon et al. 2006. 4: Aziz, Davies et al. 2007. 5: Mayer-Hamblett, Aitken et al. 2007. 6: Sagel, Chmiel et al. 2007. 7: Downey, Brockbank et al. 2007.

Methods

Subjects

Twenty one patients were recruited in Edinburgh; 19 of these were from the Scottish Adult CF Service, based at the Western General Hospital, and 2 patients were recruited from the Edinburgh Paediatric CF Service, based at the Department for Respiratory and Sleep medicine at the Royal Hospital for Sick Children.

Patients were recruited when they were started on intravenous (i.v.) antibiotic therapy for treatment of an exacerbation, as assessed by their usual clinical team and based upon a combination of symptoms and spirometry. Subjects over 10 yrs old, both inpatients and those receiving home i.v. therapy, were eligible. Patients were excluded if their FEV_1 at the time of exacerbation was less than 30% predicted. Additional exclusions were patients who were on systemic corticosteroids at study entry or in the preceding month, subjects who were pregnant or breastfeeding, and those who had undergone lung transplantation.

Study protocol

Subjects completed a number of non-invasive assessments of disease activity in a fixed order at three separate time points (see Figure 4.1):

1- Within 48 hours of commencing iv antibiotics for a pulmonary exacerbation.
2 - Within 5 days of completion of antibiotic therapy
3- On full recovery, originally intended to be between 2 and 8 weeks later, but requiring the patient to have been off treatment for at least 2 weeks.

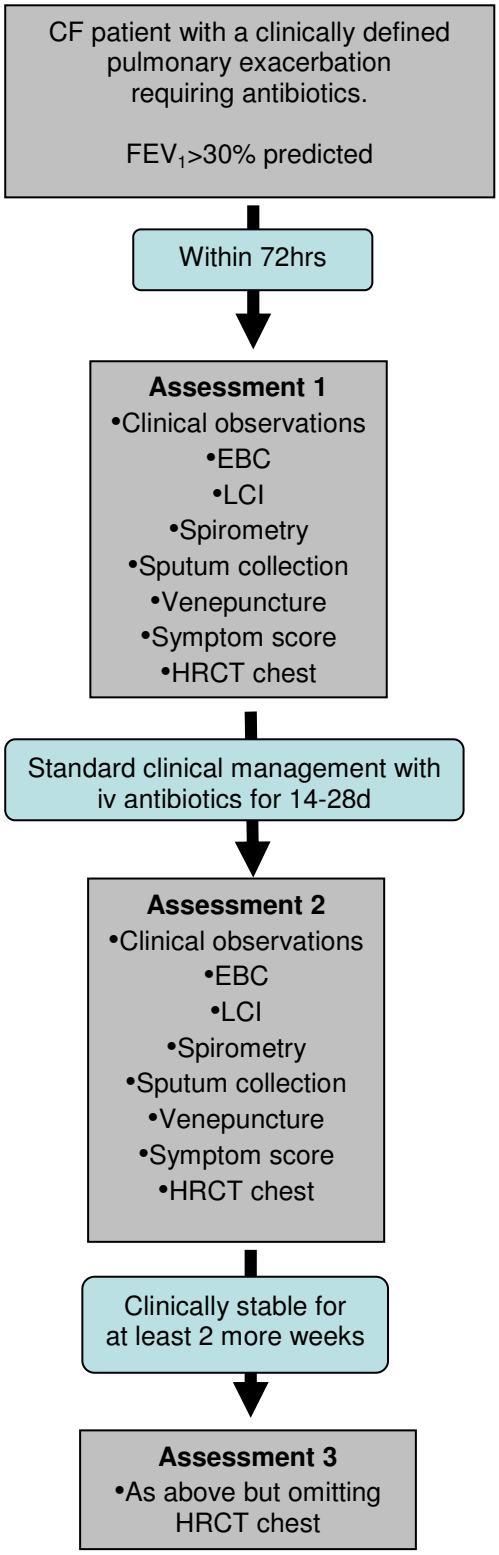


Figure 4.1: Summary of study flow

In addition, high resolution CT scan of the chest was performed at visit 1 and 2, but not at visit 3. The order of the CT scan was not fixed, so some patients had this prior to LCI and some afterwards, though all on the same day. Details of the individual assessments are given below.

This study was approved by the Lothian Research and Ethics Committee. All subjects provided informed consent. For the two subjects recruited from the Paediatric CF service, consent was obtained from parents, and the children provided assent.

Clinical observations

Pulse rate, blood pressure, respiratory rate, pulse oximetry, body temperature and weight were recorded at every visit.

Lung Clearance Index

Multiple breath washout was performed as previously described in Chapter 3, using the modified Innocor gas analyser and 0.2% sulphur hexafluoride (SF_6) as the tracer gas.

Spirometry

Spirometry was measured according to American Thoracic Society / European Respiratory Society guidelines (Miller, Hankinson et al. 2005) using an Easyone spirometer (ndd Medizintechnik AG, Zurich, Switzerland). Predicted values for FEV_1 and mid-expiratory flows are those provided by the European Community for Coal and Steel (adults≥17 years) (Quanjer, Tammeling et al. 1993) and Rosenthal et al. (children≤16 years) (Rosenthal, Bain et al. 1993). Three reproducible measures were required for a satisfactory result. The best of the three manoeuvres, defined as the result with the greatest sum of FEV_1 and FVC, was recorded. Measurements were performed without a noseclip.

Sputum collection and processing

Freshly expectorated sputum was stored on ice for a maximum of two hours and processed using a method modified from that described by Pavord et al. (Pavord, Pizzichini et al. 1997). Whole sputum was transferred to a sterile Petri dish and the sputum plugs separated out into a pre-weighed Falcon tube. The sputum plugs were treated with freshly prepared 0.1% dithiotreitol (Sigma-Aldrich, Dorset, UK) in Dulbecco's phosphate buffered saline (D-PBS), at a ratio of 4ml:1g. Each aliquot was then briefly vortexed and rotated for 15 minutes at 4^{o}C. After dilution in an equal volume of D-PBS, the sample was filtered through pre-moistened 48µm nylon gauze (Seva, Bury, UK) to remove solid debris. The sputum sol phase was obtained by centrifugation (1200rpm for 10 minutes at 4^{o}C), and the supernatant transferred to cryovials for storage at -70^{o}C.

The cell pellet was re-suspended in 0.9% D-PBS. Total cell counts were obtained by counting cells in an improved Neubauer counting chamber. For differential cell counts, four spots (25, 50, 75 and 100µl) were pipetted onto glass slides for cytology. The slides were spun at 400 rpm for 5 minutes to draw the cells onto the slides. These were then fixed and stained using a commercially available kit based on May-Grünewald Giemsa stain (Surgipath Industries, Richmond, IL, USA). Cell differentials were obtained by inspecting the slide with the optimal cell density at a magnification of 100 times, under oil. 300-500 cells were identified and counted from each slide from two different regions, and the final percentage is the mean of these two measurements.

The majority of patients were able to expectorate sputum spontaneously. However, when this was not possible, sputum was obtained by hypertonic-saline induction. Subjects were pre-treated with 2.5mg nebulised salbutamol. After a wait of 20 minutes, spirometry was checked, and the patient was then administered 3% saline via an ultrasonic nebuliser (Devilbiss, Sunrise Medical, CA, USA). After four minutes of nebulisation, subjects were asked to blow their nose and rinse their mouth with water before attempting to expectorate. This was repeated to a maximum of three saline nebulisations. Subjects repeated spirometry after every saline nebulisation to ensure no adverse effect of the procedure. Each sputum sample was collected in a fresh, pre-chilled tube, but all samples

were pooled for processing, which was identical to the procedure described for spontaneous sputum.

Venous blood sampling

Venous blood was collected in standard clinical blood collection tubes (Monovettes, Starstedt AG, Numbrecht, Germany) and analysed at the local clinical laboratories for full blood count and C-Reactive Protein (CRP). For data analysis, CRP samples where the level is below the lower limit of detection (3mg/ml) were given the value of 1mg/ml.

Symptom score

A symptom score sheet was developed to allow the patients to grade their symptoms in response to seven questions relating to different aspects of respiratory function *(*Figure 4.2). Subjects were required to tick one of 5 boxes, scored from -2 (much worse than usual) to +2 (much better than usual). The possible range of scores is therefore from -14 to +14, with no change from usual giving a score of 0.

Two additional questions, on sputum colour and change in colour hue (question numbers 6 and 7), were subsequently excluded because of difficulty in scoring these objectively.

Date:	VISIT 1	Initials: Study no:

Scoring Sheet

This questionnaire relates to how you feel on the day that you are filling it in compared to when you are stable. Please answer each question as best you can. There are no right or wrong answers. For each question, please tick the box that best describes how you feel on that day.

1. **How severe is your cough?**

Much worse than usual (-2)	Worse than usual (-1)	Same as usual (0)	Better than usual (+1)	Much better than usual (+2)

2. **How severe is your breathlessness?**

Much worse than usual (-2)	Worse than usual (-1)	Same as usual (0)	Better than usual (+1)	Much better than usual (+2)

3. **How tired or lethargic are you?**

Much worse than usual (-2)	Worse than usual (-1)	Same as usual (0)	Better than usual (+1)	Much better than usual (+2)

4. **How far can you walk easily?**

Much less far than usual (-2)	Less far than usual (-1)	Same as usual (0)	Farther than usual (+1)	Much farther than usual (+2)

5. **How much sputum are you producing?**

Much more than usual (-2)	More than usual (-1)	Same as usual (0)	Less than usual (+1)	Much less than usual (+2)

6. **What is the usual colour of your sputum?** (not scored)

Clear / white	Yellow	Green	Brown	Flecked with blood

7. **Has your sputum changed colour? (If yes, please specify)**

_____(not scored)_____

8. **Has the shade of the sputum changed?**

Much darker than usual (-2)	Darker than usual (-1)	Same as usual (0)	Lighter than usual (+1)	Much lighter than usual (+2)

9. **How thick is your sputum?**

Much thicker than usual (-2)	Thicker than usual (-1)	Same as usual (0)	Thinner than usual (+1)	Much thinner than usual (+2)

Figure 4.2: Symptom score sheet. Responses were scored from -2 to +2, with a score of 0 representing no change from usual. Questions 6 and 7 were not included in analysis due to difficulty in scoring objectively, so possible scores range from -14 to +14.

Computed Tomography

Each CT examination consisted of two scans; a volumetric high resolution CT at inspiration and interspaced high resolution CT at expiration. These were performed on a Siemens 64 slice multi-detector scanner (Siemens AG, Erlangemn, Germany) or a Siemens Sensation 16 slice scanner (Royal Hospital for Sick Children and Western General Hospital respectively). The volumetric CT comprised contiguous thin-sections (1 mm) through the entire volume of the lungs, 0.5s rotation time, 64 x 0.6mm collimation, pitch 1.4. The interspaced CT was obtained at end expiration with 1mm sections every 10mm, 0.36s rotation time, 2 x 1mm collimation. In order to limit effective dose, 100 kVp was used for both scans. mAs values were varied according to the weight of each patient: 1mAs per kg for patients up to 30kg, 30mAs for patients between 30 and 50 kg, 35 mAs for patients above 50 kg and 50 mAs for patients weighing more than 70 kg. No intravenous contrast was used.

CT analysis

Each set of CT images were saved onto a separate compact disc, with the only identifier on the disc being a four digit randomly generated number. A copy of the complete set of CTs was sent to the Royal Brompton Hospital where the images were read by two experienced radiologists blinded to the patient details. All the images were reviewed directly from workstations. The CTs were scored for eight independent features according to a scoring system devised at the Royal Brompton Hospital by Professor Hansell (Table 4.3). A semi-quantitative graded scoring system was used for extent and severity of bronchiectasis, bronchial wall thickness, small and large mucus plugging. Air trapping, consolidation and ground glass opacification were scored as a percentage to the nearest 5%. Each lobe of 6 was assessed: right upper, middle and lower lobes; left upper and lower lobes and lingula. The final score represents the sum of the individual lobe scores for that feature from both radiologists, i.e. 12 x the maximum score possible for an individual lobe. Unlike other scoring systems, the different features are only intended to be considered independently and there is no global CT score which summarises all the findings in a single number (Brody, Klein et al. 2004).

Feature	Score range	Maximum possible score
Extent of Bronchiectasis	0 = none 1 = <25% lobe involved 2 = 25-50% lobe involved 3 = 51-75% lobe involved 4 = 76-100% lobe involved	48
Severity of Bronchiectasis	0 = absent 1 = trivial dilatation 2 = >1 but <2 x diameter of accompanying vessel 3 = 2-3 x vessel diameter 4 = >3 x vessel diameter	48
Airway wall thickening	0 = absent 1 = trivial wall thickness 2 = up to 0.5x diameter of adjacent vessel 3 = > 0.5 to 1x vessel diameter 4 = > 1 to 2x vessel diameter 5 = > 2x vessel diameter	60
Small mucus plugs	0 = absent 1 = mild 2 = extensive	24
Large mucus plugs		24
Air trapping	0-100%, scored to nearest 5%	1200
Consolidation		1200
Ground glass opacification		1200

Table 4.3: Summary of CT scoring protocol. Each lobe (of 6) was scored independently and the maximum possible score represents the sum of all the lobe scores from two radiologists (i.e. 12 x the maximum single lobe score).

Sputum IL-8 ELISA assay

IL-8 assays were performed using a commercial kit (IL-8 Easia Kit, Biosource, Invitrogen, CA, USA). For sputum assays, a modification was made to the standard diluents to compensate for the constituents of diluted samples and standards and to accommodate the presence of DTT in the samples. Lower limit of detection of IL-8 was 10pg/ml.

Statistical analysis

Data were analysed using Prism (GraphPad Software Inc, CA, USA). Normal distribution was assessed using the D'Agostino and Pearson omnibus normality test. Results are quoted as mean (SD) or median (interquartile range) unless otherwise stated.

For comparison of variables at different visits, paired t-test was used for parametric data and Wilcoxon matched pairs test was used for non-parametric data. Comparisons between multiple groups were performed using a one way ANOVA (parametric data) or Kruskal Wallis (non-parametric). Correlations were assessed using the Pearson correlation coefficient (parametric data) or Spearman R (non-parametric).

A p value of below 0.05 was considered as statistically significant.

Results

Twenty one patients were recruited in Edinburgh. Seventeen of these (81%) completed three visits, 2 (9%) completed 2 visits and 2 subjects only completed a single visit. Visit 2 data from one of the patients who only attended twice were subsequently excluded because of an excessive interval between the end of antibiotic therapy and the date of the second assessment (8 days). One patient was excluded because he was commenced on oral corticosteroids following visit 1. Paired pre- and post- antibiotic data are therefore available on 17 subjects, 16 of whom also completed a third assessment. Demographic data on these 17 subjects are summarised in Table 4.4.

Visit 1 (start of exacerbation) was performed within 1 day of starting antibiotics for all but two subjects (range 0-3 days) (see Figure 4.3). Visit 2 (end of treatment) was performed a median of 1 days after the end of treatment (range -7 to 5). A single subject had the visit 2 assessment performed before her clinical review, at which time the decision was taken was to extend her treatment to a third week. These data were included however because the patient had already received two weeks of treatment and had completed the assessment, including CT, before the decision to extend treatment was made. Visit 3 (stable) was performed a median of 20 days after visit 2 (range 14-139).

Mean duration of antibiotic treatment was 16 days. 12 subjects (71%) received 2 weeks of treatment (13-16 days) and the other 5 subjects all received 3 weeks (19-22 days).

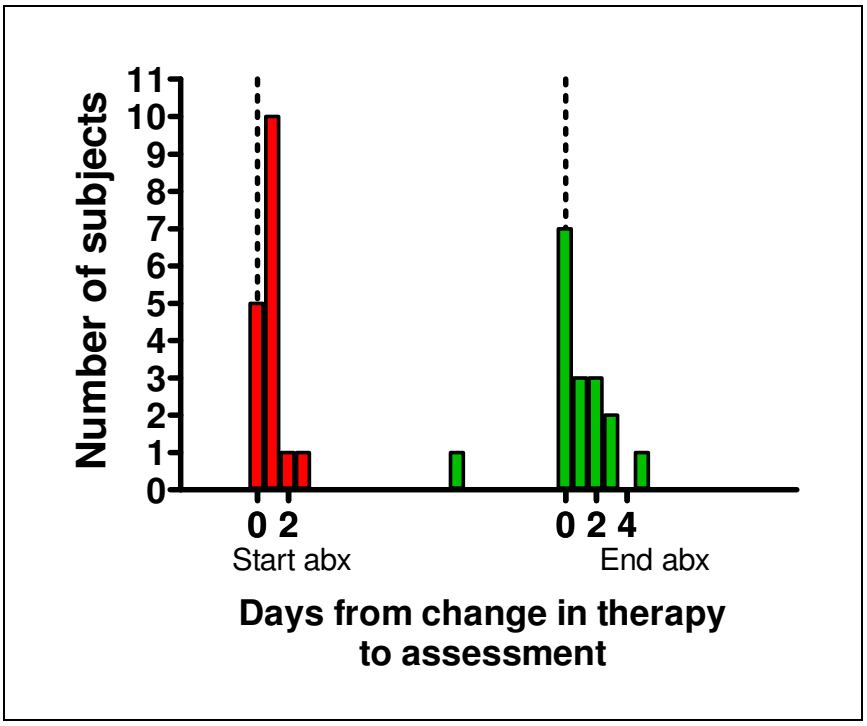


Figure 4.3: Time between assessment and start of antibiotics (red bars) or end of antibiotics (green). The dotted line represents day 0 (the day treatment was commenced or completed). A single subject had her visit 2 assessment prior to a clinical review which concluded that she required an additional 7 days of treatment to achieve a maximal response. These data have been included in the analysis.

Clinical characteristics of study population

These data are summarised in Table 4.4. The majority of subjects (11/17; 65%) had an FEV_1 at the first visit of between 40 and 60% predicted. The mean (SD) fall in FEV_1 at visit 1 was 12.4% over baseline (defined as the best FEV_1 in the last 6 months). Although the need for iv antibiotics was determined by the patient's consulting clinician, all patients had a Fuchs score (Fuchs, Borowitz et al. 1994) for definition of pulmonary exacerbation of greater than or equal to 4 at visit 1, with the exception of a single subject who scored only 1. Median Fuchs score was 5 (range 1-8).

Of the 17 subjects with paired visit data, 8 were known to have chronic colonisation with *Pseudomonas aeruginosa*, 5 with *Burkholderia cepacia,* and 5 with *Stenotrophomonas maltophilia (*see Table 4.4). 8 subjects were on DNase, 9 were on nebulised antibiotics (colomycin, tobramycin and gentamicin) and 8 were on regular azithromycin. 11 subjects were on inhaled corticosteroids or combined long-acting b-agonists and inhaled corticosteroids.

	Subjects with paired assessments pre and post antibiotic therapy	Subjects recruited into study but excluded or with a single assessment only
n	17	4
Mean age (SD) [range]	21.8 (6.8) [11 - 40]	22.3 (5.3) [18 – 29]
Mean FEV_1 % predicted at first visit [range]	57.0 (10.9) [39.0 – 86.8]	48.4 (10.6) [40.4 – 64.0]
Mean best FEV_1 % predicted in last 6 months [range]	69.4 [55.5 – 104.0]	67.9 [50.5 – 87.3]
Male / Female	11 / 6	1 / 3
Number (%) DF homozygotes	14 (82)	0
N (%) with pancreatic insufficiency	15 (88)	3 (75)
N (%) with diabetes mellitus	6 (35)	1 (25)
N (%) with chronic airway colonisation by *Ps. aeruginosa*	8 (47)	0
N (%) with chronic colonisation by *B. cepacia*	5 (29)	2 (50)

Table 4.4: Clinical and demographic features of cystic fibrosis patients with and without paired assessments before and after antibiotic therapy for a pulmonary exacerbation.

Response of individual assays to treatment of an exacerbation

1. Symptom score

At the start of treatment, patients felt worse than normal with a mean (SD) symptom score of -4.9 (3.8) (Table 4.5). A single subject scored +5 on his symptom score, but this is likely to have been completed erroneously since he also indicated deterioration in the same symptoms when questioned for completion of Fuchs scoring. Since this was only recognised some time after the event, it has not been possible to retrospectively re-score the symptom score, and this value has been retained in the analysis.

Mean symptom score improved to 3.3 (4.3) at the end of treatment ($p<0.0001$ compared to visit 1, paired t-test) (Figure 4.4). Mean symptom score at visit 3 fell to 1.1 (5.3), this was still significantly greater than visit 1 ($p=0.001$). Visit 3 symptom score was not significantly different from a theoretical mean of 0 ($p=0.41$, one sample t test). Since a score of zero means no change over usual symptoms, this indicates that the patients were, on average, stable. The range of symptom scores however was greater than either of the preceding assessments. As might be expected, the mean score at visit 1 was significantly lower than usual baseline (score of zero) ($p<0.0001$), but the score at visit 2 was greater than baseline ($p=0.006$). This indicates that the patients' symptoms had, on average, improved to a level that was better than their usual baseline after 2 weeks of antibiotics.

2. Clinical observations

The majority of clinical observations did not change significantly over the course of antibiotic treatment (see Table 4.5). Systolic BP was significantly higher at visit 3 compared to visit 1 (124.7 vs 118.5 mmHg, $p=0.004$), and diastolic BP was significantly lower at visit 2 compared to visit 1 (71.4 vs 74.4 mmHg, $p=0.033$). The magnitude of changes was modest and the significance of these observations is unclear. There was a fall in respiratory rate between visits 1 and 2 which just failed to achieve statistical significance (22.1 breaths/min at visit 1 compare to 19.3 breaths/min at visit 2, $p=0.052$) (Figure 4.5). A single subject recorded a respiratory rate of 33 at visit 2. This was an 11 yr old male, whose other clinical observations had not deteriorated. There were no significant changes in weight, temperature, pulse rate, or oxygen saturations between visits.

	Visit 1	Visit 2	Visit 3
n	17	17	16
Symptom score [range]	-4.9 (3.8) [-10 to 5]	3.3 (4.3)*** [-5 to 9]	1.1 (5.3)** [-10 to 12]
Weight (kg)	60.13 (13.46)	58.95 (11.54)	59.97 (11.90)
Temperature ^{o}C	36.40 (0.60)	36.16 (0.55)	36.16 (0.43)
Pulse rate (bpm)	87.8 (17.0)	84.4 (14.9)	86.5 (16.4)
Blood pressure (mmHg) systolic/diastolic	118.5 (108.8) 74.4 (8.4)	118.8 (13.4) 71.4 (8.1)*	124.7 (14.0)** 74.6 (11.9)
Respiratory rate (breaths/min)	22.1 (4.0)	19.3 (5.6)	20.4 (3.4)
Oxygen saturations (%)	95.9 (1.7)	95.8 (1.2)	95.8 (1.4)

Table 4.5: Change in clinical variables with antibiotic treatment (visit 1 to 2) and at stability (visit 3). Values are mean (SD).

*p<0.05 compared to visit 1

**p<0.005 compared to visit 1

***p<0.0001 compared to visit 1

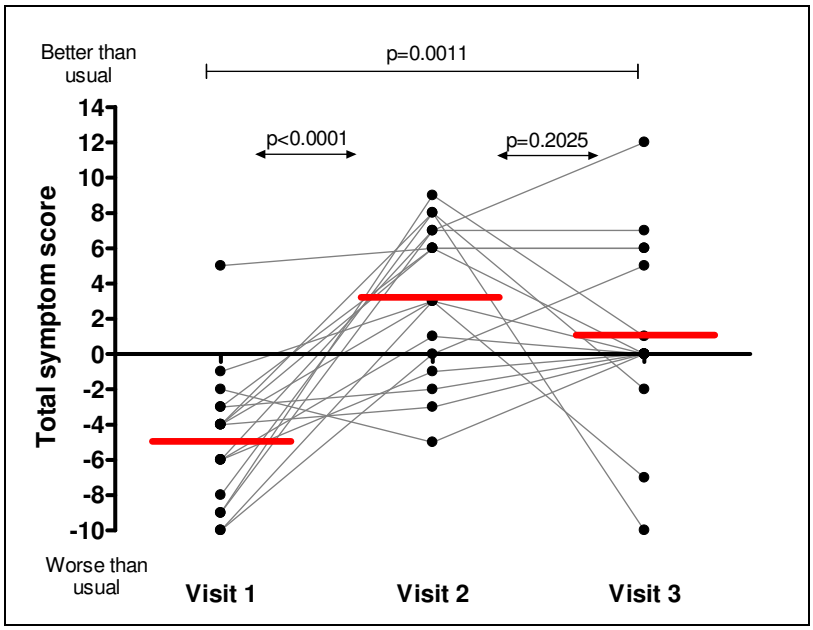


Figure 4.4: Change in symptom score with treatment of an exacerbation. Each set of points joined by a single grey line represents a single patient. A score of zero represents usual baseline symptoms. Horizontal red bars represent group mean. p values refer to significance of change in mean symptom score at the different time points (paired t-test).

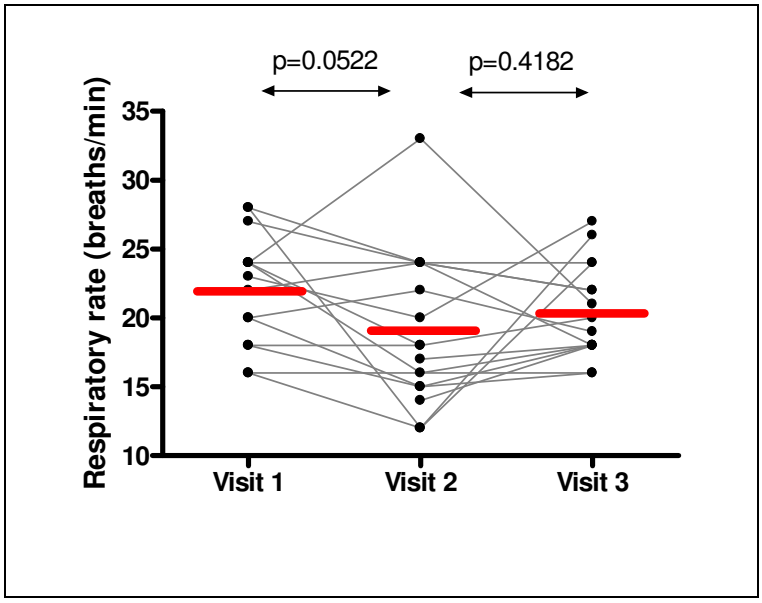


Figure 4.5: Change in respiratory rate between visits. Each set of points joined by a single grey line represents a single patient. Horizontal red bars represent group mean. p values refer to significance of change in mean symptom score at the different time points (paired t-test).

3. Spirometry

FEV_1 improved in 12 (71%) subjects between visits 1 and 2 (Figure 4.6). In 4 subjects, FEV_1 increased by over 30% between the first two visits. Between the same two assessments, mean FEV_1 increased significantly from 2.13 to 2.37 L/s (p=0.02, paired t-test) (Table 4.6). There was a further increase in mean FEV_1 at visit 3 to 2.40 L/s (p=0.003 vs visit 1, paired t-test). This represents a mean improvement of 240ml, or 11.1%, with antibiotics. When expressed as percent predicted, FEV_1 increased from 57.0 to 63.0 % predicted between visits 1 and 2 (p=0.018).

FVC also increased significantly between visits 1 and 2, from a mean (SD) of 3.21 (0.97) L to 3.49 (1.12) L, p=0.027 (paired t-test) (Figure 4.7). There was a further small increase in mean FVC at visit 3 to 3.57 (1.11) L (p=0.007 vs visit 1, ns vs visit 2). The change between visits 1 and 2 was skewed by a single outlier, but remained statistically significant even if this subject was excluded.

FEF_{25-75} percent predicted data were only analysed for flow volume loops from adult subjects, since the normal range data are only applicable above the age of 18 yrs (Quanjer, Tammeling et al. 1993). There was a non-significant improvement in mean FEF_{25-75} % predicted between visits 1 and 2, from 28.3 to 35.7% predicted, p=0.096 (paired t-test) (see Figure 4.8).

	Visit 1	Visit 2	Visit 3
FEV_1 (L)	2.13 (0.58) [1.13 – 3.33]	2.37 (0.73)* [0.90 - 3.99]	2.40 (0.71)** [1.08 – 4.03]
FEV_1 % predicted	57.0 (10.87) [39.0 – 86.8]	63.0 (14.04)* [39.3 – 91.5]	64.7 (12.57)** [47.2 – 92.9]
FEF_{25-75} % predicted	28.3 (10.20) [15.8 – 50.2]	35.7 (16.6) [18.6 – 68.5]	33.4 (14.08) [15.8 – 58.1]
FVC (L)	3.21 (0.966) [1.64 – 4.95)	3.49 (1.118)* [1.56 – 6.49]	3.57 (1.113)* [1.69 – 6.37]
Lung Clearance Index	14.0 (2.52) [11.2 – 19.6]	13.1 (2.47)* [9.7 – 17.1]	13.3 (2.83) [8.8 – 19.0]
Functional Residual Capacity (L)	2.62 (0.66) [1.55 – 3.94]	2.64 (0.67) [1.74 – 3.84]	2.50 (0.70) [1.64 – 3.89]

Table 4.6: Effect of antibiotic treatment on lung function parameters. Data are presented as mean (standard deviation) and [range].

*$p<0.05$ vs visit 1

**$p<0.005$ vs visit 1

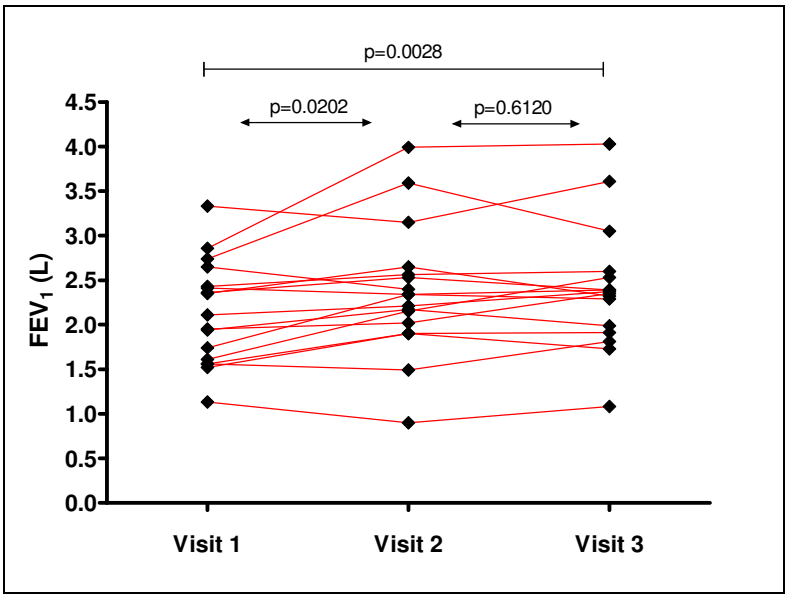


Figure 4.6: Change in FEV_1 with treatment of an exacerbation. Each set of points joined by a single red line represents a single patient. p values refer to significance of change in mean FEV_1 at the different time points (paired t-test).

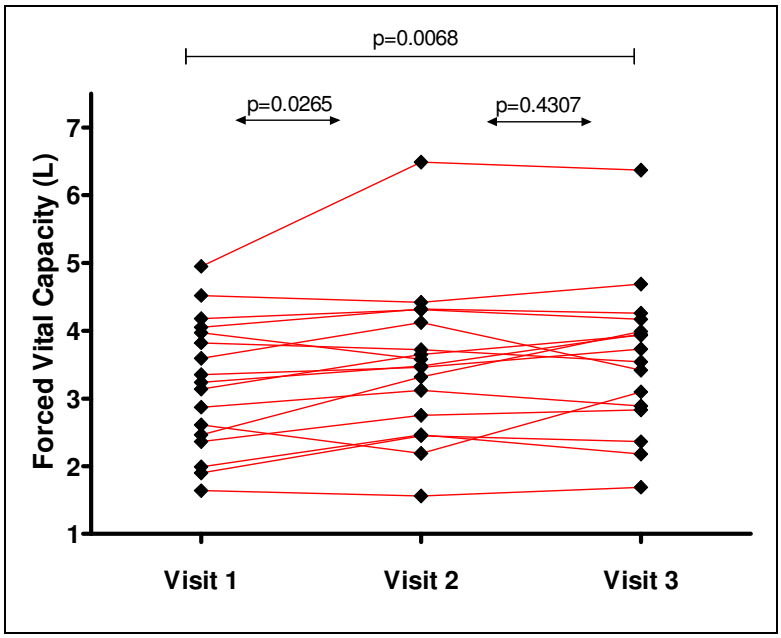


Figure 4.7: Change in forced vital capacity (FVC) with treatment of an exacerbation. Each set of points joined by a single red line represents a single patient. p values refer to significance of change in mean FVC at the different time points (paired t-test).

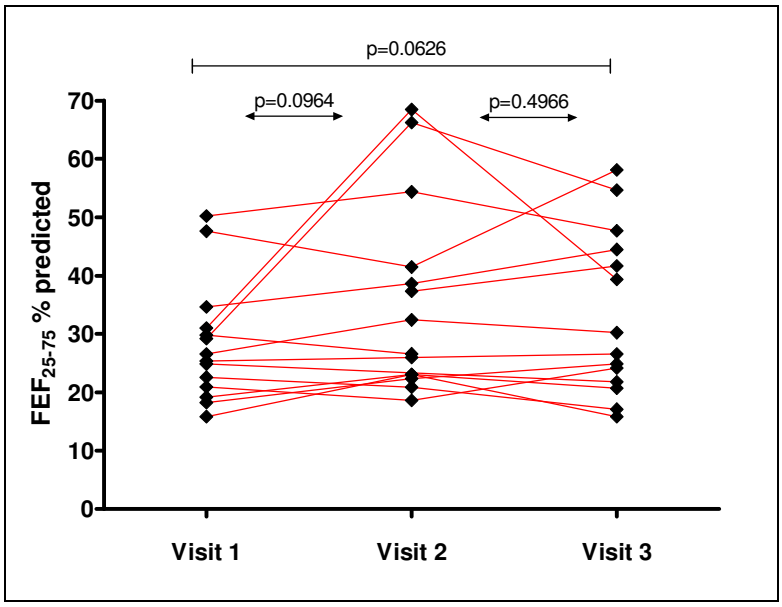


Figure 4.8: Change in FEF_{25-75} with treatment of an exacerbation.
Each set of points joined by a single red line represents a single patient. p values refer to significance of change in mean FEF_{25-75} at the different time points (paired t-test).

4. Lung Clearance Index

LCI improved in 12 (71%) of subjects between visits 1 and 2, see Figure 4.9. Mean (SD) LCI improved from 14.0 (2.52) to 13.1 (2.47) between the same two visits, p=0.0401 (paired t-test). This represents a mean improvement in LCI of just over 0.8, or 5.9% over visit 1. It can also be seen from Figure 4.9 that, even at visit 2 when the patients were symptomatically at their best, the LCI in all subjects was considerably greater than the upper limit of normal (7.45, see Chapter 3). This is in contrast with the FEV_1, which was greater than 80% predicted in 3 subjects (18%) at visits 2 and 3.

Between visits 2 and 3 there was less consistency in the change in LCI, with improvement in 9 of 16 subjects, and a greater spread of values. Mean LCI was greater (indicating a deterioration in lung gas mixing) at visit 3 than visit 2, although this difference was not statistically significant. This is similar to the change seen in FEF_{25-75} but is in contrast to FEV_1 and FVC, which continued to improve between visits 2 and 3.

5. Functional Residual Capacity

MBW measurements also generate a measure of FRC. This is the end tidal lung volume that is ventilated by quiet tidal breathing, and therefore does not include gas from regions of the lung that are not ventilated during tidal breathing. There was no significant change in mean FRC between any of the visits. Change in FRC was not correlated with disease severity, as assessed by either LCI or FEV_1 percent predicted. Mean FRC was 86 percent predicted at visit 1 and 91 percent predicted at visit 2, with a range over the three assessments of 60 to 117 percent predicted.

The relationship between the FRC and LCI change over the course of antibiotic treatment is represented in Figure 4.11. This shows individual paired FRC and LCI measurements at visit 1 (red) and 2 (green) linked by a solid line. It can be seen that there is considerable heterogeneity of change in the individual markers, both in terms of magnitude and direction of change. In addition to this, there is heterogeneity of correlation between the two variables. If a consistent relationship existed between the two variables, then the lines would show a clear tendency to be linked in the same direction, e.g. if LCI improved (fell) as FRC increased the lines would point in the direction from top left to bottom right. The reasons why this might not be the case are covered in more detail in the discussion.

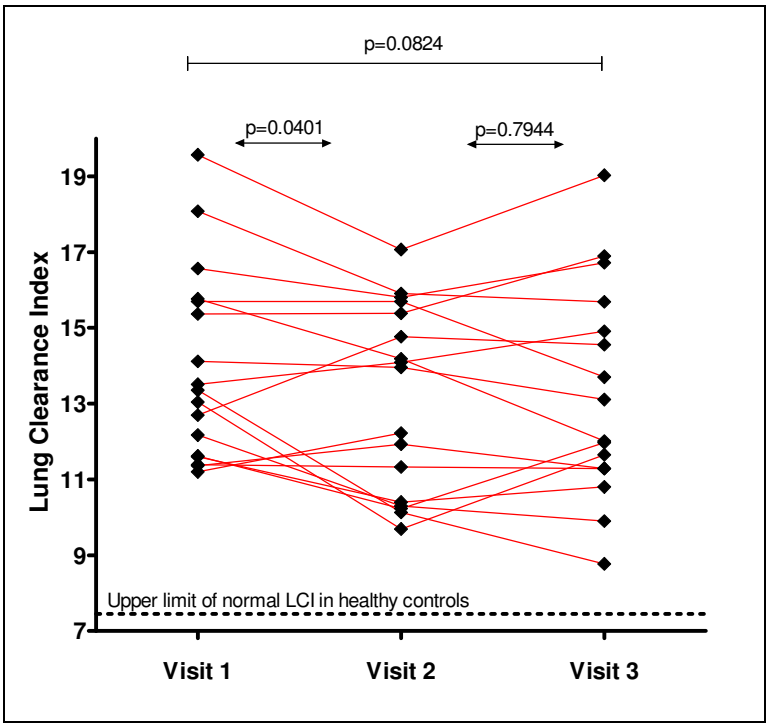


Figure 4.9: Change in LCI with treatment of an exacerbation. Each set of points joined by a single red line represents a single patient. p values refer to significance of change in mean LCI at the different time points (paired t-test). The upper limit of normal LCI for healthy controls is shown by the dotted line.

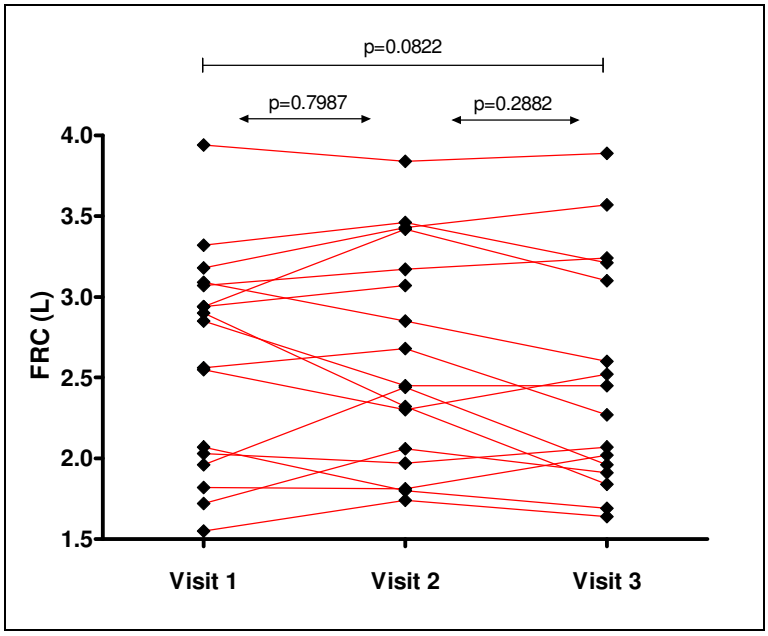


Figure 4.10: Change in FRC with treatment of an exacerbation. Each set of points joined by a single red line represents a single patient. p values refer to significance of change in mean FRC at the different time points (paired t-test)

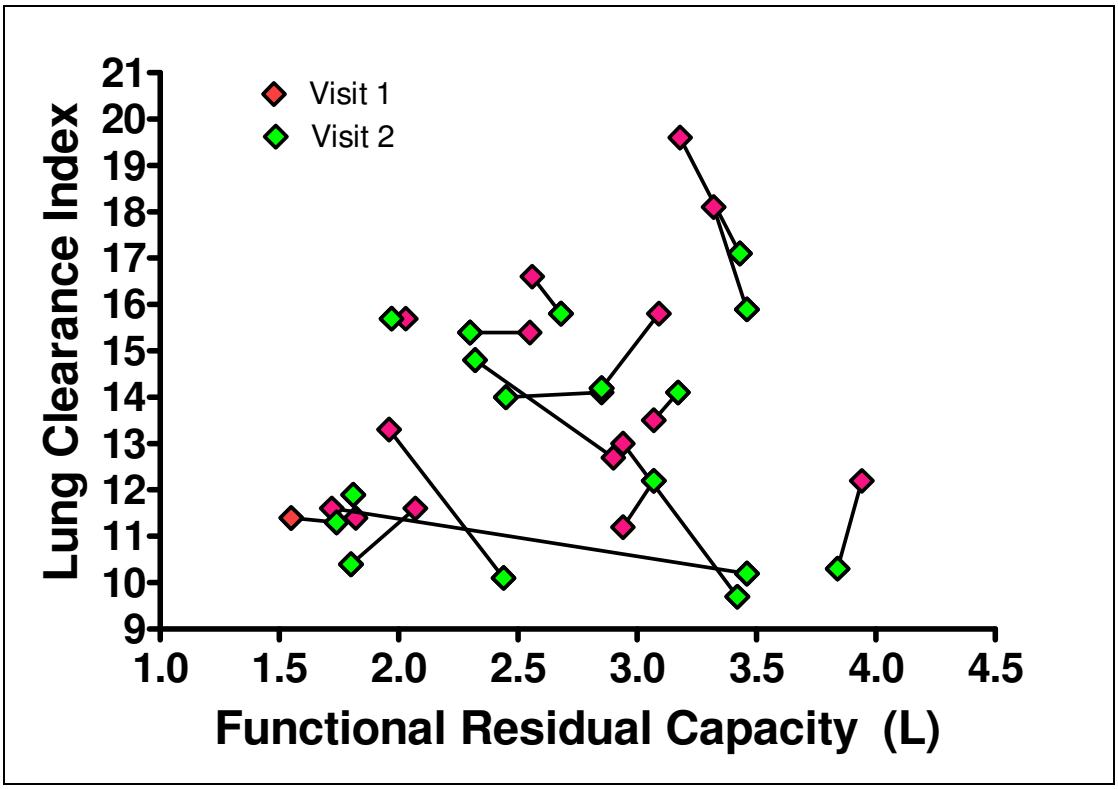


Figure 4.11: Paired mean functional residual capacity (FRC) and mean lung clearance index (LCI), derived from multiple breath washouts, for cystic fibrosis patients treated for an exacerbation. Each pair of points represents a single patient, with paired measurements at visit 1 (pre treatment) represented by a red diamond, and visit 2 (post treatment) by a green diamond linked by a solid line. There is considerable heterogeneity of individual response in terms of both magnitude and direction of change in the individual variables, as well as correlation between the two.

Correlation between different measures of lung function

In cross sectional analysis, there were significant correlations between LCI and both FEF_{25-75} percent predicted and FEV_1 percent predicted at all three time points (Figure 4.12). For FEV_1, the Pearson r values ranged from -0.53 (p=0.028) at visit 1 to -0.59 (p=0.013) at visit 2. For FEF_{25-75}, the Pearson r values ranged from -0.62 (p=0.019) at visit 1 to -0.86 (p<0.0001) at visit 3. There was no correlation between LCI and FVC percent predicted at any time point.

FEV_1 percent predicted correlated strongly with both of the other two spirometric indices. Pearson r values for correlation between FEV_1 % predicted and FVC % predicted ranged from 0.83 to 0.82, p=0.0003 to <0.0001. For correlation between FEV_1 percent predicted and FEF_{25-75} percent predicted, Pearson r values ranged from 0.70 (p=0.0052) to 0.86 (p<0.0001).

There was a significant correlation between percent change in LCI and percent change in FEV_1 (Figure 4.13), Pearson r=-0.59, p=0.012. There was also a correlation between percent change in LCI and percent change in FEF_{25-75} (Figure 4.14), Pearson r=-0.70, p=0.008. Despite these correlations, individual values showed wide discrepancy – some with large changes in LCI but no change in spirometry, and others with the opposite. There was no correlation between percent change in LCI and percent change in FVC (Figure 4.15).

Overall there was a mean improvement in LCI of 5.6%, compared to a mean improvement in FEV_1 of 11.1%, in FVC of 9.4%, and in FEF_{25-75} of 26.0%.

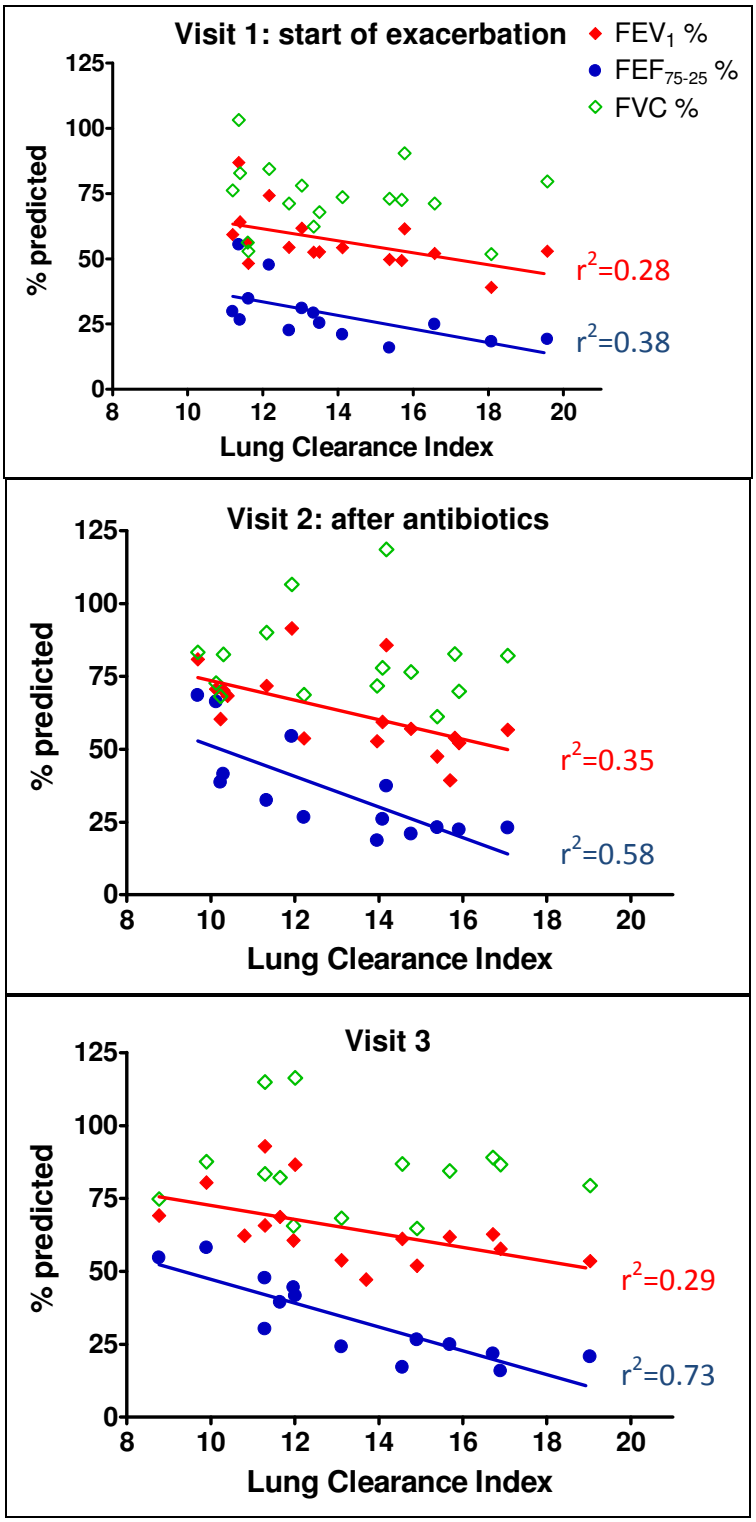


Figure 4.12: Cross sectional correlations between LCI and spirometry at the 3 visits. FEV_1 (red diamonds), FVC (green open diamonds) and FEF_{25-75} (blue circles) are all expressed as % predicted. Significant correlations are represented by solid lines in the corresponding colour.

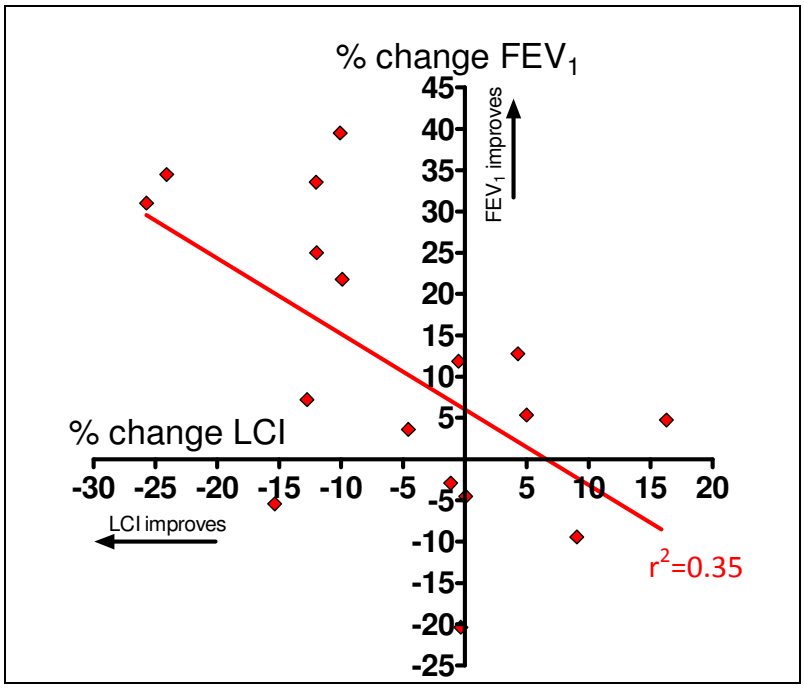


Figure 4.13: Correlation between percent change in FEV_1 and percent change in LCI. An improvement in LCI is represented as a negative change in LCI. Each point represents a single subject with paired LCI and spirometry before and after antibiotic therapy for an exacerbation. The solid line represents the regression between the two variables, r^2=0.35, p=0.012.

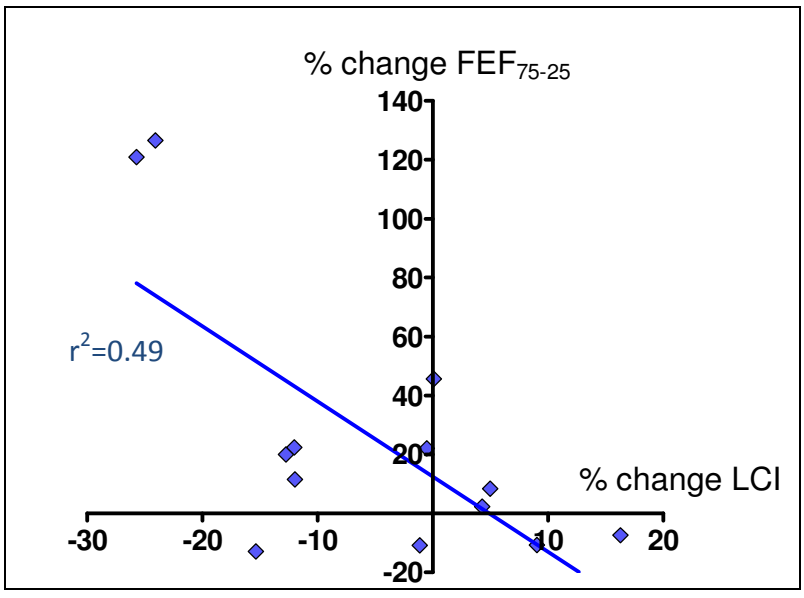


Figure 4.14: Correlation between percent change in FEF_{25-75} and percent change in LCI. Each point represents a single subject with paired LCI and spirometry before and after antibiotic therapy for an exacerbation. The solid line represents the regression between the two variables, r^2=0.49, p=0.0077.

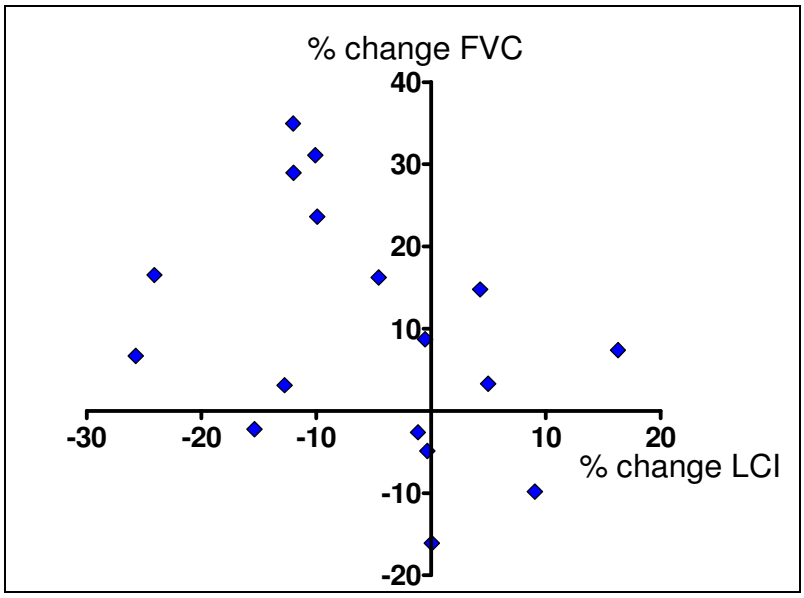


Figure 4.15: Correlation between percent change in FVC and percent change in LCI. Each point represents a single subject with paired LCI and spirometry before and after antibiotic therapy for an exacerbation. There was no significant correlation between percent change in the two variables.

6. Computed Tomography

Of the 21 patients recruited, 16 (76%) had HRCT performed at visit 1. 12 of these also had a CT performed after antibiotic treatment; two subjects only attended for a single visit, one subject missed his CT appointment but completed the remaining assessments, and one subject was excluded because he was commenced on oral steroids. Because the CT data are intended to be paired, these four CT scans have not been scored, and have therefore not been included in this analysis. Subjects who did not receive a CT at visit 1 also did not receive a CT at visit 2, since the value of the assessment was felt to be principally in obtaining paired measurements. Three subjects did not undergo CT scanning at visit 1 because they were recruited before the radiology department had confirmed the protocols, and two subjects did not undergo CT scanning because of time constraints. A single subject with paired CT scans did not receive an expiratory CT scan at visit 2, so air trapping scores for this visit are not available. Lung function assessments were completed on all 12 subjects with paired CTs.

Table 4.7 and Figure 4.16 summarise the individual CT scores at both visits for the 12 subjects with paired CT scans. There was a fall in all 8 score components after antibiotics. Statistically significant improvements were seen in the scores for: extent of bronchiectasis (38.3 after treatment vs 40.8 at visit 1, p=0.044); small mucus plugs (15.8 vs 18.3, p=0.004); and large mucus plugs (14.5 vs 16.8, p=0.022). There was no change in the severity of bronchiectasis score. Consolidated lung and ground glass appearances were present in only a minority of subjects overall. There was a trend to improvements in the wall thickness and air trapping scores.

The changes seen at CT are illustrated in Figures 4.17-4.19 using representative CT images from a single subject, EdTr013. This subject was an 18 year old female who was significantly unwell at the start of treatment, with an elevated CRP (249mg/ml) and a fall in FEV_1 of 37% from her best recorded value in the last 6 months. With treatment, she showed improvement in all of these variables (FEV_1 improved by 25% from 1.52 to 1.90) and in LCI (improvement of 12% from 11.6 to 10.2). This patient showed improvement in all CT scores except extent and severity of bronchiectasis. Large mucus plugging the left lower lobe is shown on HRCT in Figure 4.17. Following over two weeks of antibiotics and physiotherapy, this has largely cleared from the airway. Small mucus plugs, seen as nodular irregularities in small airways made visible by inflammation and mucus, are

illustrated in Figure 4.18. Finally, gas trapping is illustrated in Figure 4.19. This occurs when inflamed small airways collapse on expiration, leading to hyperinflation and decreased attenuation of the distal lung. It is seen as a mosaic attenuation pattern on expiratory CT (Hansell 2001).

	Maximum possible score	Visit 1	Visit 2	Difference (95% CI)	p value
Extent of bronchiectasis	48	40.8 (6.4) [31 – 48]	38.3 (8.2) [21 – 48]	2.0 (0.1 – 3.9)	**0.0439**
Severity of bronchiectasis	48	31.0 (8.5) [21 – 45]	30.9 (8.3) [14 – 42]	0.1 (-2.4 – 2.6)	0.942
Wall thickness	60	31.1 (7.2) [21 – 43]	27.5 (6.5) [19 – 38]	3.6 (-0.4 – 7.6)	0.0723
Small mucus plugs	24	18.3 (4.3) [12 – 24]	15.8 (3.9) [10 – 24]	2.5 (1.0 – 4.0)	**0.004**
Large mucus plugs	24	16.8 (4.9) [8 – 24]	14.5 (5.3) [5 – 21]	2.3 (0.4 – 4.3)	**0.0217**
Air trapping	1200	492.3 (230.7) [50 – 745]	436.8 (186.7) [210 – 725]	55.5 (-38.8 – 149.7)	0.219
Consolidated lung ***Median (IQ range)***	1200	12.5 (5 – 15)	0 (0 - 7.5)	-12.5	0.129*
Ground glass ***Median (IQ range)***	1200	0 (0 – 12.5)	0 (0 – 2.5)	0	0.375*

Table 4.7: Effect of antibiotic treatment on scores for eight different abnormalities seen on HRCT. The details of the score are described in the text. Values are expressed as mean (SD) [range] unless otherwise stated. p values refer to comparison of group means from before and after treatment (visits 1 and 2 respectively), calculated using paired t-tests or Wilcoxon signed rank pairs (indicated with an asterisk: *). p values less than 0.05 are highlighted in bold.

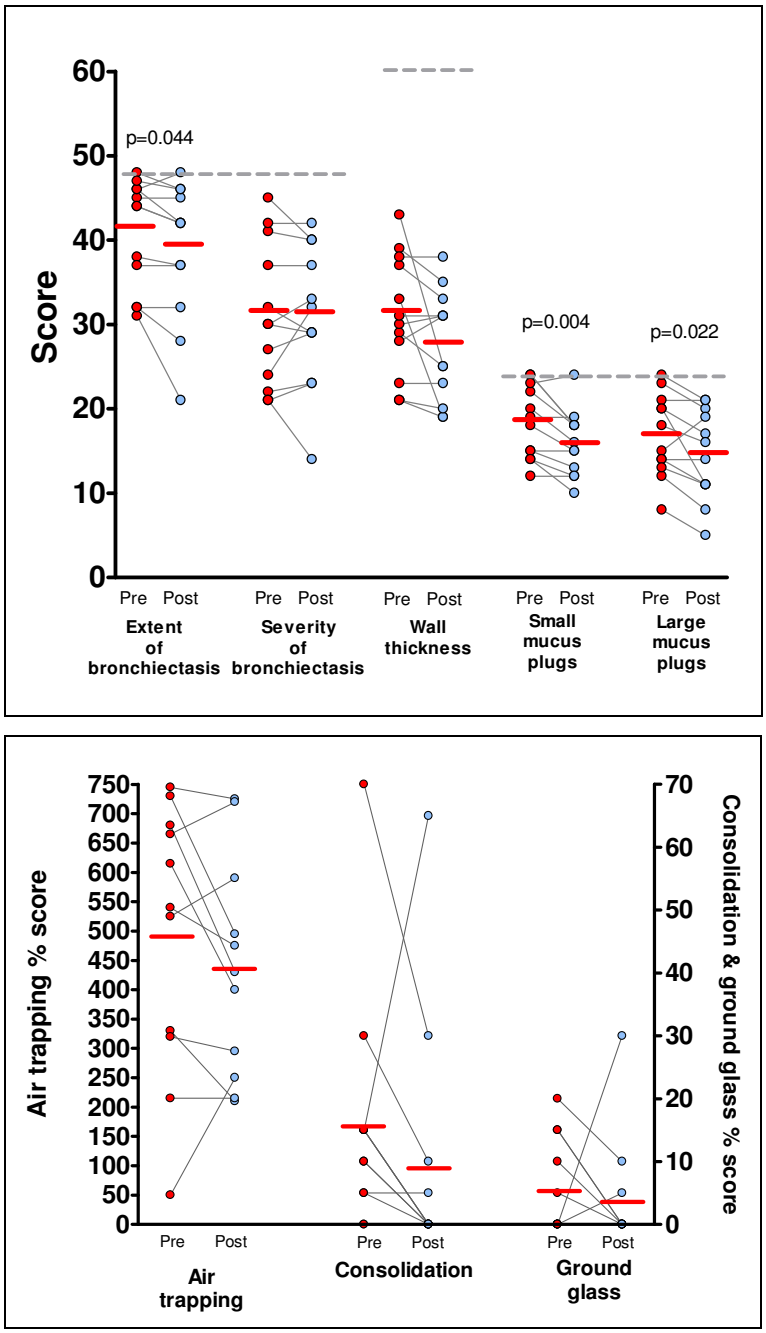


Figure 4.16: Effect of antibiotic therapy on lung CT scores. Each set of points represents a single CF patient with paired assessments before (red circle) and after (blue circle) antibiotic therapy for an exacerbation. Group means are shown by the horizontal red line and p values refer to comparison of group means before and after treatment ($p > 0.05$ are not shown). Horizontal dotted grey lines in upper panel indicate maximum possible score for that feature. The scores for consolidation and ground glass appearances (lower panel) were zero at both visits in the majority of subjects. Maximum possible score for all features in lower panel was 1200.

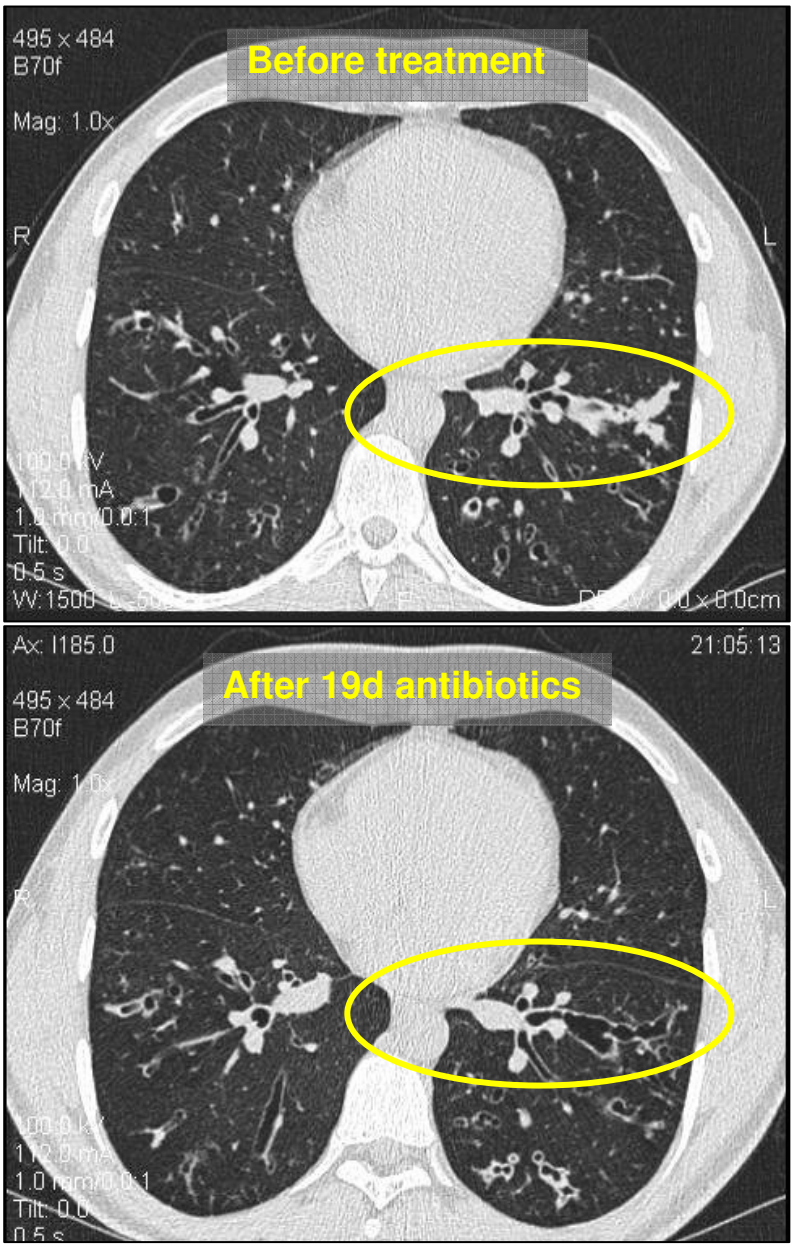


Figure 4.17: Large mucus plugs seen on HRCT in a bronchus in the left lower lobe (circled) of an 18 year old female with CF at the start of an exacerbation (top) and after 19 days of intravenous antibiotics. Following treatment, the airway has been largely cleared of the obstructing mucus. For this subject, overall mucus plug score improved from 20 to 11.

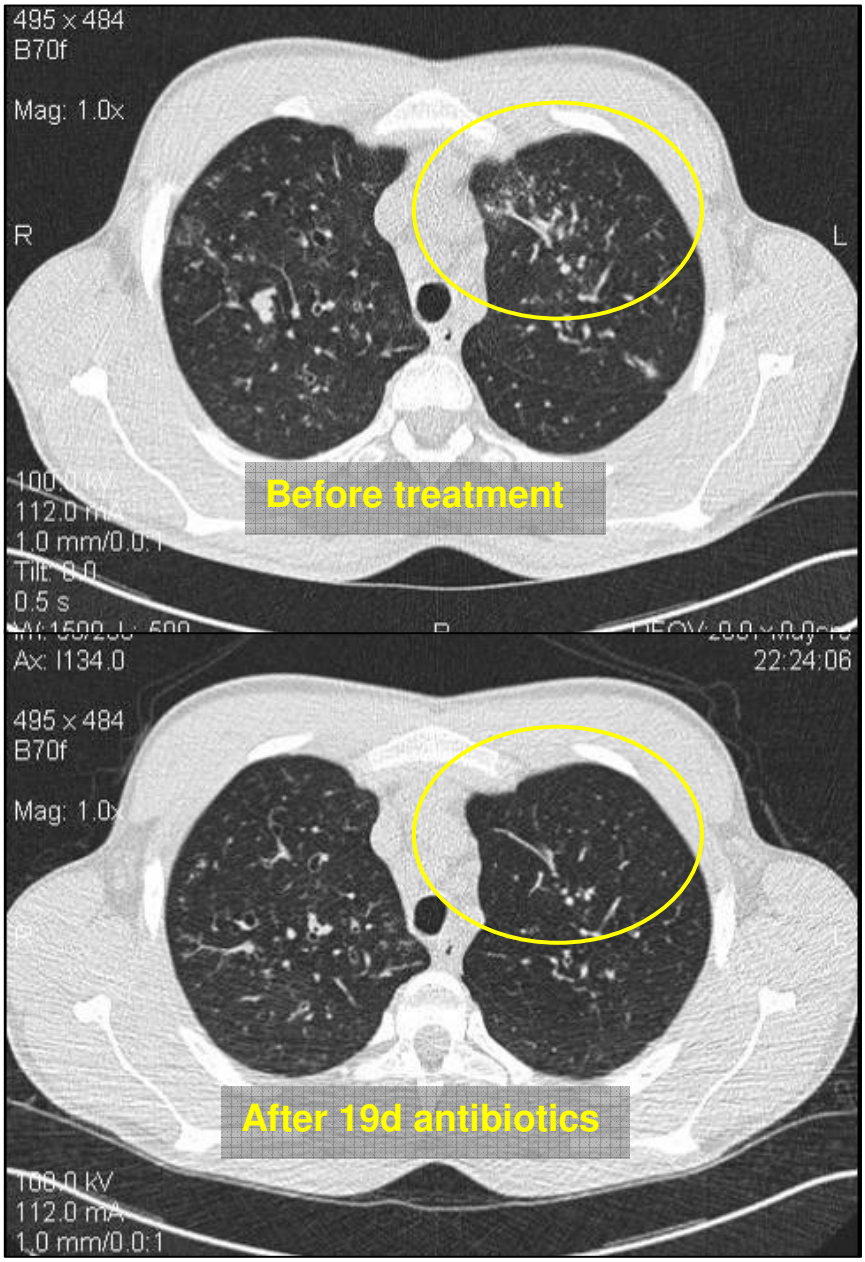


Figure 4.18: Small mucus plugs seen on HRCT in the lung of the same subject described in Figure 4.17. Small plugs are seen as nodular irregularities in the left upper lobe (circled); these represent small airways made visible by plugging with mucus and inflammatory secretions. In this subject, the small mucus plugs score improved from 22 to 18.

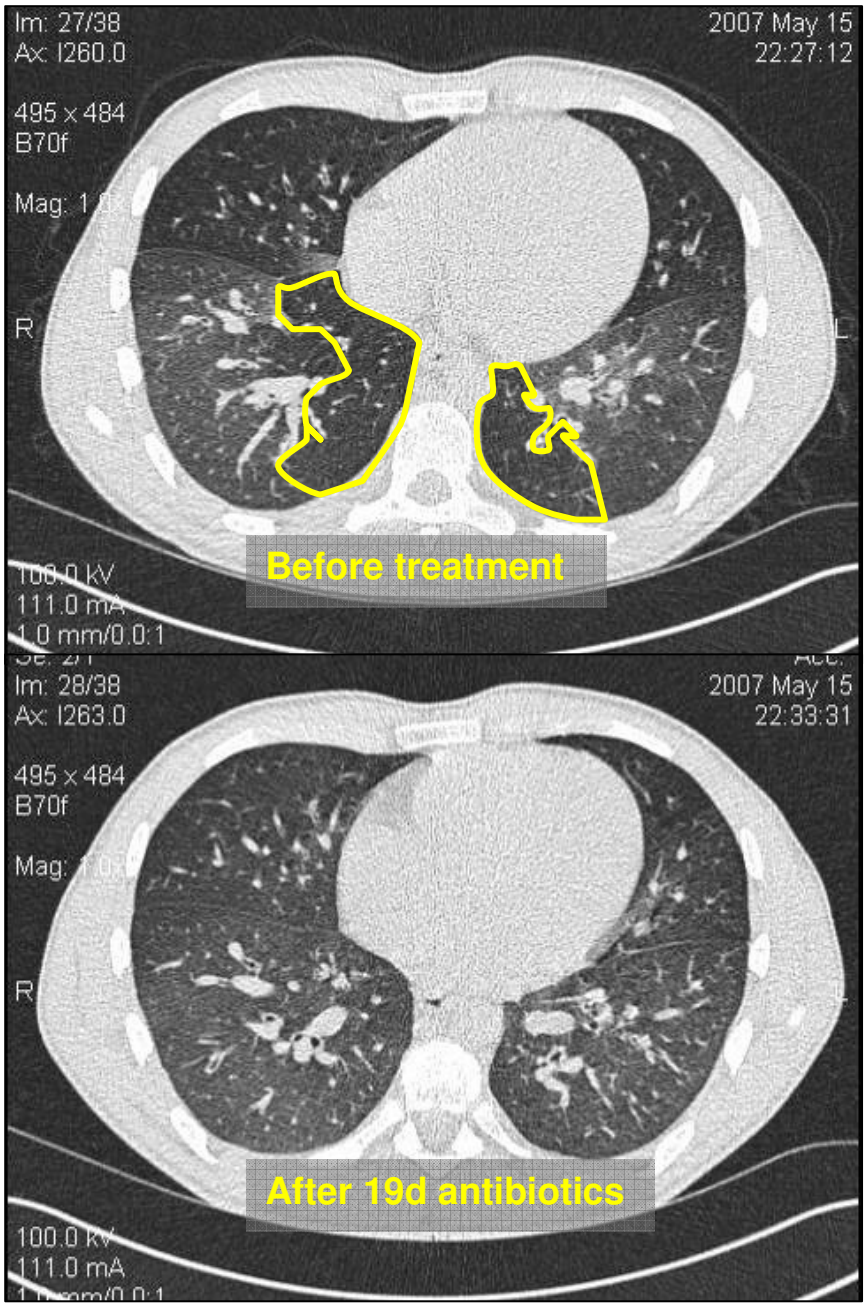


Figure 4.19: Expiratory CT scan showing air trapping in the lungs of the same CF patient as shown in Figures 4.17 and 4.18. A mosaic attenuation pattern is seen before treatment, with the dark areas representing regions of gas trapping (two of these have been highlighted, but there is additional gas trapping anteriorly bilaterally). After treatment, the dark areas are no longer visible. In this subject the air trapping score improved from 680 to 430.

Correlation of functional and structural assessment

Correlations between the structural measurements derived from CT scores and the physiological assessments can be assessed in two ways. The first method is to look at cross sectional comparisons, with each of the two visits considered separately, and the second method is to look at how the changes in different variables over the course of treatment correlate with each other. Both of these will be reviewed.

Table 4.8 shows the cross sectional correlations between the different CT features and both LCI and FEV_1, for both visits. At visit 1, LCI only showed a weak correlation with a single feature - extent of bronchiectasis (Pearson r=0.62, p=0.03). At visit 2 however, there were significant correlations between LCI and wall thickness score (r=0.73, p=0.008), large mucus plugs (r=0.64, p=0.02) and air trapping (r=0.68, p=0.02). These are illustrated in Figure 4.20.

FEV_1 percent predicted showed significant correlations at visit 1 with three features: extent of bronchiectasis (Pearson r=-0.65, p=0.02), small mucus plugs (r=-0.70, p=0.01) and large mucus plugs (r=-0.60, p=0.04). At visit 2, FEV_1 percent predicted showed significant correlations with 5 of the CT features: extent of bronchiectasis (r=-0.85, p=0.0005), severity of bronchiectasis (r=-0.70, p=0.01), wall thickness (r=-0.68, p=0.02), small mucus plugs (r=-0.61, p=0.03), and large mucus plugs (r=-0.75, p=0.005). See Figure 4.21.

Neither LCI nor FEV_1 showed any correlation with the scores for percent consolidated lung or ground glass shadowing.

When the absolute and percent changes in lung physiology were compared to the absolute and percent changes in the CT scores, there were almost no significant correlations. The single exception was a correlation between percent change in FEV_1 and percent change in air trapping score (Pearson r=-0.61, p=0.045). FEV_1 did not correlate with air trapping in cross sectional analysis, and this positive correlation was distorted by a single outlier with a 400% increase in air trapping score at visit 2 (Figure 4.24). Graphs of percent change in the CT variables which showed change with antibiotic therapy, versus LCI and FEV_1, are presented in Figures 4.22 to 4.24.

	Visit	Lung clearance index	FEV_1 % predicted
Extent of bronchiectasis	1	**0.62 (0.03)**	**-0.65 (0.02)**
	2	0.58 (0.05)	**-0.85 (0.0005)**
Severity of bronchiectasis	1	0.03 (0.92)	-0.10 (0.75)
	2	0.46 (0.14)	**-0.70 (0.01)**
Wall thickness	1	0.17 (0.59)	-0.46 (0.13)
	2	**0.73 (0.008)**	**-0.68 (0.02)**
Small mucus plugs	1	0.40 (0.20)	**-0.70 (0.01)**
	2	0.47 (0.12)	**-0.61 (0.03)**
Large mucus plugs	1	0.33 (0.30)	**-0.60 (0.04)**
	2	**0.64 (0.02)**	**-0.75 (0.005)**
Air trapping	1	0.42 (0.20)	-0.27 (0.42)
	2	**0.68 (0.02)**	-0.34 (0.31)
Consolidated lung*	1	-0.46 (0.13)	-0.32 (0.31)
	2	-0.13 (0.68)	0.15 (0.63)
Ground glass*	1	0.33 (0.29)	-0.54 (0.07)
	2	0.48 (0.18)	0.18 (0.56)

Table 4.8: Correlations between individual CT score components and LCI or FEV_1. Both visits are presented; visit 2 (post treatment) are shown in grey. Correlations are shown as the Pearson correlation coefficient with the p value in brackets. Non-parametric (Spearman) correlations were used for non-normally distributed data (indicated by an asterisk: *). Significant correlations ($p<0.05$) are highlighted in bold.

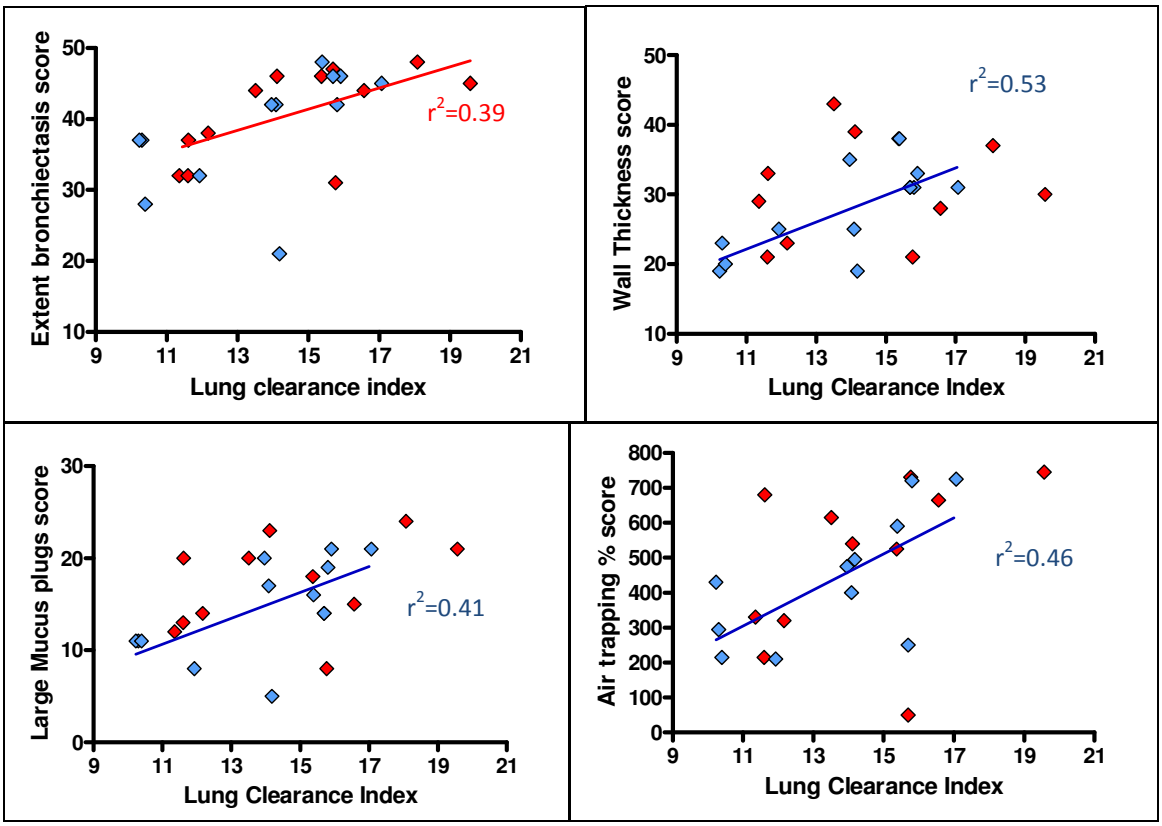


Figure 4.20: Correlation between LCI and CT score for extent of bronchiectasis (top left), airway wall thickness (top right), large mucus plugs (bottom left) and percent gas trapping (bottom right). Visit 1 data are shown in red, visit 2 in blue. Statistically significant correlations are represented by the regression line in the corresponding colour.

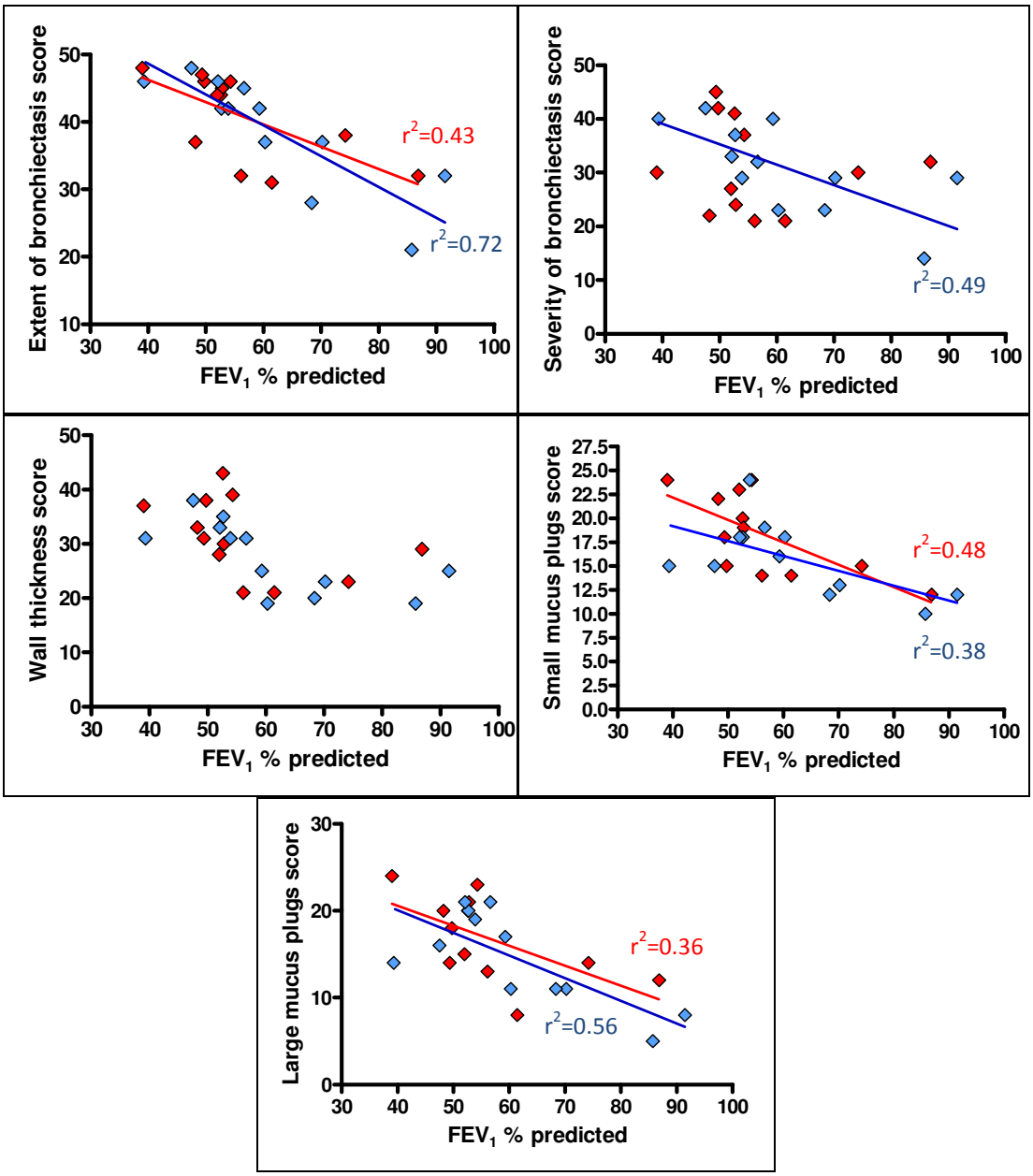


Figure 4.21: Correlation between FEV_1 % predicted and CT score for extent of bronchiectasis (top left), severity of bronchiectasis (top right) and airway wall thickness (middle left), large mucus plugs (middle right) and small mucus plugs (bottom).Visit 1 data are shown in red, visit 2 in blue. Statistically significant correlations are represented by the regression line in the corresponding colour.

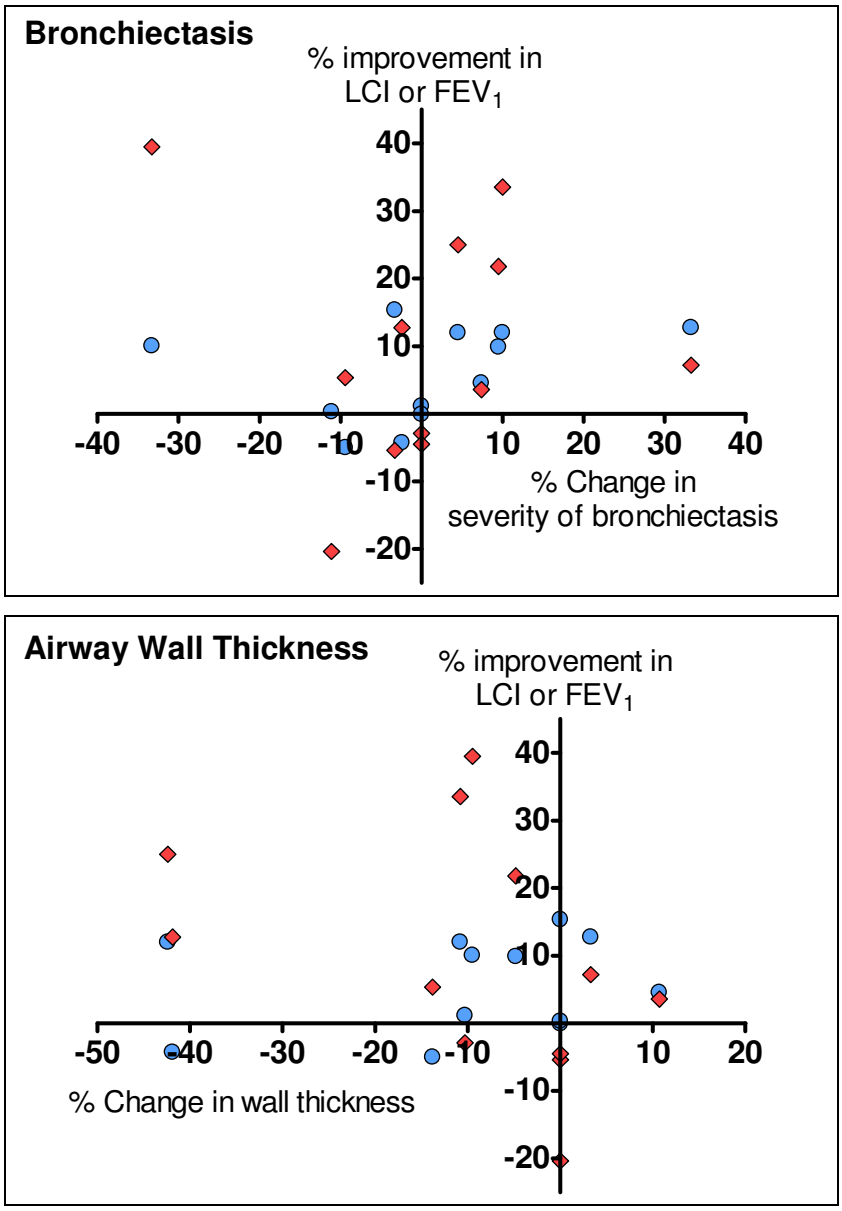


Figure 4.22: Percent change in severity of bronchiectasis score (top panel) and airway wall thickness score (bottom panel) after antibiotics, against percent improvement in LCI (blue circles) and FEV_1 (red diamonds). Improvements in FEV_1 and LCI are both positive, improvement in CT score is negative (i.e. a fall in the score).

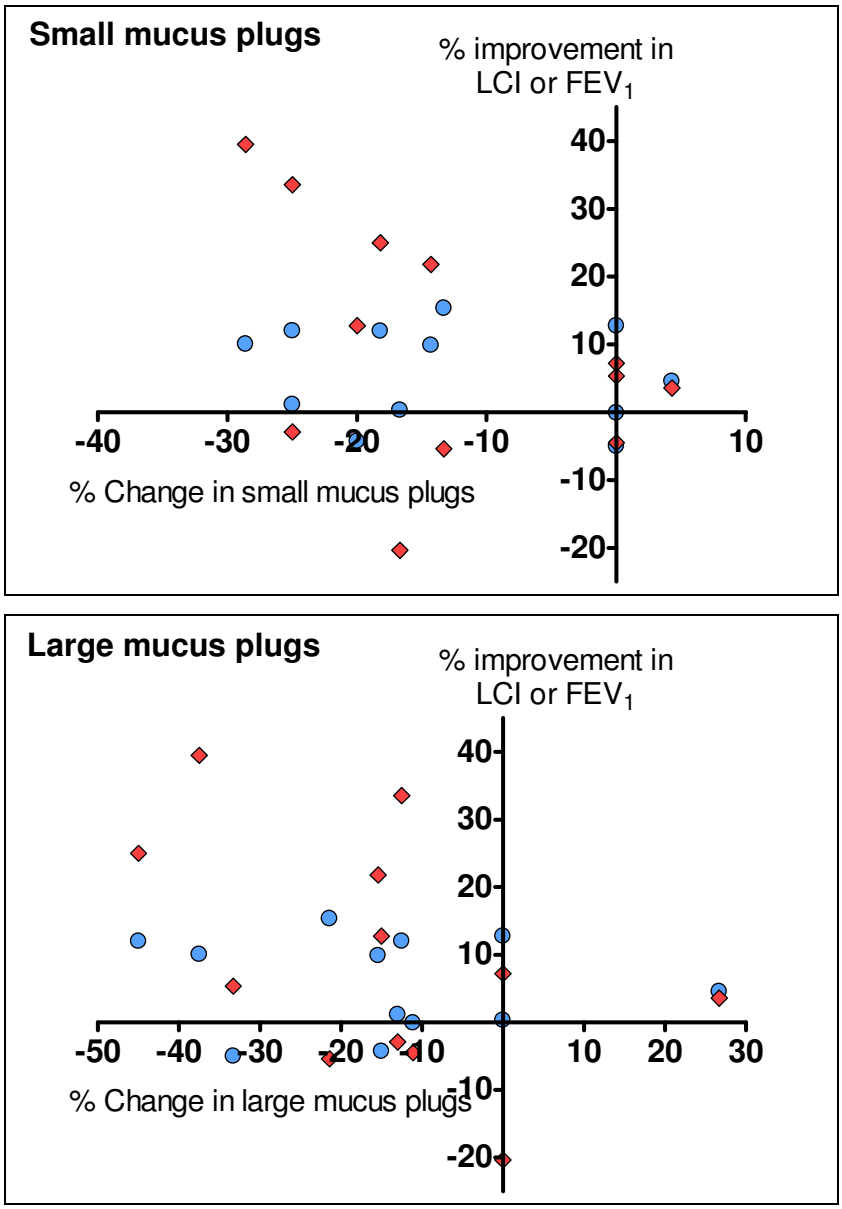


Figure 4.23: Percent change in small mucus plugs score (top panel) or large mucus plugs score (bottom panel) after antibiotics, against percent improvement in LCI (blue circles) and FEV_1 (red diamonds).

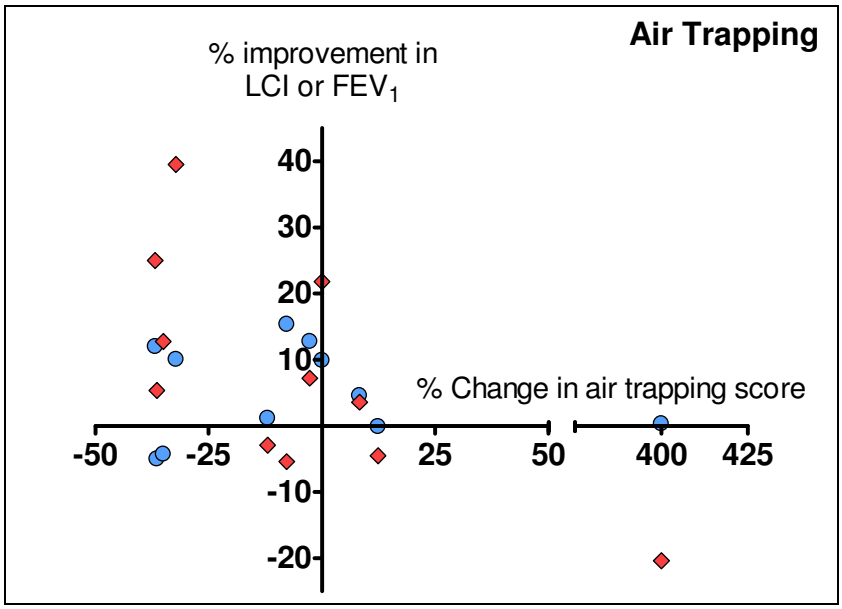


Figure 4.24: Percent change in percent air trapping score after antibiotics, against percent improvement in LCI (blue circles) and FEV_1 (red diamonds).

7. Response of sputum markers of inflammation.

Sputum was obtained by spontaneous expectoration from all 17 patients at visit 1. Two patients were unable to produce sputum at visit 2, and required induction of sputum by saline nebulisation. At visit 3, all 16 of the subjects who attended for this assessment were able to expectorate spontaneously.

Sputum cell differentials were performed on all subjects, but adequate total cell counts were incomplete due to a lab error. Cell counts were therefore only obtained from 7 samples at visits 1 and 2, and from 9 samples at visit 3. Summary cell differential and cell count data are presented in Table 4.9. There was no significant change in any of the cell differentials with treatment (Kruskal-Wallis 1 way ANOVA). There was significant change in the total cell count between the visits, from a median of 3.4 $x10^6$/ml at visit 1, to 0.9 $x10^6$/ml at visit 2 (p=0.03 versus visit 1), and 2.6 x 10^6/ml at visit 3 (p=0.03 versus visit 1). Therefore, despite there being no change in the percentage of neutrophils, there was a fall in the absolute neutrophil count after antibiotics (p=0.039), see Figure 4.25. There was no change in the absolute numbers of the other cell types. Given the small numbers of subjects involved, the cell count data should be interpreted cautiously.

Sputum IL-8 is a recognised marker of inflammation, and has been previously shown to be elevated in patients with CF, and correlates with lung function and other sputum inflammatory markers (Mayer-Hamblett, Aitken et al. 2007). In this cohort there was no significant change in sputum IL-8 with antibiotics (Figure 4.25). Mean (SD) sputum IL-8 at the start of the exacerbation was 15.4 (10.7) ng/ml. At visit 2, sputum IL-8 was 19.8 (15.2) ng/ml, and at visit 3 it was 15.2 (12.4) ng/ml (p=0.62, 1 way ANOVA).

	Visit	Neutrophils	Lymphocytes	Macrophages	Eosinophils
Cell differentials (%)	**1 n=17**	91.7 [83-99]	6 [0.9-14]	0.7 [0-5.3]	0.5 [0-5.3]
	2 n=17	91.9 [67-99]	6 [0.4-16]	0 [0-4.7]	1 [0-17.2]
	3 n=16	93.2 [75-98]	5 [0.9-12.5]	0 [0-1.5]	0.8 [0-12.5]
Cell counts x10^6/ml	**1 n=7**	304.6 [157-354]	16.6 [6-39]	1.8 [0-11.3]	1.2 [0-2.7]
	2 n=7	75.5* [25-203]	4.7 [2-16]	0 [0-2.3]	0.7 [0-9.0]
	3 n-9	244.2*+ [52-611]	13.8 [2-29]	0 [0-2.0]	1.8 [0-22.6]

Table 4.9: Median [range] cell differentials and total cell counts for sputum obtained before and after antibiotics from CF patients.

* p=0.03 versus visit 1

+ p=0.03 versus visit 2

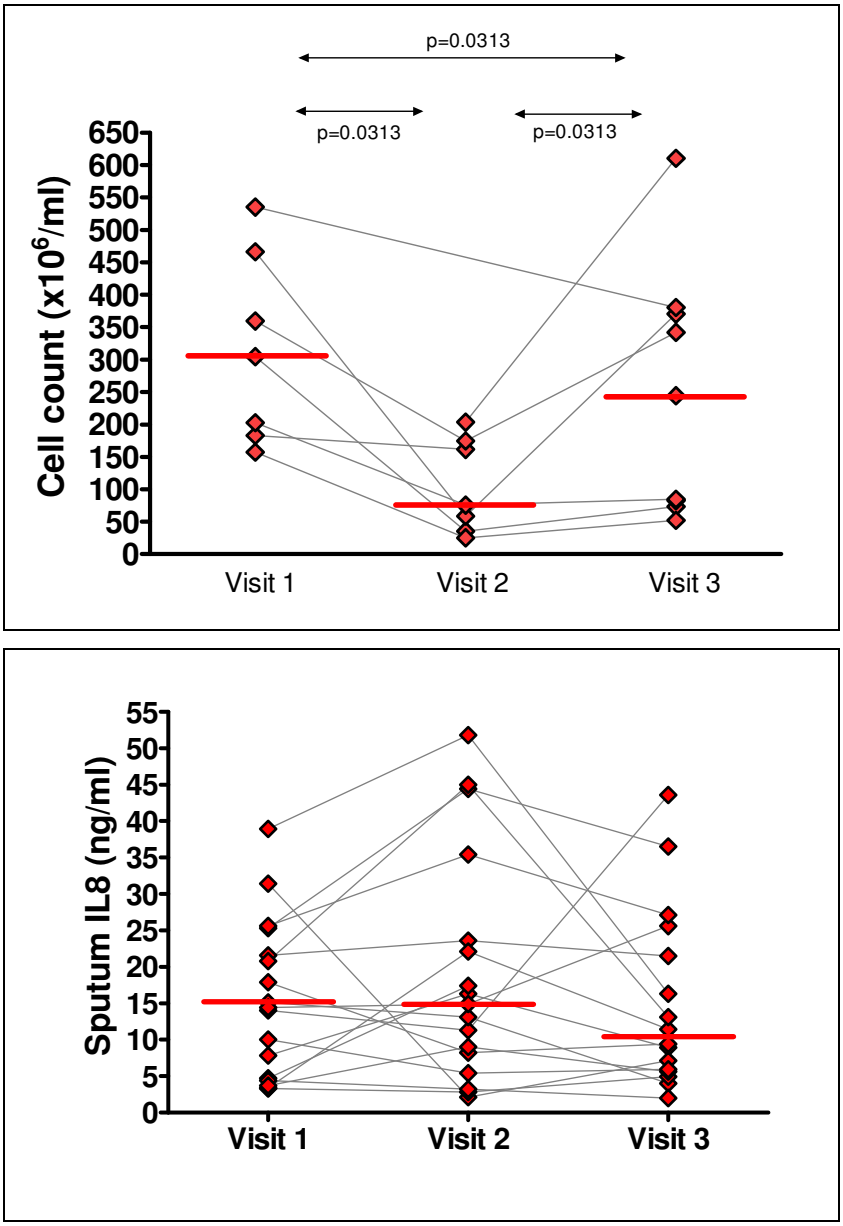


Figure 4.25: Change in total sputum neutrophil count (top panel) and sputum IL-8 concentration (bottom panel) with treatment of a CF exacerbation. Each set of points joined by a line represents a single patient. Red bars represent group medians (top) and group means (bottom).

Correlation between change in clinical and physiological measures of CF

The following parameters, selected on the basis of significant change and clinical relevance, were assessed for correlations between each other:

Change (between visits 1 and 2) in:-

- LCI (percent change)
- FEV_1 (percent change)
- Sputum IL-8
- Total sputum neutrophil count
- Peripheral blood white cell count
- Serum CRP
- Symptom score

As previously described, there was a significant correlation between the percent change in LCI and the percent change in FEV_1 between visits 1 and 2 (Pearson r=-0.59, p=0.01). LCI was not however significantly correlated with any of the other variables described above. FEV_1 percent change was also correlated weakly with change in symptom score (Pearson r=0.49, p=0.046), but not with any of the other variables. Change in sputum IL-8 was correlated with change in symptom score (Spearman r=-0.60, p=0.02), but this was due to the effects of a single outlier. There were no other significant correlations between any other of the variables.

Time to next exacerbation

It was hypothesised that low responders, in terms of magnitude of change in LCI or improvement in symptoms, might have improved only poorly and would exacerbate again quickly. The date of the next course of antibiotics, either oral or intravenous, was therefore retrieved from the patients' notes after the end of the study. This was compared to the percent change in LCI and to the change in symptom score between the preceding two visits. A single subject (EdTr012) has two entries because he was prescribed antibiotics both between visits 2 and 3 and after visit 3. These data are summarised in Table 4.10.

There was no consistent relationship between either change in LCI or change in symptoms, and time to next exacerbation (Figure 4.26). EdTr010 showed a significant elevation in LCI at visit 2. Despite an improvement in his symptom score at this time, he was sufficiently unwell to restart intravenous antibiotics 5 days later. However, when EdTr011 restarted antibiotics on the day of his third assessment, his symptom score had fallen by 18 points from visit 2, to -10, yet his LCI and FEV_1 were both better than at the post-antibiotic assessment. Apart from these two subjects, no other antibiotics were prescribed within 2 weeks of an assessment.

Overall, there was an average improvement in LCI of 0.8 between visits 1 and 2. When the data in Table 4.10 were divided on the basis of a rise (deterioration) in LCI between visits of greater than 0.8 (n=7) and a fall (improvement) of greater than 0.8 (n=6), there was no difference between the two groups in terms of median time to next exacerbation (59 days for those with an improvement in LCI, vs 62 days for those whose LCI deteriorated). The median change in symptom score was a fall of 3 points for subjects with an improvement in LCI and a rise of 5 points for those with a fall in LCI.

Time to next exacerbation was actually longer in those who had reported more symptoms at the preceding visit. When subjects were divided on the basis of an improvement or deterioration in symptom score of +/- 4 or more, there was a median time to next antibiotics of 72 days in those whose symptom score had deteriorated (n=5), and only 61 days in those whose score had improved (n=6) (p=0.44).

Subject number	Last visit before antibiotics	Time between visit and start antibiotics (d)	% Change in LCI at last visit	Change in symptom score
EdTr001	3	35	-0.5	1
EdTr003	3	93	5.0	-8
EdTr004	3	184	-25.7	-3
EdTr005	3	61	4.3	5
EdTr007	3	92	-15.4	0
EdTr008	3	72	-24.1	-6
EdTr009	3	80	-12.8	0
EdTr010	2	5	13.0	7
EdTr011	3	0	-1.1	-18
EdTr012	2	16	-14.1	10
EdTr012	3	70	-12.0	5
EdTr013	3	51	-12.0	-10
EdTr014	2	62	6.5	13
EdTr015	3	231	-10.1	-10
EdTr016	3	74	0.1	3
EdTr017	3	104	-4.6	5
EdTr051	3	46	-0.3	2
EdTr052	3	57	-9.9	0

Table 4.10: Time to next exacerbation in days, and change in LCI and symptom score at the last visit prior to starting antibiotics. There are two entries for EdTr012, corresponding to two different courses of antibiotics (between visits 2 and 3, and after visit 3). Percent change in LCI and change in symptom score refers to the difference between the preceding two assessments.

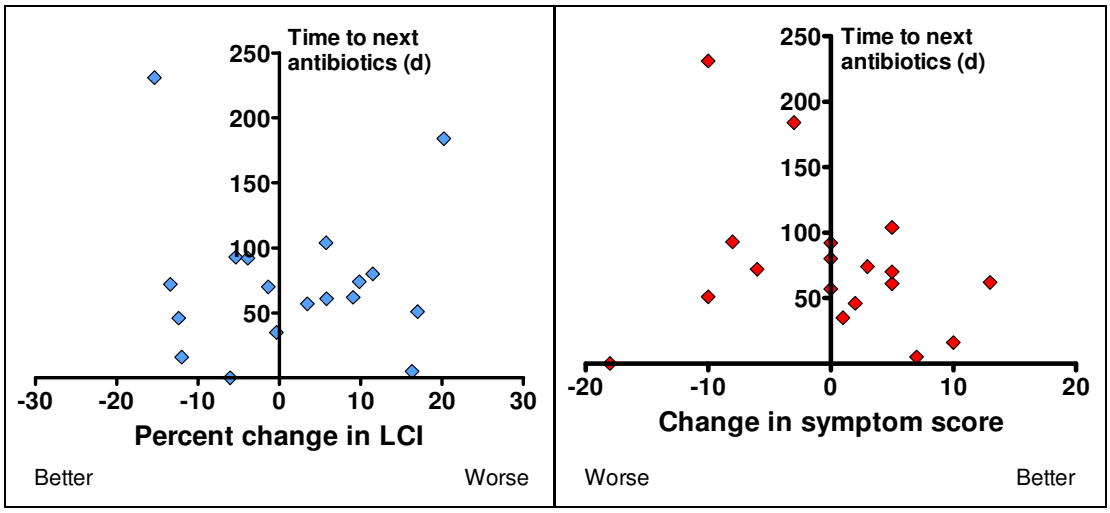


Figure 4.26: Time between last assessment and next course of antibiotics for a pulmonary exacerbation (days) plotted against the percent change in LCI (left, blue diamonds) or change in symptom score (right, red diamonds) between the preceding two visits. There was no consistent relationship between either the direction or magnitude of change in either of these variables and the timing of the next antibiotic course.

Discussion

This study represents the first time that LCI has been measured in CF before and after an intervention that is known to improve lung function. Previous investigators have only shown improvement in LCI in response to short term interventions, such as inhaled β-agonists in asthmatics (Gustafsson 2007). The improvement of LCI with antibiotics in CF is an important finding, since the ability of LCI to change in accordance with clinical status is vital if it is to be considered as an endpoint for therapeutic trials.

There are however a number of important differences between this study and the proposed gene therapy studies. The most obvious difference is that in this study the patients were unwell at the start, and have undergone assessment of LCI before and after treatment. This is a different scenario to that proposed for gene therapy studies. Patients with an exacerbation are known to have suffered a deterioration in lung function, and we anticipate that this will be reversible with treatment. FEV_1 in the subjects described here was 12% lower than their best measurement in the last 6 months. With gene therapy however, we will be treating stable patients and hoping to detect an improvement above their usual baseline. How easy that will be depends upon how much of the abnormality in gas mixing is reversible.

LCI is a measure of overall ventilation heterogeneity, which is in turn affected by a number of different processes in the CF lung. These include fixed abnormalities in airway and parenchymal structure due to fibrotic and destructive processes, as well as modifiable abnormalities due to regional differences in inflammation and mucus retention. This can be seen clearly in the CT images in Figures 4.17-4.19. Antibiotics have resulted in an improvement in the inflammatory processes, such as mucus retention (Figure 4.17) and small airway plugging (Figure 4.18), but the appearances of the underlying bronchiectasis are unchanged. Neither severity nor extent of bronchiectasis scores changed in this subject, and nor would we necessarily expect them to. Thus there is an "offset" in ventilation heterogeneity due to fixed abnormalities in lung architecture that are not amenable to improvement with antibiotics, and are unlikely to be improved even with reversal of the genetic defect.

In gene therapy studies there is particular interest in delivering treatments to subjects with the mildest disease. This is because the presence of mucus and pus filled airways in

those with more severely affected lungs may reduce transfection efficiency. Also, a long term aim of gene therapy would be to deliver to younger subjects, before disease becomes established. Adults with mild disease are the best available model of these patients. LCI is particularly useful in subjects with mild airways disease, and in Chapter 3 it has been shown to be a sensitive marker of airways disease in these adults. However, it is not known how much of the ventilation heterogeneity that causes this elevation in LCI is due to the effects of airway remodelling, and how much is due to the reversible effects of inflammation.

Subjects with mild airways disease are not well represented in the current cohort because they tend to suffer fewer exacerbations. Three patients had FEV_1 within the normal range at the end of treatment, and all had considerable elevation in LCI. All subjects also had measurable bronchiectasis, mucus plugging and air trapping at visit 2, although the lack of a matched healthy control population means that it is not possible to make judgements about how significant these findings are. However, it is encouraging that improvements in LCI were also seen in subjects with milder disease, not simply in those with the most severely affected lungs. Indeed if the cohort is divided into two groups, based upon the LCI at visit 2, the mean LCI improves by 9.2% in the half with the best (lowest) LCI at visit 2, but only improves by 2.2% in those with the highest LCI.

An improvement in LCI with treatment suggests that lung gas mixing is becoming more homogenous. Given that the mean FRC is unchanged, this suggests that ventilation is being improved to regions of the lung poorly ventilated at the start of the exacerbation. This is an important observation, since it is also possible that LCI could increase in treated patients if the treatment served to open up regions of the lung previously unventilated, though it is not known what volume of lung would be sufficient to cause a measurable change in LCI. As shown in Figure 4.11 however, there is considerable heterogeneity of response, with some subjects showing an increase in FRC and in LCI. This suggests that some less well ventilated lung regions are indeed being opened up, causing overall inhomogeneity to increase, and thus leading to a rise in LCI as a result of successful treatment (see Figure 4.27C). Indeed an increase in CT score for inhomogeneity was seen in CF patients treated with inhaled tobramycin solution, despite an improvement in the overall CT score (Nasr, Gordon et al. 2006). Figure 4.27 represents this diagrammatically. Although clearly an oversimplification, it represents two possible

outcomes of therapy, both increasing the volume of lung ventilated by tidal breathing (i.e. the FRC measured by washout) but one reducing and the other increasing ventilation heterogeneity. In vivo, the interactions between different lung units are not independent (Venegas, Winkler et al. 2005), and the effects on LCI and FRC of mucus clearance likely to be complex and unpredictable (Mentore, Froh et al. 2005).

Changes in other markers

LCI is the only endpoint that has not been assessed previously in CF patients over the course of treatment for an exacerbation. Previous studies have shown improvements in spirometry and CRP with treatment of an exacerbation, similar to the data presented here (Bell, Bowerman et al. 2000; Cunningham, McColm et al. 2003; Downey, Brockbank et al. 2007). These markers provide useful confirmation that the patients in this study were similar to previously reported studies, were unwell at the start of therapy, and improved with treatment.

IL-8 was included in this study because it is considered to be an important chemo-attractant in the CF airways, and is known to be induced by both elastase and Tumour Necrosis Factor-α (TNFα) (Konstan and Berger 1997). IL-8 has been shown to correlate with FEV_1 in a large cross sectional analysis, combining 269 patients from 4 different studies (Mayer-Hamblett, Aitken et al. 2007). IL-8 has also recently been listed as a candidate biomarker of CF airways inflammation (Sagel, Chmiel et al. 2007). However, despite being elevated in CF, IL-8 has not previously been shown to fall significantly with treatment. Cunningham et al. reported a non-significant decrease in sputum IL-8 in 14 children undergoing parenteral treatment for a pulmonary exacerbation (Cunningham, McColm et al. 2003). More recently, Downey et al. could find no change in a number of sputum sol mediators, including IL-8 , in 16 adult patients after 2 weeks of treatment for an exacerbation (Downey, Brockbank et al. 2007). A different kit was used to assess IL-8 in the current study, which may explain why the levels of IL-8 were so much lower than in the study by Downey et al.. Nonetheless, the failure of IL-8 to improve with therapy is in agreement with previously published observations, and casts a shadow over its potential as a useful biomarker in interventional studies.

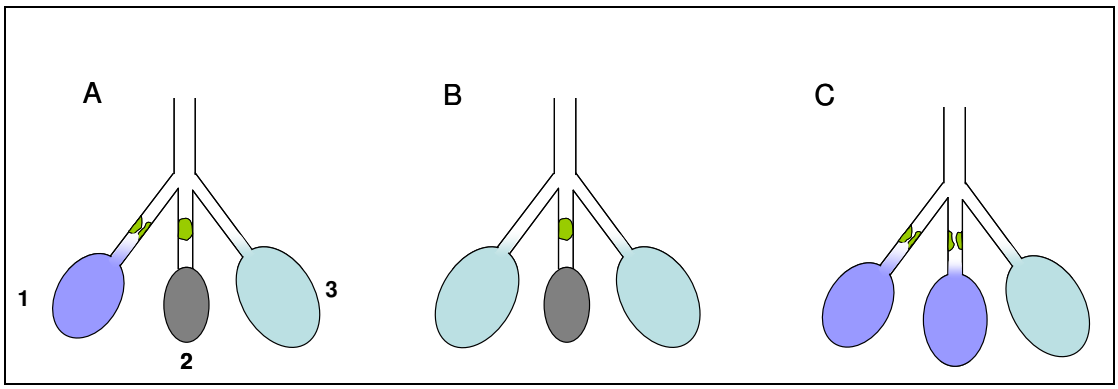


Figure 4.27: Possible effects of mucus clearance in the lungs on ventilation heterogeneity (LCI) and ventilated lung volume (FRC).

The figure represents three lung units of the same size. Figure 4.27A shows the units pre-treatment; 1: partially obstructed and poorly ventilated, 2: completely obstructed and unventilated, 3: unobstructed.

Figure 4.27B represents one possible outcome of treatment on the same three lung units: the partially obstructed unit has been cleared and is now normally ventilated. This should reduce ventilation heterogeneity (and hence LCI) and increase FRC.

Figure 4.27C represents another possible outcome. The unventilated unit has now been partially opened up and is poorly ventilated. This will therefore also increase FRC but will increase ventilation heterogeneity, leading to an increase in LCI.

Downey et al. also reported a fall in absolute neutrophil count, although this did not achieve statistical significance. Similar to this study, they reported no change in percent cell counts or absolute counts of the other cell types (Downey, Brockbank et al. 2007).

Over the last few years HRCT has attracted interest in the evaluation of CF lung disease, including as an endpoint in interventional studies. It is known to be more sensitive than traditional measures of lung function, and a number of scoring systems have been developed and been shown to have good inter-observer reproducibility (Aziz, Davies et al. 2007). Changes on CT have been shown to correlate with important clinical outcomes, such as pulmonary exacerbations (Brody, Sucharew et al. 2005), and to correlate with regional inflammation, assessed by percentage neutrophils and IL-8 levels on bronchoalveolar lavage (Davis, Fordham et al. 2007). Global HRCT scores have also been shown to improve in 16 CF patients treated with inhaled tobramycin solution, whereas in the placebo group there was no change (Nasr, Gordon et al. 2006). The CT scans included in this study were analysed by some of the leaders in this field, and their analysis has also shown good reproducibility (Aziz, Wells et al. 2007). The scoring system used however is one that they have developed themselves. Although this contains very similar components to those of previously described systems, this does not permit direct comparison with previous studies nor with previously reported healthy controls. CT appearances before and after antibiotics have been reported in 19 CF subjects by Shah et al. (Shah, Sexauer et al. 1997). They observed a reduction in airway wall thickness in 2 subjects, and in mucus plugging in 6, but no change in severity and extent of bronchiectasis, or air trapping. These observations are similar to those reported here, where the most significant changes were in scores for mucus plugging. Unlike Shah et al., air trapping and wall thickness did improve in an appreciable percentage of subjects, which may reflect the sensitivity or the scoring system, the resolution of the scans, or the experience of the observers.

Visit 3 and time to next exacerbation

The inclusion of a third visit was intended as an assessment of the patients at stability, and this assessment was intentionally deferred if the patient did not feel well. This was actually quite difficult to achieve in a number of patients, as evidenced by the extended delay between visit 2 and visit 3 in several cases. This is also illustrated in the spread of symptom scores at visit 3, far greater than at visit 2 when the majority of subjects actually felt better than "normal". Although severity of symptoms is subjective, and influenced by a range of psychological and sociological factors (Riekert, Bartlett et al. 2007), it is clear that an assessment of what is "normal" or stable is hard to achieve in some patients with CF. There is also a discordance between symptoms and other measures of well being, including measures of lung function. Despite the symptom score being focussed on respiratory symptoms, mean FEV_1 actually improved between visits 2 and 3, yet symptom score fell.

The change in LCI at visit 3 is also important. If LCI is to prove useful in long term monitoring of subjects, then it needs to be relatively stable when the subjects are well. At visit 3, the mean LCI was not significantly different from that at visit 2, but there was considerable individual variation in this. In healthy subjects, LCI has good reproducibility between two visits, and the intra-visit coefficient of variation for repeat assessments is less than 5% in both controls and CF patients (Chapter 3). In CF however, the reproducibility may be poorer between visits on different days due to changes in inflammation or mucus distribution. The question of how reproducible LCI is when subjects are stable, or as near to this as is realistically possible, is important because the level of variability affects the statistical power of LCI to detect real changes in lung physiology in response to treatment. This question cannot be addressed by this study, which involves relatively small numbers of subjects, between only 2 visits, at a time of changes in therapy and clinical status. A longitudinal study of many subjects, over a several months, is required to address these questions, and is currently in progress.

The other important feature of the third assessment was whether deterioration in LCI would be able to predict clinical deterioration. Clinical deterioration was measured as the time to the next course of antibiotics prescribed for a pulmonary exacerbation. No consistent relationship was seen between the change in LCI, the change in symptom score

or time to next exacerbation. The time to next exacerbation data should be interpreted with caution however, since only two subjects received antibiotics within 2 weeks of the assessment – one of these showed a significant deterioration in LCI and the other did not. It would be unreasonable to expect any assessment to predict how well or otherwise a CF patient would feel more than 2 weeks later. Also, presentation of respiratory symptoms in CF patients is complex, and the request for antibiotics is influenced by a variety of external factors. Thus, some subjects may have qualified for treatment earlier than that actually prescribed, but deferred or failed to present. These factors were not assessed in the present study.

Lung physiology and structural lung disease

Cross sectional comparisons of lung function assessments, including LCI, and HRCT chest have already been described. Gustafsson et al. reported on retrospective data collected on 44 CF patients aged up to 20 yrs (Gustafsson, de Jong et al. 2007). They used the Brody score, which evaluates 8 features of CF airways disease, and expresses the sum of the extent and severity of these as a percentage of the maximum possible score. Overall, LCI was more sensitive than either FEV_1 or FEF_{75} at predicting an abnormal CT score, and was particularly sensitive to an abnormal gas trapping score (>30%), with a sensitivity of 94%. These were patients with well preserved lung function, the majority having FEV_1 within the normal range, and almost a third having LCI within the normal range. In the subjects described here, all subjects had elevated LCI, and only three had FEV_1 within the normal range. There was still a significant correlation with the gas trapping score at visit 2 however, of a similar magnitude to that described by Gustafsson et al. (Gustafsson, de Jong et al. 2007). There were also significant correlations with the scores for airway wall thickness and large mucus plugs, again similar to the findings reported by Gustafsson et al.. Unlike the earlier study however, in this study there were more, and stronger, correlations between CT score and FEV_1, which probably at least partially reflects the greater number of abnormal FEV_1 measurements. FEV_1 showed strongest correlations with CT features of large airways disease, including extent and severity of bronchiectasis, airway wall thickening and large mucus plugs. There was no

correlation between air trapping, believed to be a feature of small airways obstruction, and FEV_1, though there was a weak correlation between the small mucus plugs score and FEV_1.

No convincing associations were seen between changes in CT score and changes in conventional lung function measurements. This may be related to the nature of the CT scoring system used, which considers each feature as independent and does not produce an overall CT score. The lack of correlation between CT and lung function changes may therefore be due to the fact that there is no single structural feature that is disproportionately responsible for the deterioration in lung function seen at the start of an exacerbation. Instead there is a combination of abnormalities that all contribute. It is possible that these associations would become more apparent, and statistically significant, if greater numbers of subjects were included in the analysis.

Relationship between LCI and FEV_1

In Chapter 3, LCI was shown to be a more sensitive indicator of airways dysfunction in CF patients than FEV_1. In this study, on the face of it, LCI has performed less well than FEV_1, with a mean precentage change of only around half that seen in FEV_1, and far less than that seen in FEF_{25-75}. To a certain extent this is to be expected. Deterioration in FEV_1 is one of the few objective criteria routinely employed in the decision to start antibiotics. The mean percent fall in FEV_1 (from best baseline) at the start of the study was almost exactly the same as the percent improvement in FEV_1 after treatment (12.4% versus 11.1% respectively). An improvement in FEV_1 therefore confirms the success of the antibiotic treatment. Furthermore, since FEV_1 falls with worsening lung function, any improvement is measured against the initial (lowest value). LCI on the other hand rises with deterioration in gas mixing, and improvement is measured against the higher value (LCI improves by 7.3% if measured against the visit 2 value). In addition, there is an offset to LCI, equivalent at least to the lower limit of the normal range (5.95, see Chapter 3), below which LCI cannot fall, no matter how well the patient. Although FEV_1 also possesses a lower limit, this is considerably lower, relative to normal range, than that of LCI. This must be borne in mind when considering the percent change in LCI. Finally, FEV_1 is technique and effort dependent (Krowka, Enright et al.

1987), and unwell patients often struggle to complete the manoeuvre, with maximal effort, to obtain three reproducible repeats. LCI however is independent of effort, and is not affected by how systemically well the patient feels. These factors may all contribute to the smaller percent improvement seen with LCI as opposed to FEV_1.

However, it is important to note that LCI is not proposed as a replacement for FEV_1. The two measures are quite distinct, and measure different, and complementary, aspects of lung physiology. FEV_1 is a less physiological manoeuvre than multiple breath washout, and is insensitive to early airways disease. This has been recognised for some time (as discussed in Chapter 1). That does not mean however that spirometry is not a valid assessment of airways function, particularly in those with more marked airways disease and measurable impairment in FEV_1. In these subjects, LCI is less useful since, from a practical point of view, it takes longer to perform, and there is a trend towards less reproducibility. Certainly, in subjects with an FEV_1 below 40% predicted it becomes considerably more difficult to perform accurately and may not be an appropriate physiological measurement in this population.

There is a significant correlation between FEV_1 and LCI, and between the change in these two measurements. Since both measure aspects of lung physiology, correlation between them is to be expected. However, they are affected to differing extents by disease of different regions of the bronchial tree, and this is emphasised by the features on CT that they show strongest correlations with. If a strong correlation existed between FEV_1 and LCI, this would be both surprising and would make the newer and more complex measurement less useful.

Multi-centre use of Innocor to measure LCI

From the point of view of the CF Gene Therapy Consortium, the establishment of equipment and expertise to measure LCI on multiple sites was an important objective of this study. Although this thesis only includes data collected in Edinburgh, it is nonetheless worthwhile considering some of the lessons learnt. Despite being used in three centres by three different operators, the system was largely trouble-free. This was in no small part due to the robustness of the equipment, which meant that there were no major technical challenges during the eight months that this study was recruiting. The only minor

technical problem occurred with the machine used at the Royal Brompton Hospital. There was evidence of occasional interference in the SF_6 signal in a handful of washouts (not more than three), that looks much like an electronic pulse, such as that generated by a mobile phone. This was resolved by excluding the affected portions of the breaths from analysis, but cannot be done without some impact on accuracy.

It was also apparent that there was a learning curve associated with the performance of washout tests, and a degree of operator-dependency. In addition, unwell CF patients may be less receptive to being taught new techniques, especially when they are protracted. The process is made more difficult because Innocor does not offer an effective real time feedback. The operator is not provided with a last-breath expiratory volume display, and the SF_6 signal display is a rolling average, delayed 1.5 seconds by the flow-gas delay, rather than a real time feedback of the actual SF_6 concentration. Furthermore the readout available to view washouts whilst they are progressing is too small to do much more than look for leak and to grossly confirm adequacy of wash-in and washout. With experience, these limitations can be managed, but occasionally problems with washout quality are only apparent after the test is complete and the data are exported for analysis.

In order to confirm the integrity of the washout procedures, a Standard Operating Procedure was written both for the conduct of the washout tests and for the analysis of data (Appendix A & B). Accuracy of data analysis amongst the three operators was confirmed by a reciprocal sharing and analysis of data from each of the three sites (Macleod, Horsley et al. 2007).

Limitations of this study

The limitations of the use of time to next course of antibiotics as an outcome have already been discussed. The major limitation of the data presented in this chapter are the small numbers involved. This makes it difficult to generate statistically meaningful correlations, particularly in the case of the CT data where there are only 12 subjects with paired data. It also means that the data are particularly susceptible to the effects of outliers. Despite this, the correlations between CT score and lung physiology at visit 2 are

similar to those previously reported in a much larger cross sectional study, which supports the validity of the findings (Gustafsson, De Jong et al. 2008).

The data presented here are only those from the Edinburgh patients, although another 25 subjects were recruited at the Royal Brompton Hospital (of whom 21 have at least 2 visits). These data have not been included in the analysis presented here, for a number of reasons. These data will be jointly analysed at a later date following consensus review.

Summary

Lung clearance index improves with antibiotic treatment in CF patients with a pulmonary exacerbation. This is despite no significant change in the ventilated lung volume, suggesting that treatment improves the ventilation of regions of the lung poorly ventilated during an exacerbation. LCI is complementary to spirometry as a measure of lung function, and is more closely correlated with measures of small airway function (CT score for air trapping) than FEV_1.

This confirms that LCI is able to change appropriately in response to an intervention – an important observation for the development of LCI as a biomarker of airway function in CF.

Chapter 5 - Effects of cystic fibrosis lung disease on gas mixing indices derived from alveolar slope analysis

Introduction

The preceding two chapters have involved the use of lung clearance index (LCI) as the primary outcome measure of the multiple breath washout tests. The washout curves however contain more information than is necessarily summarised by a single figure derived from the cumulative expired volume required to wash out the tracer. Over the years a number of attempts have been made to refine washout analysis in order to extract more of the information. Phase III slope analysis was first described in a clinical study by Verbanck et al. over 10 years ago (Verbanck, Schuermans et al. 1997). This analysis is particularly significant because it proposes that the ventilation heterogeneity can be divided into two components, reflecting processes in different parts of the bronchial tree.

The analysis is explained in more detail in the following section but, in summary, involves plotting the concentration normalized phase III slope of the individual breaths of the washout against the lung volume turnover (obtained by dividing the cumulative expired volume by the FRC) (see Figures 5.1 and 5.2). This profile is then divided into two separate components, each reflecting a different aspect of gas mixing. The first component is called $S_{cond,}$ since it is considered to be determined purely by convective gas mixing in the conducting airways. The second component is considered to originate from interaction between diffusion and convection in the acinar zone in healthy lungs, and is labelled S_{acin}.

In children with well controlled asthma, LCI was significantly higher than controls, despite no difference between the cohorts in baseline FEV_1 or FeNO, and remained higher after nebulised salbutamol (Macleod, Horsley et al. 2008). S_{cond} correlated with LCI, though the difference between controls and asthmatics was not statistically significant. In adult asthmatics, S_{cond} is a predictor of airways hyper-responsiveness and responds to treatment with bronchodilators, whilst in smokers S_{cond} shows a persistent improvement with smoking cessation (Verbanck, Schuermans et al. 1999; Verbanck, Schuermans et al. 2006; Downie, Salome et al. 2007). In these studies, abnormalities in S_{acin} appear to be

less reversible than those of S_{cond}. In COPD, S_{acin} correlates with gas transfer, a marker of alveolar integrity (Verbanck, Schuermans et al. 1998; Verbanck, Schuermans et al. 2004).

Phase III slope analysis has become increasingly prominent over the last few years, and has recently been the subject of editorials in major respiratory journals (Cosio 2006; Venegas 2007). It promises to offer a method of assessing effects of treatment on conducting airway function, an appealing proposition for CF gene therapy studies. However, the analysis has so far largely remained restricted to a single unit and their collaborators. More importantly, it has not been performed previously in adults with CF. Because the derivation of S_{cond} and S_{acin} was based upon experimental observations and modelling of gas mixing processes in the distal airways of healthy adults (Paiva and Engel 1984; Crawford, Makowska et al. 1985), it is not known how the very different pathologies present in the CF lung would affect it.

Alveolar function is known to be preserved in CF until late in the disease. The original hypothesis behind this study was thus that CF would primarily affect the conducting airways, and therefore S_{cond}, and that this would correlate with LCI as a measure of overall ventilation heterogeneity, and with lung function measurements that reflect conducting airway function (e.g. FEV_1 and R_{aw}).

Aims:

The aims of this study were to explore how ventilation distribution is affected by CF lung disease across a range of severities.

1. To characterise how S_{cond} and S_{acin} change with increasing severity of CF lung disease, as defined by worsening FEV_1 and LCI.
2. To correlate S_{cond} and S_{acin} with other markers of lung function, specifically transfer factor (as a marker of alveolar function), airways resistance (R_{aw}), and RV/TLC (a measure of gas trapping).

In addition, data collected by Dr Kenny Macleod on S_{cond} and S_{acin} in children have also been included in order to better explore the relationship between disease severity and phase III slope indices.

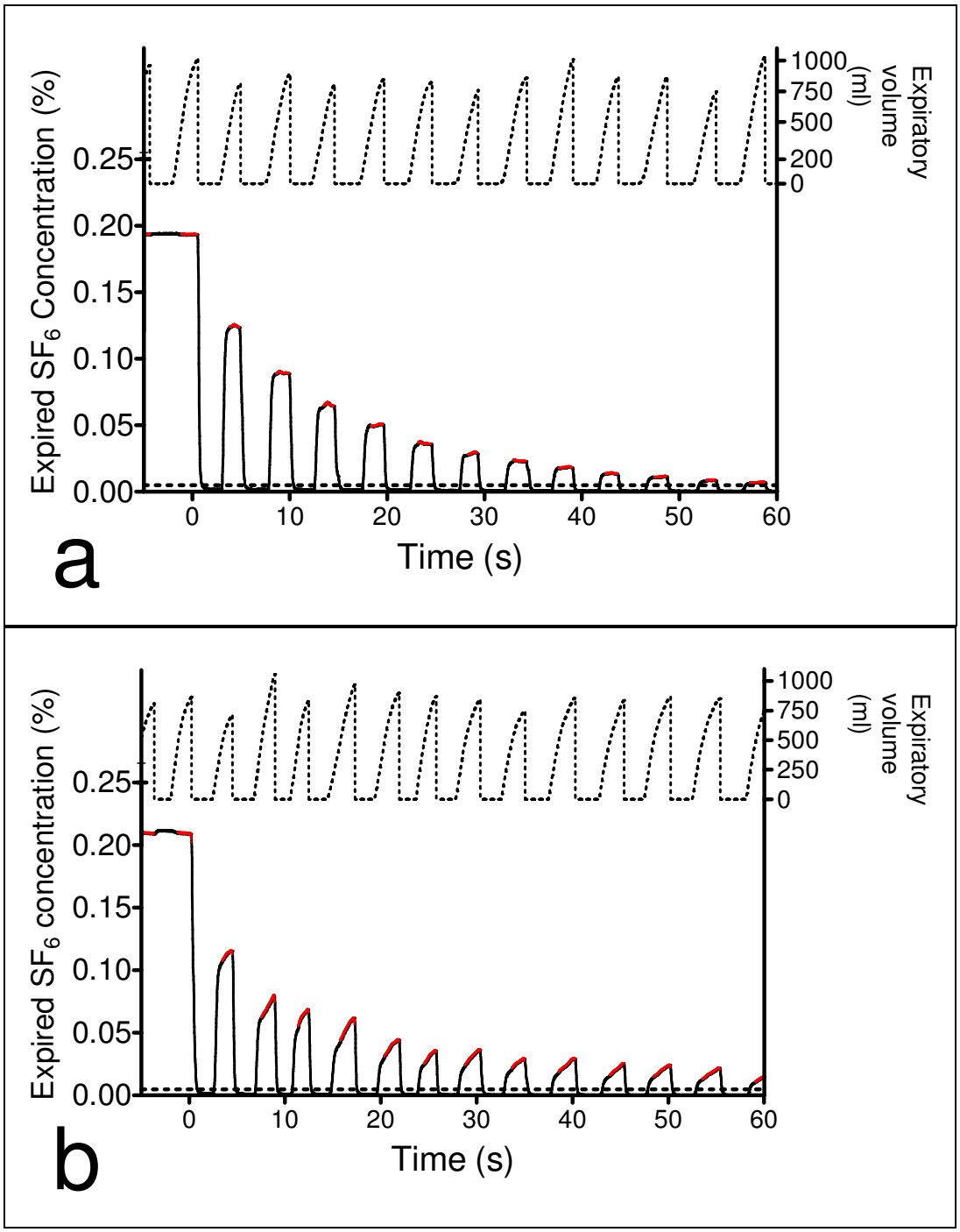


Figure 5.1a (top): Washout of a healthy subject (FEV_1 103% predicted, FRC 1.9L, LCI 7.0). SF_6 concentration is shown with phase III portion highlighted in red. Expiratory volume is shown by the dotted tracing in the upper part of the graph.

Figure 5.1b (bottom): Washout of a subject with moderate CF (FEV_1 58% predicted, FRC 2.3L, LCI 14.3). The phase III slope is much steeper, both at the first breath and over subsequent breaths. A volume-concentration plot of an individual breath from this same washout, illustrating how the phase III slope is calculated, is shown in Figure 5.2.

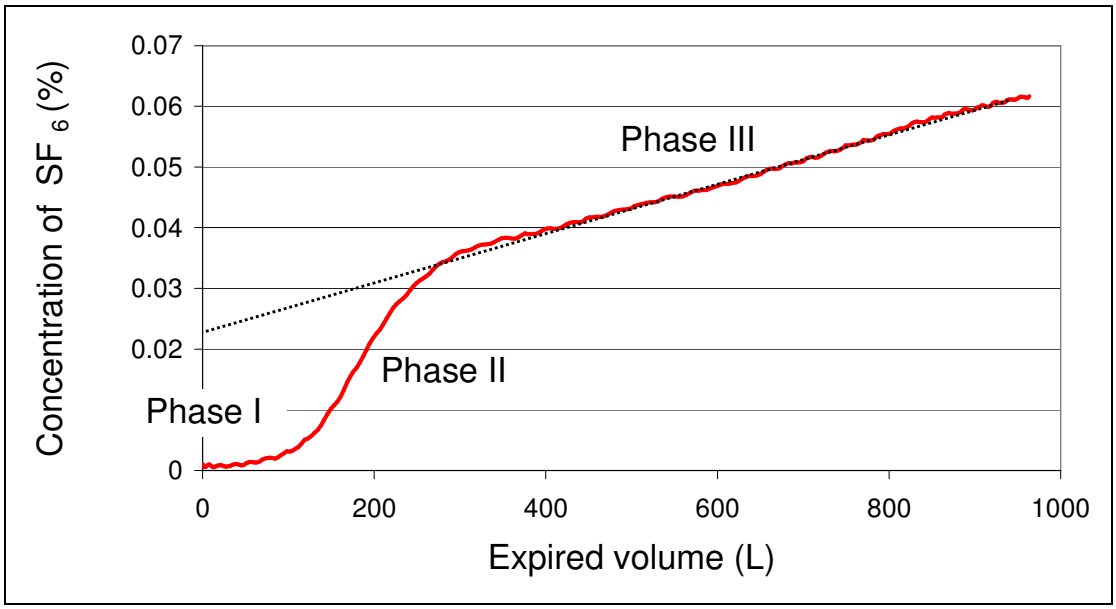


Figure 5.2: Identification of phase III slope. A single breath of the washout in Figure 5.1b is shown. Phase I of the washout represents anatomic deadspace, with little or no tracer gas concentration. Phase II represents the mixed alveolar and bronchial tracer gas, as the concentration of tracer rises rapidly to the sloping phase III ("alveolar") plateau. In some subjects an additional phase IV slope is seen as a steep upturn in the slope at the end of the breath. This represents the closing volume and the expiration of tracer from the least well ventilated regions of the lung. Linear regression of the SF_6 concentration over the final third of the breath volume (65-95% expired volume) is used to calculate the phase III slope, shown by the dotted line.

In addition, airway dead space can be calculated from analysis of phase I and II slopes, according to the method described by Fowler (1948). This is covered in more detail later in the chapter.

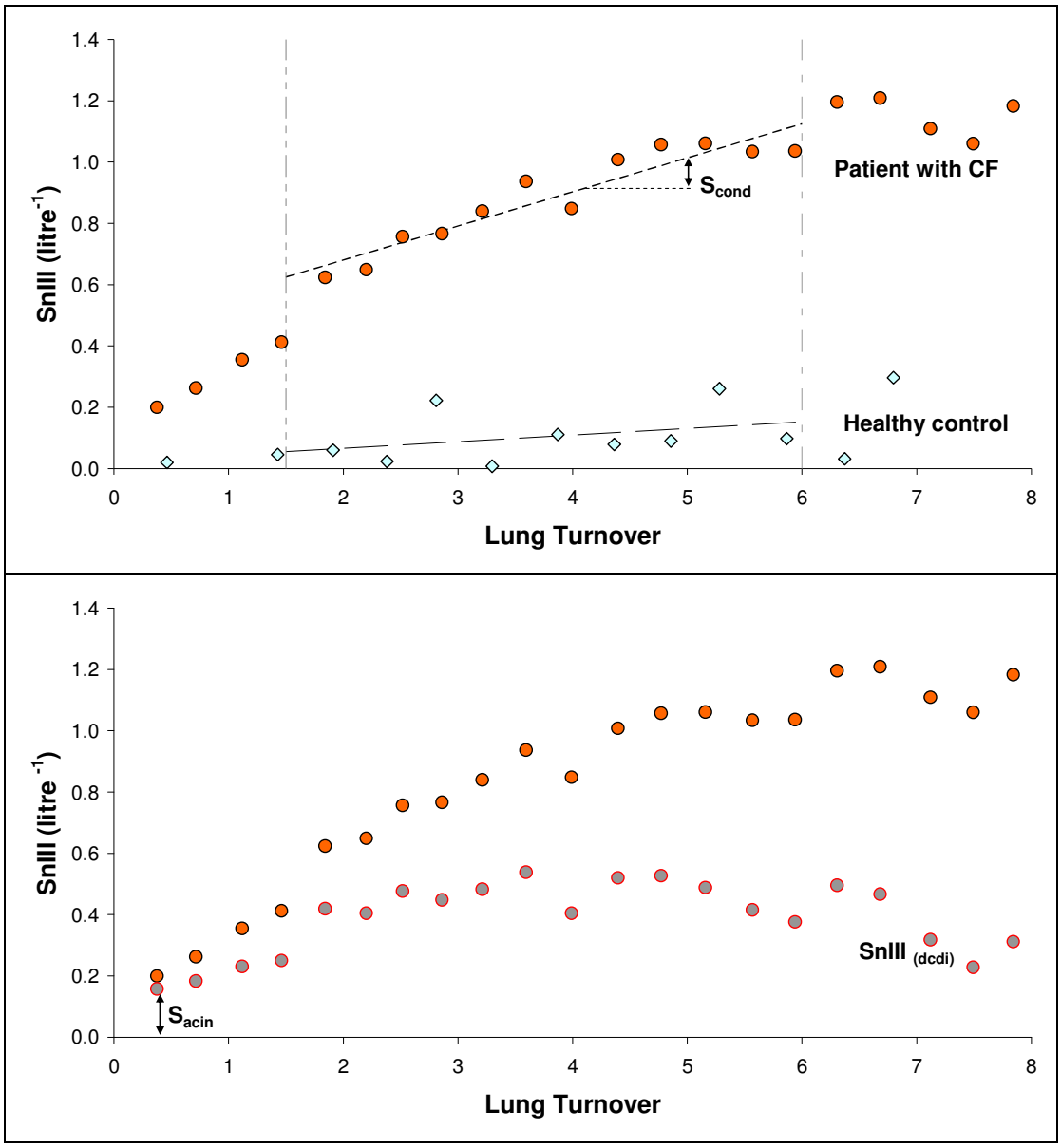


Figure 5.3a (top): Sn_{III} is plotted against lung turnover (TO) for the whole of the washout for a patient with cystic fibrosis (red circles) and for a healthy volunteer (blue diamonds). There are clear differences between the two subjects, relating both to the slope of the plot and the offset. S_{cond} is derived by linear regression of the portion of the slope between lung TO =1.5 to 6.

Figure 5.3b (bottom): This refers to further analysis of the CF data in Figure 5.3a. The convection dependent contribution to the total Sn_{III} is derived by multiplying S_{cond} by the lung turnover. This is then subtracted from the total Sn_{III} to give a measure of diffusion-convection dependent inhomogeneity ($Sn_{IIIDCDI}$, plotted as grey circles). The value of $Sn_{IIIDCDI}$ for the first breath is S_{acin}.

Phase III slope analysis

Using the single breath washout (SBW) test, ventilation heterogeneity is determined from the slope of the alveolar plateau, also referred to as the phase III slope (see Figure 5.2). Although SBW tests are useful clinical tools (Estenne, Van Muylem et al. 2000), they are not able to inform us about the mechanisms responsible for the observed inhomogeneity. Both diffusive and convective ventilation heterogeneity are present in the normal lung (e.g. gravity dependent variations in ventilation distribution (Milic-Emili, Henderson et al. 1966)) but are affected to varying extents by different disease processes. Within the normal human airways, diffusive gas mixing is the major contributor to ventilation distribution in the acinus (Dutrieue, Vanholsbeeck et al. 2000). Since diffusion is related to the molecular mass of the gas molecule, SBW tests can be refined by using helium and sulphur hexafluoride (SF_6) together. This allows inferences to be made about the ventilation of the most peripheral air spaces (Gronkvist, Emery et al. 2002).

Phase III slope analysis of multiple breath washout is an alternative analysis that aims to separate the contribution of the convection and diffusion to overall gas mixing (Verbanck, Schuermans et al. 1997; Aurora, Kozlowska et al. 2005). The technique of phase III slope analysis used in this study is similar to that originally described by Verbanck et al. (Verbanck, Schuermans et al. 1997). The alveolar slope of each breath of the washout is determined by linear regression of the alveolar plateau. This is initially identified from the final third of the expired volume, but can also be manually adjusted to allow for closing volume and physiological variability (see Figure 5.2). The phase III slope is then divided by the mean expired SF_6 concentration over the corresponding portion of the expiration, in order to account for the dilution of the marker gas, to give a concentration-normalised phase III slope (Sn_{III}) for SF_6. When plotted against lung turnover (TO) (i.e. cumulative expired volume divided by FRC), there is a progressive rise in Sn_{III} in those with abnormal gas mixing. The two different measures of ventilation heterogeneity are calculated from this plot of lung TO vs Sn_{III} (Figure 5.3).

When washouts are performed with gases of differing diffusivity, the difference in Sn_{III} values (ΔSn) between the two gases increase over approximately the first five breaths, but remain constant thereafter (Crawford, Makowska et al. 1985). According to this model, this indicates that the change in Sn_{III} over the middle portion of the washout

(TO 1.5 to 6) is independent of diffusive gas mixing - this is known as S_{cond}. Multiplication of S_{cond} by the TO number of a breath generates a measure of the convection dependent inhomogeneity (S_{CDI}) contribution to the total (measured) Sn_{III} for that breath. This can be subtracted from the total Sn_{III} of the breath to generate a measure of the diffusion-convection dependent inhomogeneity (S_{DCDI}), i.e. that occurring in the zone of the diffusion front, where convective and diffusive processes interact to produce a phase III slope (see Figure 5.3).

The two measures of ventilation heterogeneity have been given designations based upon the supposed anatomical location of the gas mixing process (Verbanck, Schuermans et al. 1997). Hence, the slope of Sn_{III} versus lung TO (between TO 1.5 and 6) is labelled S_{cond}, because it is believed to reflect convective gas mixing, which is considered to occur in the conducting airways. The S_{DCDI} value for the first breath is labelled S_{acin}, because this is considered to best represent diffusion-convection interaction. In healthy adults, modelling suggests that this occurs around division 20-21 of the bronchial tree for SF_6, within the acinus (Dutrieue, Vanholsbeeck et al. 2000).

Methods

Subjects

Seventeen healthy non-smokers (less than 10 pack years smoking history) with no known lung disease and on no regular respiratory medications were recruited as normal volunteers. Twenty nine healthy child controls, with no history of wheeze, asthma or prematurity (<34 weeks), were recruited from amongst those attending follow-up of stable upper-limb fractures as well as children of hospital staff. Twenty two CF adults were recruited from the Scottish Adult CF Service, and eighteen children with CF were recruited from the paediatric respiratory service at the Royal Hospital for Sick Children in Edinburgh. The diagnosis of CF was based upon a combination of clinical presentation and sweat testing and confirmed by genotyping. All volunteers and patients or guardians provided informed consent. Children too young to provide formal consent provided assent. This study was approved by the Lothian Research and Ethics Committee.

Multiple Breath Washout

LCI was measured by multiple breath inert gas washout, using the modified Innocor gas analyser and 0.2% SF_6 as the tracer gas, as described in Chapter 3. Unlike the studies by Verbanck et al. (Verbanck, Schuermans et al. 1997), subjects were not restricted to an expired volume of 1L. In children, the requirement for 1L breaths is impractical. When this was tried in adults, it was found that patients with CF experienced difficulty maintaining this artificial breathing pattern, which was also prone to induce coughing. Exhaled volume was displayed to the adult subjects on a separate screen, so that tidal volume could be targeted to between 500-1000 ml, but this was not used for children.

Figure 5.1 shows raw data illustrating the washout and the difference in phase III slopes between controls and patients.

Data analysis

The custom-built software described in Chapter 2 that is used for calculation of FRC and LCI is also able to analyse phase III slopes by linear regression of the concentration-volume plot of each breath of the washout. The software also calculates the mean expired SF_6 concentration over the same portion of the breath, and generates values for Sn_{III}. The default setting is for the alveolar slope to be identified as lying between 65 and 95% of the exhaled breath volume. Each breath is visually inspected to ensure that the linear portion of the phase III slope is selected and to achieve an accurate rendition of the phase III slope. In particular, in cases where a phase IV slope was visible on the tracing, this was not included in the calculation.

The software is only able to generate values for Sn_{III} for each breath, and does not calculate S_{cond}. Derivation of S_{cond} and S_{acin} from breath volume and SnIII data was therefore performed in Excel (Microsoft, Redmond, WA, USA) on a table generated by the MBW analysis software, containing breath number, breath volume and Sn_{III}. Plots of Sn_{III} versus lung volume turnover were inspected to ensure that there were no extreme outliers (e.g. from very small or rapid breaths), before linear regression between lung TO 1.5 and 6 to generate a value for S_{cond}.

Breath volume correction

Before calculation of S_{cond}, Sn_{III} values were transformed by multiplying by the corresponding breath volume in litres. This method was described by Aurora et al. (Aurora, Kozlowska et al. 2005) as a method of reducing the dependence of SnIII on expired volume. In a study of phase III slopes in 63 children, aged 2-17 yrs, the authors recognised that a strong hyperbolic relationship existed between first breath SnIII and expired volume, and furthermore that a large proportion of SnIII variability was explained by subject variables such as age, height and gender. By multiplying SnIII by expired volume for that breath (in litres), this relationship was abolished, and the association between S_{cond} and age disappeared.

Volume correction is a mathematical device which reduces the variability associated with breaths of different sizes, and also allows comparison between individuals of different ages and with previous studies. The approach is appropriate in the studies presented here because of the wide age range of subjects included. For assessments in adults alone, it would be much harder to justify the use of this correction. In the studies presented here however, the relationship between uncorrected and volume-corrected variables is strong, and the conclusions are unchanged if the uncorrected values are used in adults (see Results). To make it clear when the transformed data have been used, phase III slope indices derived by this method have been designated $S_{condVTc}$ and $S_{acinVTc}$ (V_Tc = tidal volume corrected).

Tests were performed in triplicate and LCI is quoted as the mean of at least two reproducible washouts. As a quality control measure, tests where the measured FRC differed by more than 10% from both of the other two repeats were excluded (Wanger, Clausen et al. 2005).

Lung function

All other lung physiology parameters were performed on standard lung function laboratory equipment according to ATS/ERS standards (Macintyre, Crapo et al. 2005; Miller, Hankinson et al. 2005; Wanger, Clausen et al. 2005). Plethysmography was performed on a Zan 500 USB plethysmograph (Ferraris Respiratory, Hertford, UK) or a Jaeger Masterlab plethysmography (Erich-Jaeger, Hoechberg, Germany). Lung volumes are quoted as the mean of three reproducible repeats (within 5%). Airways resistance was calculated as the specific resistance at 0.5L/s, R_{aw} (0.5), and is quoted as the mean of at least two reproducible repeats (within 10%). Diffusing capacity was assessed on a Collins Pulmolab (Ferraris Respiratory, Hertford, UK) using the single breath technique (Macintyre, Crapo et al. 2005). Measurements of KCO are quoted as the mean of two repeats. Reference ranges were taken from Quanjer et al. (Quanjer, Tammeling et al. 1993).

FEV_1 and FVC are quoted as the highest of three repeat manoeuvres (Miller, Hankinson et al. 2005). FEV_1 data are expressed as z-scores, as described by Stanojevic et al. (Stanojevic, Wade et al. 2008). For reference, percent predicted values for FEV_1 are also presented (Quanjer, Tammeling et al. 1993). In this regard, the presentation of spirometry differs from the other chapters in this thesis, which have employed the conventional percent predicted notation for FEV_1. The Stanojevic reference ranges were published after all these studies contained within this thesis had been completed, and after the studies in Chapters 3 had been published. The studies presented in the current chapter however were published after the new reference ranges. Since the published and peer reviewed manuscript derived from these data contains the Stanojevic z-score notation for FEV_1, this has been maintained in the current chapter, but the percent predicted values are also quoted for comparison with other chapters and published studies. The advantages of the Stanojevic reference ranges are that they provide more accurate reference ranges, particularly in adolescence, and improved definition of the lower limits of normality. They are particularly for useful for studies involving a wide age range of subjects (Stanojevic, Wade et al. 2008).

Lung function testing was performed within 3 hours of LCI measurement, and though the order of the tests was not fixed there was at least a 30 minute interval between

the completion of lung function testing and start of MBW. Exhaled gas volumes were converted to body temperature, ambient pressure, and saturated water vapour (BTPS) conditions.

Paediatric volunteers completed triplicate washouts followed by spirometry only, using an Easyone spirometer (Ndd Medizintechnik, Bern, Switzerland), according to ARTP guidelines (1994). Full lung function was not performed in these patients. FEV_1 data have been presented and analysed as z-scores (Stanojevic, Wade et al. 2008), but percent predicted values are also presented for reference (Rosenthal, Bain et al. 1993).

Statistical analysis

Data were analysed using Prism (GraphPad Software Inc, CA, USA). Parametric data are quoted as mean (SD), unless otherwise stated, and were compared using t-tests. Non-parametric data, and small datasets, are quoted as median (inter-quartile range), and compared using the Mann-Whitney U test. Correlations were analysed using Spearman's rank correlation. A p value of 0.05 was considered significant. A Bonferroni correction for multiple comparisons of independent variables was applied to the correlations in Table 5.3, and for this analysis a p value of below 0.01 was considered statistically significant. The upper limit of normality for S_{acin}, S_{cond} and LCI was defined at the mean + 1.96 x standard deviation of the control population.

Results

Twenty two CF adults completed MBW, spirometry and diffusing capacity assessments. Plethysmography was not completed in one adult patient because of technical problems with the apparatus. Seventeen adult healthy volunteers completed inert gas washout, and diffusing capacity. Plethysmography was not completed in 5 controls. A single CF adult was excluded from Sn_{III} analysis because she was only able to produce two reproducible washouts, which due to variability in breathing pattern could not be analysed accurately for Sn_{III} analysis.

Eighteen children with CF and 29 healthy child volunteers completed MBW and spirometry. No other lung function assessments were performed in children.

Demographics and lung function data for all 39 adult subjects are presented in Table 5.1 and for all 47 paediatric subjects in Table 5.2. Based upon the combined control populations, the upper limit of normality for LCI was calculated as 7.50, for $S_{acinVTc}$ was 0.230 and for $S_{condVTc}$ was 0.048.

	Healthy Adults	CF adults	Difference (95% CI)
Number	17	22	
Male / Female	10 / 7	13 / 9	
Age (yrs)	31.3 (6.0) [21-47]	28.9 (10.1) [17-47]	-2.4 (-8.0 to 3.2)
FEV_1 percent predicted	106.2 (8.2) [90.5-119.2]	66.4 (18.2) [29.4-106.8]	-39.9* (-49.5 to -30.2)
FEV_1 z-score	0.04 (0.76) [-1.26 – 1.77]	-3.03 (1.62) [-6.33 – 0.79]	-3.1* (-3.9 to -2.2)
FEV_1/FVC %	80.0 (5.6) [69.6-89.7]	63.0 (13.6) [34.8-87.1]	-17.0* (-24.2 to -9.9)
LCI	6.7 (0.6) [5.9 – 7.9]	12.8 (3.3) [6.2-17.6]	6.15* (4.46 to 7.84)
Mean expired volume (ml)	822 (154)	836 (140)	14 (-82 to 111)
Mean (SD) FRC (L) [mean percent predicted]	1.94 (1.10) [93%]	2.79 (0.77) [92%]	-0.13 (-0.76 to 0.49)
R_{aw} (0.5) (L/s)	0.12 (0.08) [0.03-0.29]	0.31 (0.13) [0.11-0.57]	0.18^{+} (0.10 to 0.27)
RV/TLC	22.6 (4.5) [16.5-33.0]	39.5 (9.5) [20.4-56.9]	16.8* (10.9 to 22.8)
Mean (SD) DL_{CO} [mean percent predicted]	10.42 (1.97) [96.5]	9.12 (2.58) [86.3]	-1.30 (-2.82 to 0.23)
Mean (SD) DL_{CO}/V_A [mean percent predicted]	1.62 (0.61) [95.0]	1.8 (0.28) [105.0]	10.0** (-0.29 to 20.30)
$S_{acinVTc}$	0.112 (0.055) [0.012 – 0.271]	0.366 (0.208) [0.052 – 0.742]	0.254*
$S_{condVTc}$	0.010 (0.015) [-0.028 – 0.032]	0.086 (0.030) [0.044 – 0.148]	0.076*

Table 5.1: Demographics and lung function of patients and controls. Values are means (standard deviation) and [range of values], unless otherwise stated.
R_{aw} (0.5) = Airways resistance; RV/TLC = Residual volume / total lung capacity; DL_{CO} = Diffusing capacity, DL_{CO}/V_A = diffusing capacity adjusted for alveolar volume.
*p<0.0001, $^{+}$p=0.0001, **p=0.03 (t-tests) compared to controls

	Healthy Children	CF Children	Difference (95% CI)
Number	29	18	
Male / Female	18 / 11	12 / 6	
Age (yrs)	11.1 (3.3) [5.3 – 16.2]	12.5 (3.5) [7.8-16.7]	1.4 (-0.7 to 3.4)
FEV_1 percent predicted	90.6 (11.9) [64.1 – 116.6]	89.9 (32.0) [47.7 – 164.8]	0.9 (-14.1 to 12.3)
FEV_1 z-score	-0.81 (0.97) [-3.32 – 1.23]	-1.32 (2.38) [-5.04 – 4.75]	-0.51 (-1.50 to 0.49)
LCI	6.2 (0.5) [5.1 – 7.1]	7.3 (2.3)$^{+}$ [4.8 – 14.0]	1.0^{+} (1.9 to 0.2)
Mean expired volume (ml)	538 (253)	487 (149)	-51 (-184 to 82)
Mean (SD) FRC (L) **[mean percent predicted]**	2.14 (1.02) [110.7]	2.01 (0.83) [120.7]	-0.139 (-0.73 to 0.45)
$S_{acinVTc}$	0.117 (0.062) [0.019 – 0.286]	0.192 (0.123) [0.038 – 0.497]	0.076* (0.021 to 0.130)
$S_{condVTc}$	0.015 (0.019) [-0.031 – 0.058]	0.068 (0.029) [0.022 – 0.124]	0.053** (0.039 to 0.067)

Table 5.2: Demographics and lung function of paediatric patients and controls. Values are means (standard deviation) and [range of values], unless otherwise stated.

$^{+}$p=0.022, *p=0.0074, **p<0.0001 compared to controls

Comparison between CF adults and healthy controls

CF adults had lower mean FEV_1 z-scores than healthy controls (-3.03 vs 0.04, $p<0.0001$), and higher mean LCI (12.8 vs 6.7, $p<0.0001$).

CF patients also had higher mean $S_{acinVTc}$ (0.366 vs 0.112, $p<0.0001$) and higher mean $S_{condVTc}$ (0.086 vs 0.010, $p<0.0001$) than healthy controls. There was no statistically significant difference in mean K_{CO} % between the two groups.

Comparison between CF children and healthy controls

There were no statistically significant differences in spirometry between the patients with CF and healthy controls. As with the adult subjects, children with CF had higher mean LCI (7.3 vs 6.2 in controls, $p=0.022$), and higher mean $S_{acinVTc}$ (0.192 vs 0.117, $p=0.007$) and mean $S_{condVTc}$ (0.068 vs 0.015, $p<0.0001$).

Comparison between CF children and adults

There was no significant difference between mean $S_{condVTc}$ in CF children and in CF adults ($p=0.06$), but mean LCI and mean $S_{acinVTc}$ were both significantly lower in CF children than CF adults ($p<0.0001$ and $p=0.0036$ respectively).

Association between LCI and phase III slope indices

The relationships between LCI and $S_{condVTc}$ and $S_{acinVTc}$ are presented in Figures 5.4 and 5.5 respectively. Elevation of $S_{condVTc}$ appears to be an early event in CF, occurring in both adults and children with normal LCI (Figure 5.4). However, $S_{condVTc}$ did not increase further with increasing disease severity, and appeared to reach an asymptote with a maximum value of 0.15. $S_{acinVTc}$ on the other hand, was within the normal range in the majority of children (Figure 5.5). In CF adults, $S_{acinVTc}$ showed a significant correlation with LCI (Spearman $r=0.86$ ($p<0.0001$)) but remained within the normal range until LCI was greater than 10.

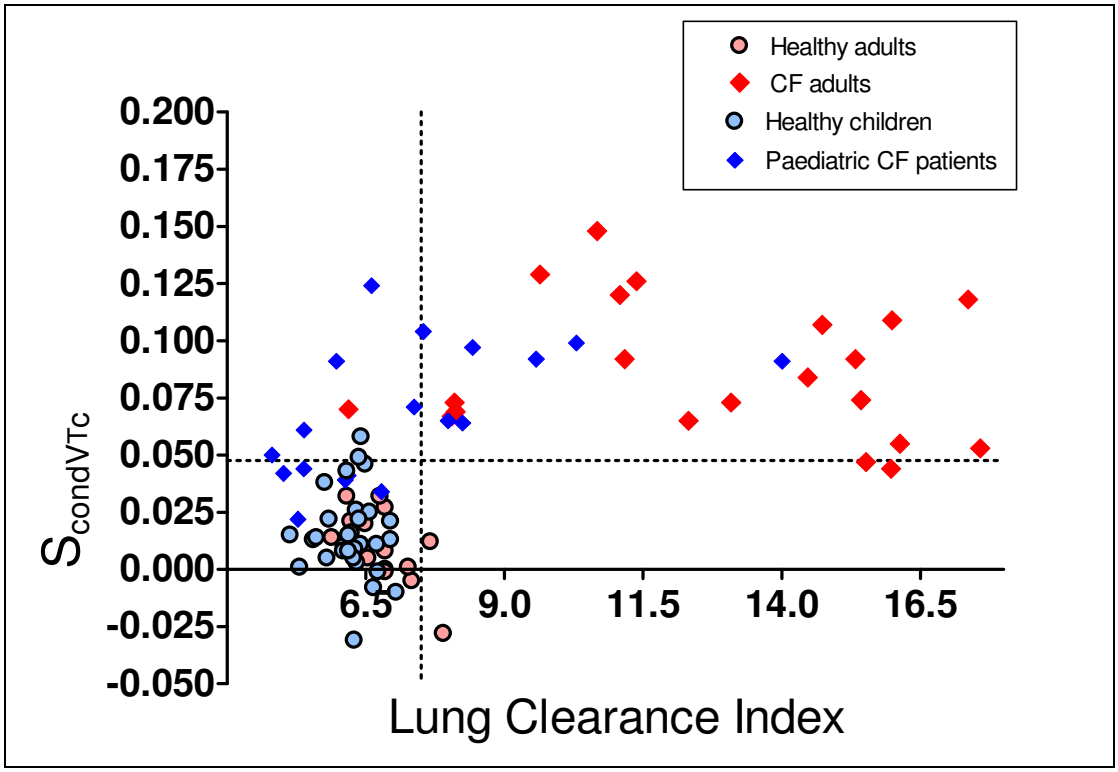


Figure 5.4: Relationship between $S_{condVTc}$ and LCI for healthy subjects and cystic fibrosis patients.

Healthy adults are represented as pale red circles and healthy children as pale blue circles. CF adults are represented as red diamonds and CF children as blue diamonds. The horizontal and vertical dotted lines represent the upper limits of normal $S_{condVTc}$ and LCI respectively.

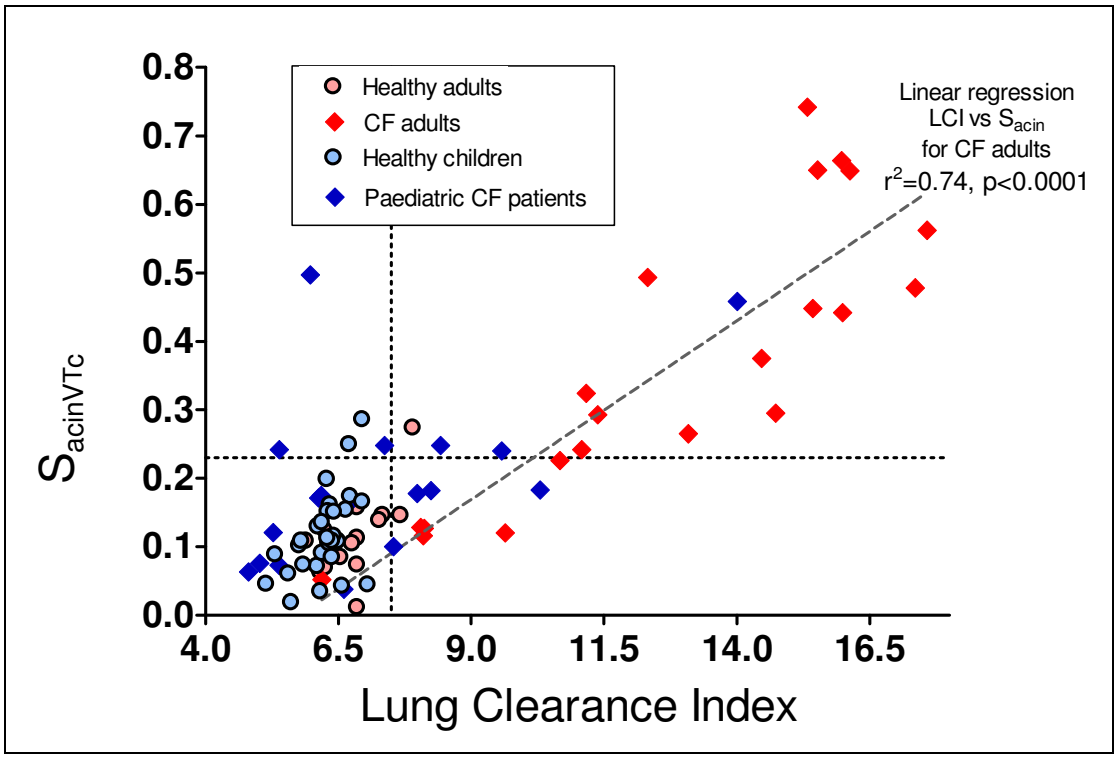


Figure 5.5: Relationship between $S_{acinVTc}$ and LCI for healthy subjects and cystic fibrosis patients.

Healthy adults are represented as pale red circles and healthy children as pale blue circles. CF adults are represented as red diamonds and CF children as blue diamonds. The horizontal and vertical dotted lines represent the upper limits of normal $S_{acinVTc}$ and LCI respectively. Linear regression of $S_{acinVTc}$ versus LCI for CF patients is represented by the diagonal line.

Association between S_{cond}, S_{acin} and other markers of lung function

In CF adults, $S_{acinVTc}$ showed significant correlations with RV/TLC (r=0.72, p=0.0003), a measure of gas trapping, and with FEV_1 z-scores (r=-0.73, p=0.0002). $S_{acinVTc}$ was also correlated with R_{aw} (r=0.56, p=0.01) but there was no association between $S_{acinVTc}$ and diffusing capacity in either CF adults or controls. In CF adults, $S_{condVTc}$ was not significantly correlated with any other measures of lung function. Table 5.3 shows how the main outcome measures correlate with each other for CF adults.

DL_{CO}/V_A percent predicted is the quoted measure of diffusing capacity since this takes into account the often abnormal alveolar volume (V_A) in CF patients. The predicted DL_{CO}/V_A was derived from predicted DL_{CO} and predicted V_A equations in the reference data of Quanjer et al. (Quanjer, Tammeling et al. 1993). When the correlations were performed against DL_{CO}, DL_{CO} percent predicted, or DL_{CO}/V_A, the findings were unchanged and there were no statistically significant correlations seen (all p>0.05).

Relationship between age and phase III slope analysis

There was no relationship between age and either $S_{acinVTc}$ (p=0.98) or $S_{condVTc}$ (p=0.22) for all healthy control subjects. These data are presented in Figures 5.6 and 5.7.

	LCI	$S_{acinVTc}$	$S_{condVTc}$	FEV_1% z-score
$S_{acinVTc}$	**0.87 <0.0001**			
$S_{condVTc}$	-0.22 ns	-0.38 ns		
FEV_1 z-scores	**-0.86 <0.0001**	**-0.73 0.0002**	0.15 ns	
R_{aw} (0.5) (L/s)	0.42 ns	0.56 0.011	-0.13 ns	-0.42 ns
RV/TLC	**0.73 0.0002**	**0.72 0.0003**	-0.21 ns	**-0.80 <0.0001**
DL_{CO}/V_A % predicted	0.10 ns	0.03 ns	0.48 0.03	-0.19 ns

Table 5.3: Summary of correlations between different markers of lung function in patients with cystic fibrosis. Correlations were assessed using Spearman's rank correlation coefficient. p values of greater than 0.05 are recorded as ns (not significant). Correlations with a p value less than 0.01 are highlighted in bold.

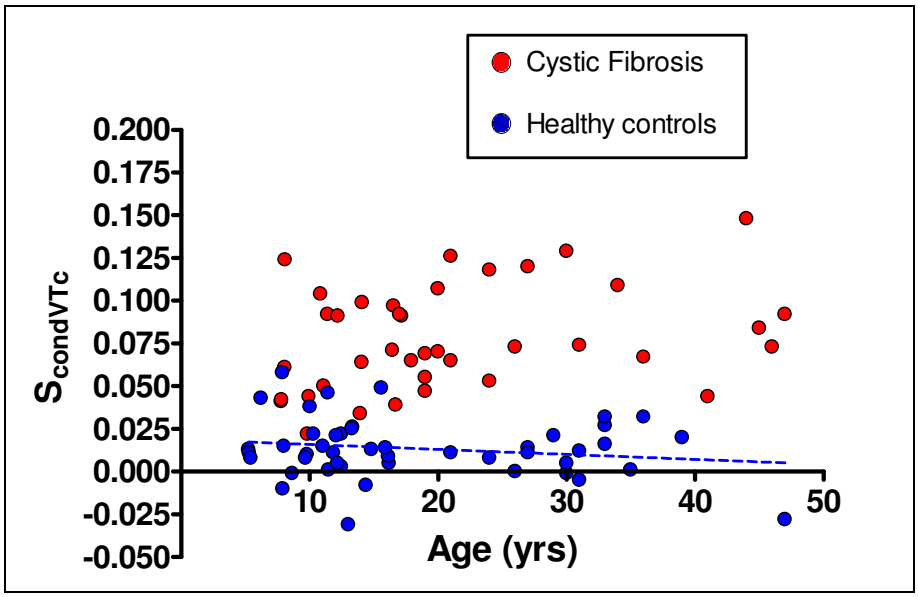


Figure 5.6: Age versus $S_{condVTc}$ for all subjects. Patients with cystic fibrosis are shown in red and control subjects in blue. The blue dotted line represents linear regression of the control data (p=0.22).

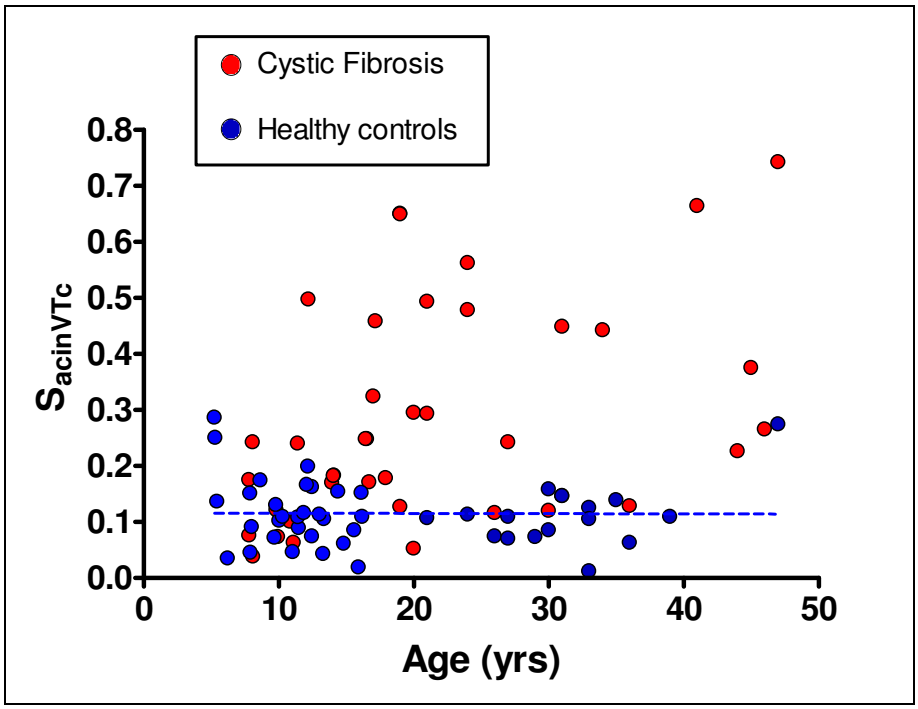


Figure 5.7: Age versus $S_{acinVTc}$ for all subjects. Patients with cystic fibrosis are shown in red and control subjects in blue. The blue dotted line represents linear regression of the control data (p=0.98).

Reproducibility and reliability of Phase III slope indices

In healthy controls the phase III slope was often essentially horizontal with each breath, and changed little over the course of a washout. This made the calculation of a "slope" of S_{cond} difficult, since all the individual phase III slopes showed essentially minor variation around zero. It also meant that negative values for S_{cond} could be obtained, something that has also been previously described in the calculation of phase III slopes in children using 4% SF_6 as the tracer gas (Aurora, Kozlowska et al. 2005). In contrast, with CF patients the anticipated progression of the phase III slope was clearly seen, and allowed calculation of a slope with reasonable certainty. This is illustrated by calculating the r^2 value of the slope. For healthy adult controls (n=12 with complete full lung function tests), overall median (IQ range) r^2 value was 0.14 (0.07 to 0.18). For CF patients on the other hand, median r squared was significantly greater at 0.64 (0.51 to 0.75), $p<0.0001$ (Mann Whitney U test).

Coefficient of variation (CoV) of intra-subject repeats is an alternative measure of reproducibility, that has already been reported for LCI (see Chapter 3). For healthy adult volunteers, the median (IQ range) CoV of $S_{condVTc}$ was 59.5 % (32.0 – 87.4), and for $S_{acinVTc}$ was 18.9% (10.0 – 30.0). The large CoV of $S_{condVTc}$ in healthy subjects reflects the poor reproducibility of the technique in this population; the very low values of S_{cond} mean that small variations in slope have a large impact on measures of reproducibility. For CF adults, median CoV of $S_{condVTc}$ was 32.7 % (14.0 – 41.4), and for $S_{acinVTc}$ was 22.4 (10.6 – 22.1).

Effects of altering the Phase III slope analysis

All data presented here are for $S_{condVTc}$ and $S_{acinVTc}$, the breath volume corrected derivations of S_{cond} and S_{acin} described by Aurora et al. (Aurora, Kozlowska et al. 2005). When the uncorrected data were analysed, a similar pattern of associations and correlations was seen. This is because both $S_{condVTc}$ and $S_{acinVTc}$ correlate strongly with their uncorrected values ($r=0.80$ and $r=0.91$, $p<0.0001$ respectively for adults).

There are however a number of differences between the analysis presented here and that performed in previous studies on phase III slopes. The first, and most obvious, is the lack of tight expiratory volume control. The reasons for this have already been covered, but in order to establish the effect of this on the calculation of S_{cond}, washouts were reanalysed according to a number of different criteria. The effects of these alterations were primarily assessed on S_{cond}, since this variable is also used to derive S_{acin}.

Association between Phase III slope indices and mean tidal volume

Previous studies have indicated that in normal subjects, LCI is affected by extremes of breath volume (Gronkvist, Bergsten et al. 2002). In order to investigate whether this could influence the results, measures of ventilation heterogeneity were compared to mean tidal volume (V_T), normalised for mean FRC or for body surface area (BSA, calculated using the Mosteller formula (Mosteller 1987)). Neither LCI, S_{cond} nor S_{acin} correlate with V_T, V_T/FRC or V_T/BSA. Because of the multiple comparisons involved, the level of significance for this analysis was set at 0.01. This is illustrated in Figure 5.8. The lack of correlation between measures of ventilation heterogeneity and breath volume, suggest that these data are not adversely affected by breath volume.

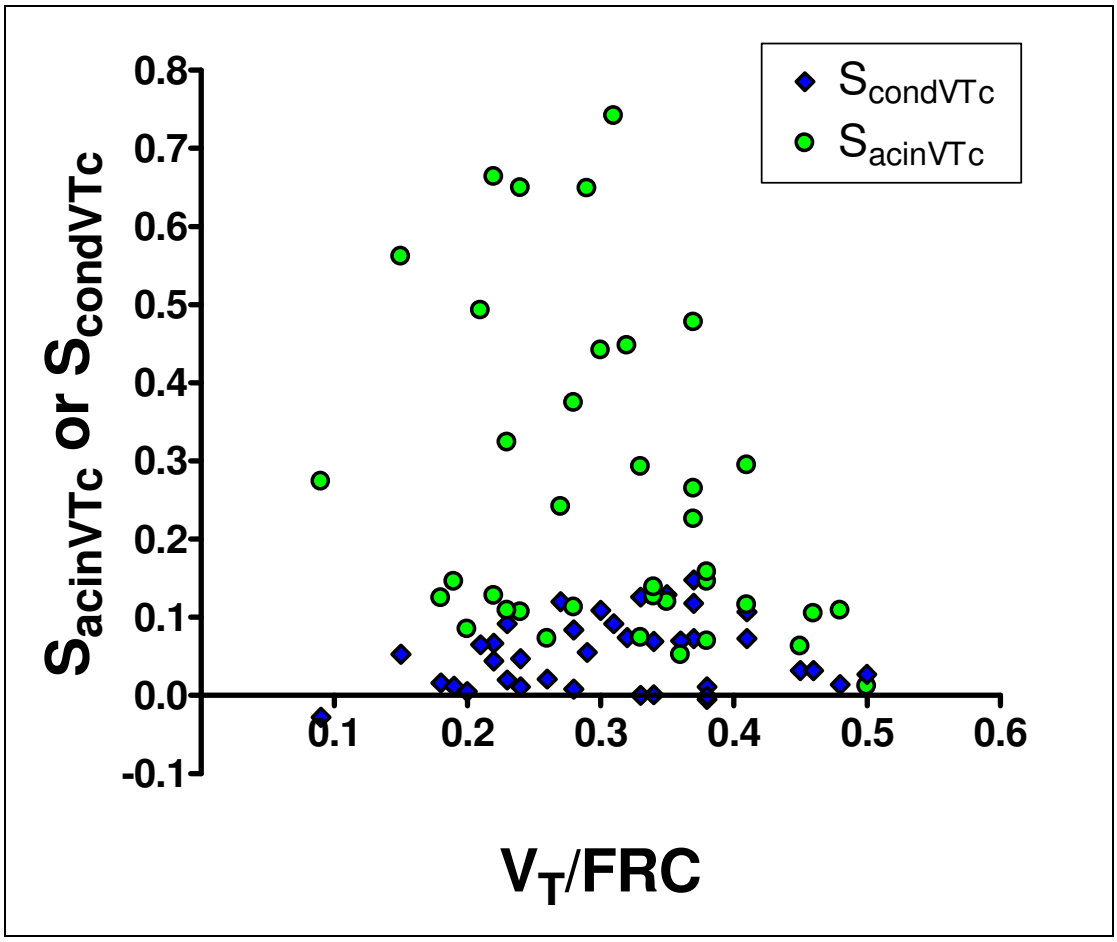


Figure 5.8: Relationship between the individual subject's mean tidal volume (V_T) to FRC ratio (averaged over 3 washout repeats) and phase III slope analysis, for all adult subjects. There was no statistically significant correlation between V_T/FRC and either $S_{acinVTc}$ or $S_{condVTc}$.

Re-analysis of Phase III slope indices based on breath volume

The effect of small tidal volumes on S_{cond} was assessed in two ways for the washouts performed in adult subjects. Firstly, in order to address the criticism that phase III slopes cannot be calculated from smaller breaths, phase III slopes were re-calculated only for breaths of greater than 850ml. No upper limit on breath volume was set, but the tidal volume correction was applied to these calculations to allow for this. If there were fewer than 4 breaths of greater than 850ml within the TO range 1.5 to 6, no attempt was made to calculate S_{cond}. Only subjects with 2 or more washouts where S_{cond} could be calculated were included in this analysis. This led to the exclusion of 5 of 12 healthy volunteers and 10 of the 21 CF patients. Mean (SD) VT of all subjects was 854 (139) ml, whereas for the washouts reanalysed with a minimum volume of 850ml, mean VT was 955 (85) ml. The effect of this on $S_{condVTc}$ is shown in Figure 5.9. This also shows the standard $S_{condVTc}$ values, as already shown in Figure 5.4, where a minimum breath volume of 400ml was accepted, here denoted as $S_{condVTc}$ (400). For CF patients, mean $S_{condVTc}$ (850) was 0.078, significantly lower than mean $S_{condVTc}$ (400) for the same subjects (0.108), p=0.0012 (paired t-test). The inclusion of more and smaller breaths into the phase III slope analysis therefore led to an increase in the calculated S_{cond}, and is thus not sufficient to explain the failure of S_{cond} to increase with increasing ventilation heterogeneity.

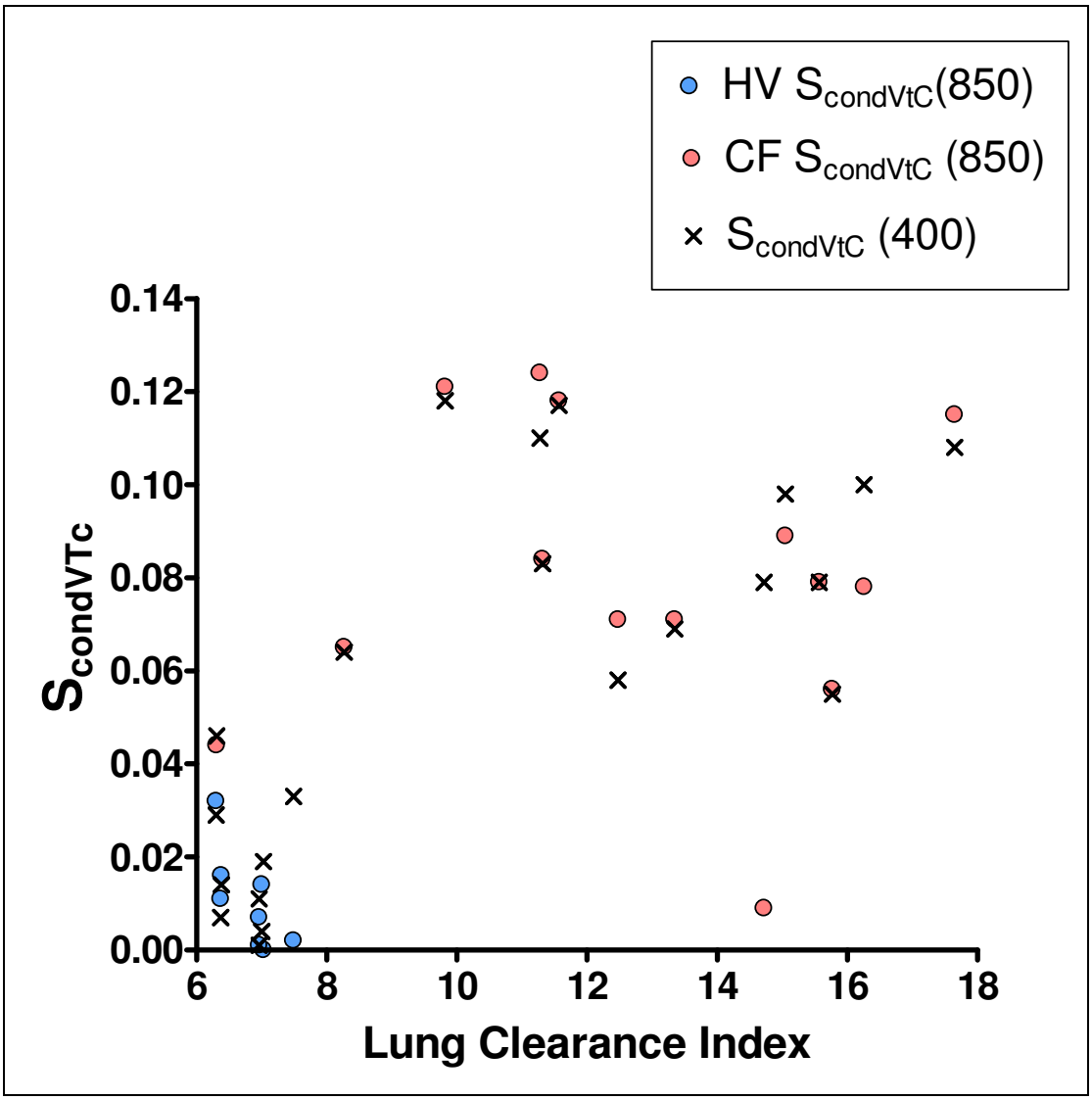


Figure 5.9: $S_{condVTc}$ calculated only from those breaths of the washout greater than 850ml in volume: $S_{condVTc}$ (850). This is plotted against LCI for healthy volunteers (HV) (blue circles) and CF patients (red circles). In addition, for the same subjects $S_{condVTc}$ calculated from all breaths greater than 400ml, as described in the Methods and previously illustrated in Figure 5.4, is also shown as black crosses: $S_{condVTc}$ (400).

An alternative analysis was also performed to calculate S_{cond} using only the most robust Sn_{III} values. Firstly, all washouts with a mean tidal volume over the TO range 1.5 - 6 of less than 850ml were excluded. Secondly, all washouts where the expiratory volume variability (measured as the within-washout CoV of expiratory volume) was greater than 20% were excluded. Finally, only the two washout repeats with the closest values for $S_{condVTc}$ were included, and a mean $S_{condVTc}$ generated from these. $S_{condVTc}$ values for 9 CF patients and 6 healthy controls met these inclusion criteria. Mean (SD) tidal volume was 953 (66) ml, and mean intra-washout coefficient of variation of tidal volume was 9.6% (range 1.9 – 18.7%). Despite this selection, the graph in Figure 5.10 remains very similar to that in Figure 5.4. There is a progression of $S_{acinVTc}$ with deteriorating LCI, but no further progression of $S_{condVTc}$.

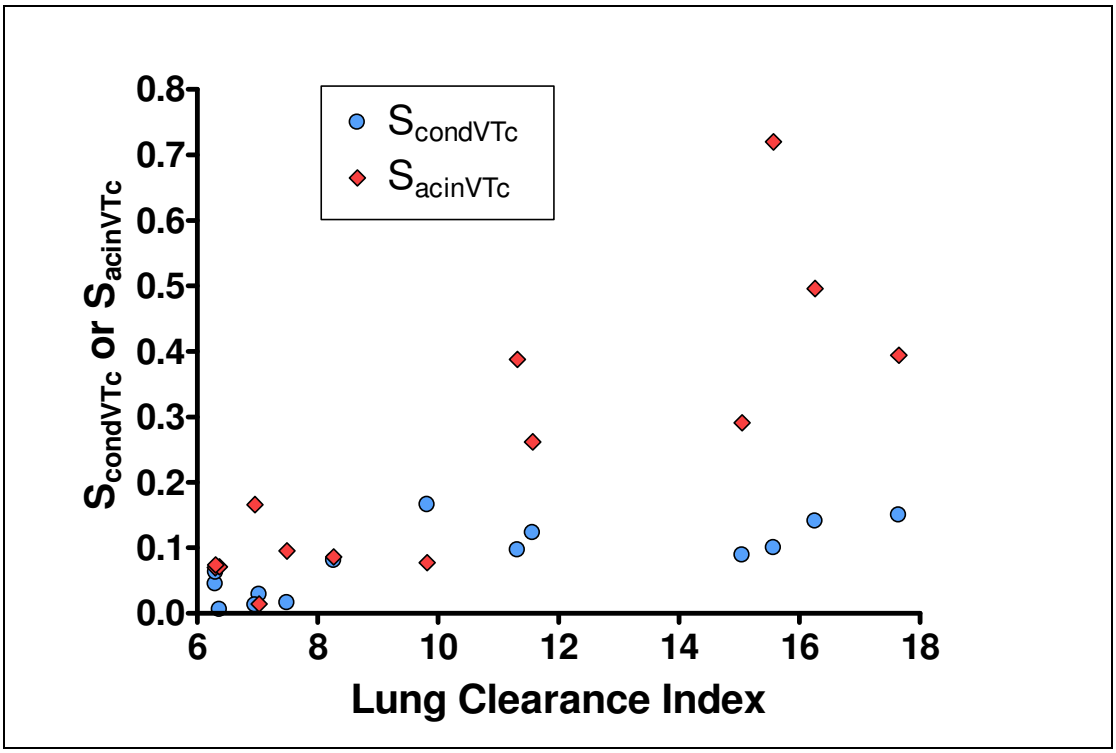


Figure 5.10: $S_{condVTc}$ and $S_{acinVTc}$ from selected washouts plotted against LCI. Washouts were only included if they conformed to strict criteria on mean and standard deviation of washout breath volume, as described in the text.

Effect of altering the TO range on S_{cond}.

It was apparent during data analysis that in some washouts from CF patients there was a levelling off of the Sn_{III} values within the TO range 1.5 to 6. This is illustrated in Figure 5.11, a plot of Sn_{III} vs TO for a washout from a 19 year old male with CF, FEV_1 61% predicted and LCI of 15.8. Because the curve flattens off before TO=6, calculation of linear regression up to TO=6 is erroneously diminished. A solution to this is to use a lower TO range. The lower limit (TO=1.5) is set by the behaviour of diffusive gas mixing, and therefore remains unaltered. The upper limit of the range however has been reduced to 4 for calculation of S_{cond}. Any further reduction risks leaving too few data points in the range of interest for accurate calculation.

All 99 washout repeats from the 21 adult CF patients and 12 adult healthy controls were reanalysed and S_{cond} re-calculated from TO range 1.5 to 4, S_{cond} (TO4). A Bland-Altman plot of S_{cond} (TO4) and the standard method of calculating S_{cond} between TO 1.5 and 6, S_{cond} (TO6), is presented in Figure 5.12. With increasing S_{cond}, there is a tendency for S_{cond} (TO6) to be lower than S_{cond} (TO4), as would be expected from looking at plots such as that in Figure 5.11.

A plot of the adjusted $S_{condVTc}$ data (utilising the TO range 1.5 to 4) versus LCI is shown in Figure 5.13. The original data (to TO=6) are also shown as black crosses. This adjustment to the calculation of S_{cond} had little effect on S_{acin}, which is therefore not shown separately. In a single CF patient, flattening occurred before TO=4 and this subject's data were therefore excluded.

Mean $S_{condVTc}$ was higher when the reduced TO range was employed (0.102 vs 0.088), but this difference was not statistically significant (p=0.25, paired t-test). Overall, this adjustment had little effect on the overall shape of the LCI vs S_{cond} graph, which continued to show restriction of progression of S_{cond} with worsening LCI.

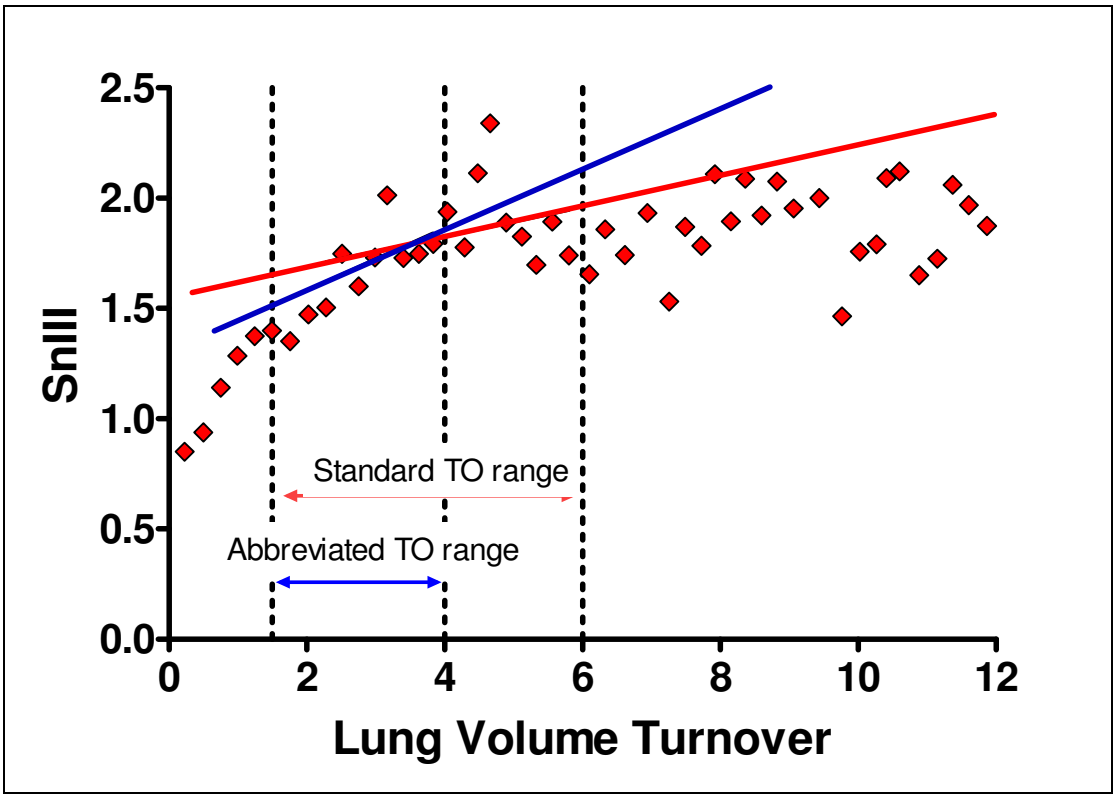


Figure 5.11: Sn_{III} vs lung volume turnover for a single washout of a patient with moderately severe CF lung disease. Although there is a clear progression of Sn_{III} with sequential breaths, this flattens out within the range of lung volume turnover (TO) used to calculate S_{cond} – shown as the red linear regression line. If a lower upper limit for TO range is used, plot appears to be more linear and the regression line (blue) is steeper.

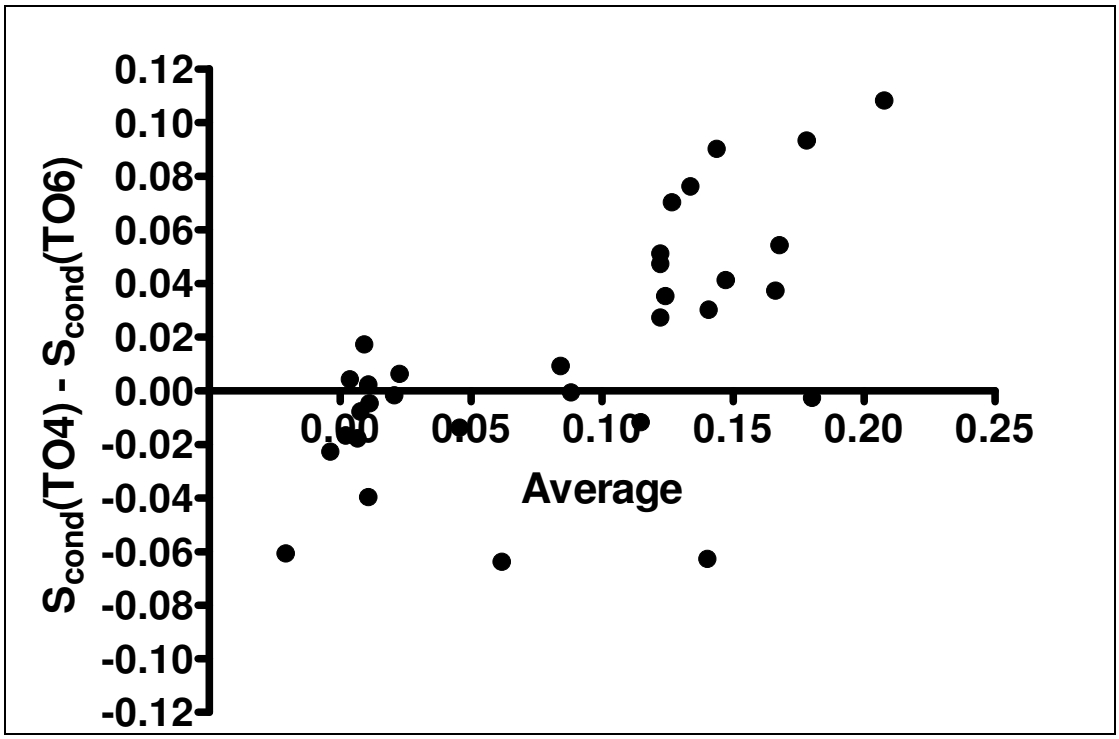


Figure 5.12: Bland-Altman plot of S_{cond} generated from a lung volume turnover range of 1.5 to 4, S_{cond} (TO4), and the standard range of 1.5 to 6, S_{cond} (TO6).

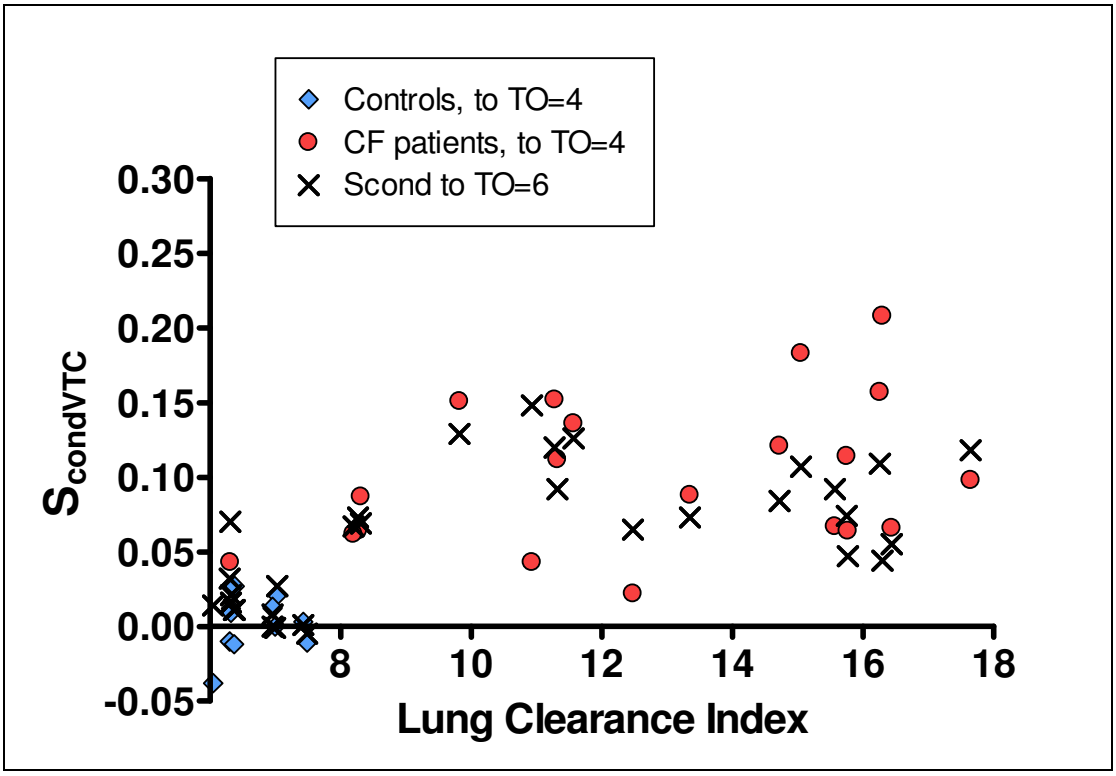


Figure 5.13: $S_{condVTc}$ (blue circles) and $S_{acinVTc}$ (red diamonds), restricted to the lung volume turnover range 1.5 to 4 for calculation of S_{cond}. This is plotted against lung clearance index. For reference, the original $S_{condVTc}$ values are shown as black crosses.

For patients with CF, there was no significant correlation between LCI and either $S_{condVTC}$ calculated to TO=4 (r^2=0.12, p=0.14) or to TO=6 (r^2=0.006, p=0.75).

Airway dead space and LCI

Airway dead space was calculated according to the method described by Fowler for the first three breaths of each washout (Fowler 1948). A custom software application written using Igor Pro 6.1 (Wavemetrics Inc, USA) was prepared by Dr Nick Bell and used to calculate the expired volume at which the area under the SF_6 expirogram (area A in Figure 5.14) equalled that between the SF_6 expirogram and the line extrapolated from the phase III slope (area B in Figure 5.14), for each of the first three breaths of each washout.

Predicted values for Fowler dead space were calculated using the height and gender-dependent equations described by Hart et al. (Hart, Orzalesi et al. 1963). These data are summarised in Table 5.4.

Although airway dead space was significantly higher in the healthy subjects (179 vs 159 ml, p=0.033), there was no significant difference between the percent predicted values or the dead space: tidal volume ratios (V_D/V_T). Moreover, the values of V_D/V_T were lower than those previously shown to have an effect on LCI (Schmalisch, Proquitte et al. 2006).There was no correlation between airway dead space or percent predicted dead space and LCI or $S_{condVTc}$. There was no correlation between LCI and V_D/V_T, but a weak correlation between $S_{condVTc}$ and V_D/V_T was statistically significant (Pearson r=-0.42, p=0.016). Inspection of the graph however did not reveal a convincing association between the two variables (Figure 5.15). In addition, this was in the opposite direction to that which would be expected if this was a true effect, i.e. one would expect an increase in V_D/V_T to cause an increase in $S_{condVTc}$.

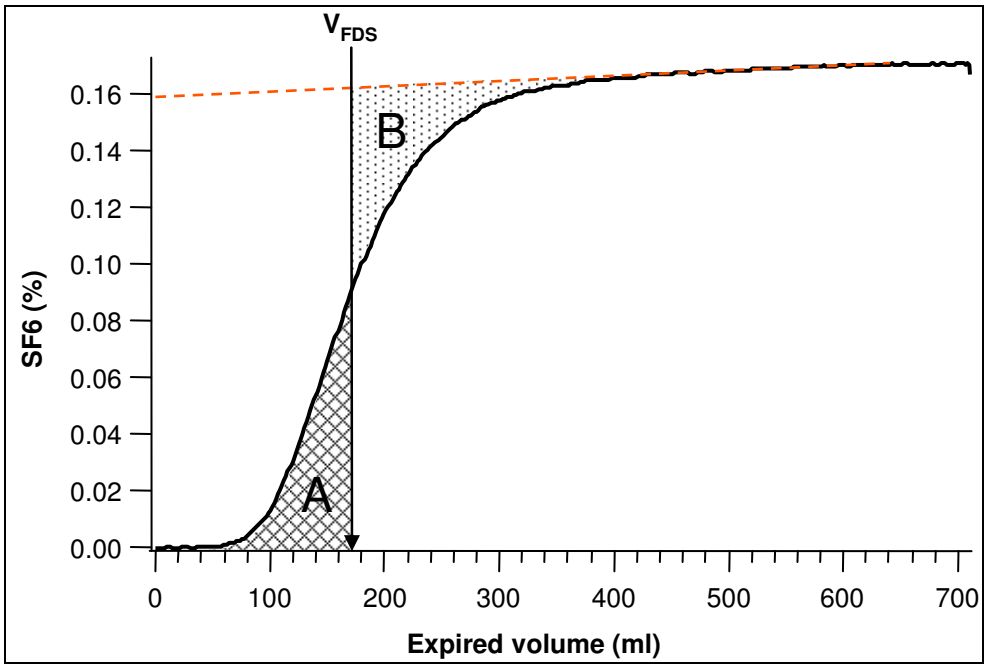


Figure 5.14: Calculation of airway dead space as described by Fowler (1948). The phase III slope is extrapolated back to meet the rising SF_6 concentration on the SF_6 expirogram. The expired volume at the point that (area A) = (area B) is the airway dead space. This was calculated for each of the first three breaths of each washout.

	Healthy adults n=12	CF adults n=21
Fowler dead space (ml)	179 (29) [132 - 247]	159 (23)* [109 - 186]
Percent predicted Fowler dead space	119 (13.8) [99.1 – 146.2]	111.2 (16.1) [84.4 – 150.0]
V_D/V_T	0.222 (0.038) [0.160 – 0.269]	0.203 (0.025) [0.172 – 0.268]

Table 5.4: Comparison of airway dead space, calculated from the first three breaths of each washout, for CF patients and healthy controls. Percent predicted dead space is taken from the equations derived by Hart et al. (Hart, Orzalesi et al. 1963).
*p=0.033

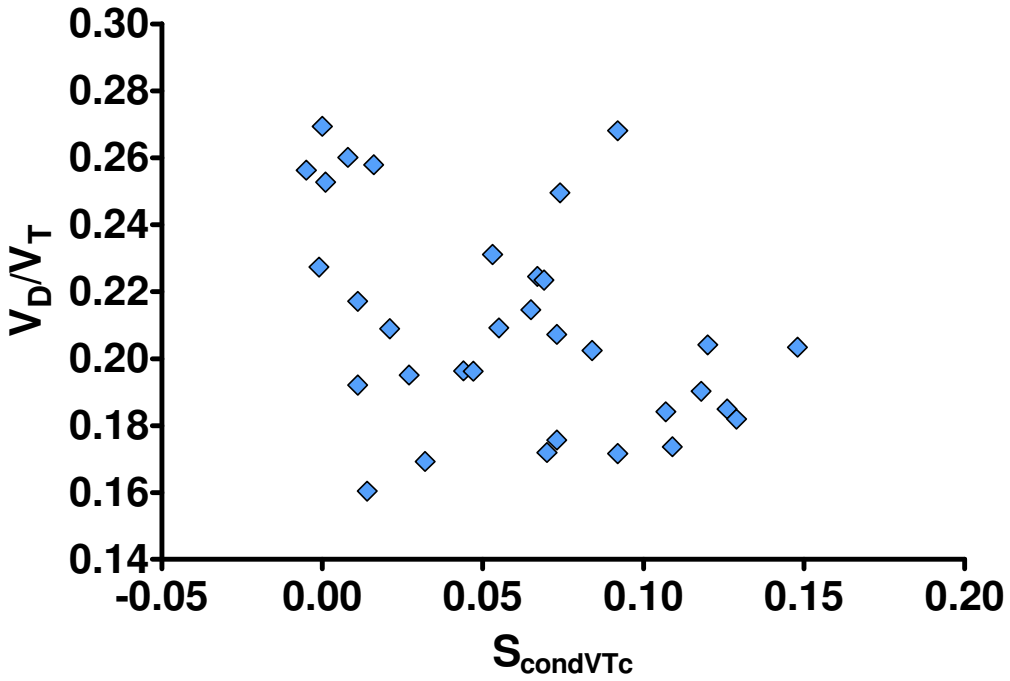


Figure 5.15: Correlation between $S_{condVTc}$ and dead space to tidal volume ratio (V_D/V_T). There is a statistically significant correlation between $S_{condVTc}$ and V_D/V_T (Pearson r=-0.42, p=0.016).

Curv analysis

An additional analysis of washout curves has also been proposed. This involves plotting lung TO against log mean expired tracer gas concentration for the first 6 lung volume turnovers. The ratio of the slopes of these points between TO 3 to 6 over TO 0 to 3 has been termed the curvilinearity, or Curv, of the washout (Verbanck, Schuermans et al. 2008). While Curv is a measure of specific ventilation heterogeneity, irrespective of the sequential emptying of these heterogeneously ventilated lung units, S_{cond} is dictated both by specific ventilation heterogeneity and sequential emptying (Verbanck, Schuermans et al. 2008). Hence, it would be expected that Curv and S_{cond} correlate to some extent.

Curv was calculated for all the washouts. Overall mean (SD) Curv was lower in CF than in controls; 0.632 (0.161) vs 0.879 (0.061), $p<0.0001$. $S_{condVTc}$ showed a significant correlation with Curv when all the data were combined (Pearson $r=-0.69$, $p<0.0001$), Figure 16. Curv also showed significant correlations with LCI ($r=-0.88$, $p<0.0001$) and FEV_1 z-score ($r=0.69$, $p<0.0001$).

The literature on Curv is less exhaustive than that on LCI or phase III slope analysis. If the assertion that Curv exclusively reflects specific ventilation heterogeneity is correct (Verbanck, Schuermans et al. 2008), then this would appear to account for a significant proportion of the early rise in S_{cond}. However, S_{cond} levels off whilst Curv continues to fall. This supports the proposal that S_{cond} is limited intrinsically by the nature of its derivation, rather than by the degree of conducting airway abnormality in CF (see discussion of main manuscript).

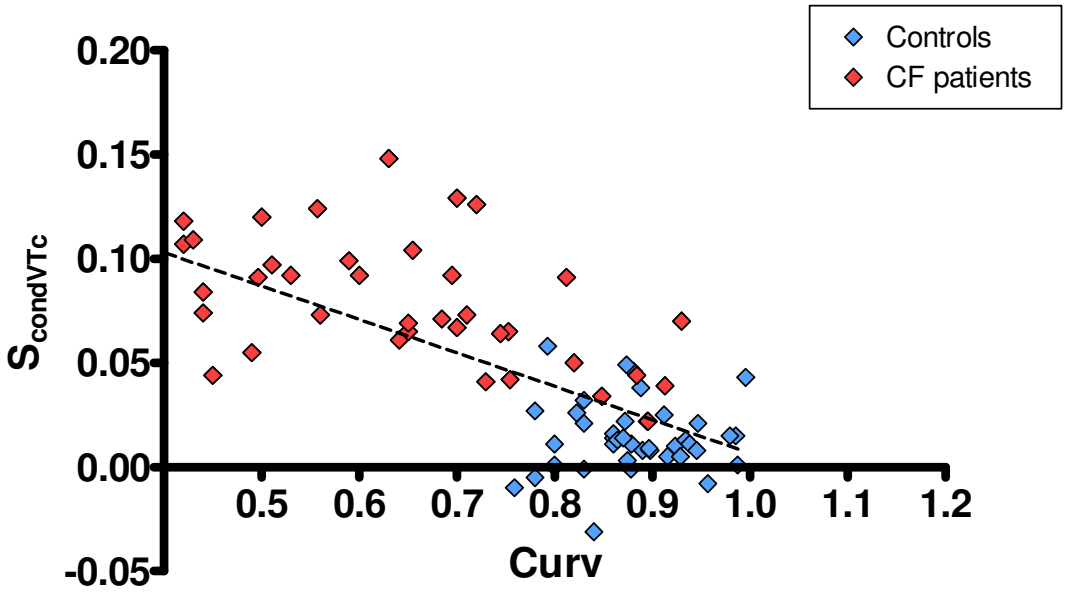


Figure 5.16: Curvilinearity (Curv) plotted against $S_{condVTc}$ for all CF patients (red) and controls (blue). The graph includes data from both children and adults. The dotted line represents linear regression of all $S_{condVTc}$ versus Curv, Pearson r=-0.69, p<0.0001.

Discussion

From first principles it might be expected that CF patients with more severe lung disease would have more inhomogeneous convective gas mixing. However, this is not what has been demonstrated using a method based upon phase III slope analysis. Although the convection dependent component (S_{cond}) was elevated in almost all CF subjects, including children with mild disease and normal LCI, S_{cond} did not continue to rise with increasing disease severity (as expressed by deteriorating FEV_1 or LCI) and appeared to reach an early asymptote. In contrast, the contribution to the normalised phase III slope of diffusion-convection interaction (S_{acin}) was correlated with severity of lung disease and hyperinflation (RV/TLC). Furthermore, increases in heterogeneity of gas mixing appeared to occur largely in the S_{acin} component. These findings were seen even after eliminating potential methodological differences between this and previously published studies.

In addition, whilst Verbanck et al. found a relationship between S_{acin} and diffusing capacity in COPD patients (Verbanck, Schuermans et al. 1998; Verbanck, Schuermans et al. 2004), this was not evident in this study of CF patients. It is likely that this reflects differences in the pathology. Verbanck looked at asthmatics and smokers, in whom diffusing capacity is a valid marker of alveolar integrity (McLean, Warren et al. 1992). In CF however, alveolar structure and diffusing capacity are well preserved until late in the disease (Sobonya and Taussig 1986; Espiritu, Ruppel et al. 2003), and this is reflected in the large number of normal diffusing capacity measurements reported here. Another important difference between the multiple breath washout analysis and the assessment of diffusing capacity is that the latter requires a vital capacity breath, which may serve to open up additional regions of the lung poorly ventilated at tidal volume. If the two tests were assessing different regions of the lung, they would not be expected to exhibit close correlations.

S_{acin} does however show a convincing correlation with RV/TLC, measured at plethysmography. This is a measure of hyperinflation which, particularly in those with normal alveolar function, one would expect to be caused by disease of the small conducting airways (measured by S_{cond}) (Macklem, Thurlbeck et al. 1971; Cosio, Ghezzo et al. 1978). This was demonstrated recently by King et al., who looked at the effect of

methacholine challenge on airways resistance (measured by forced oscillation technique) and Sn_{III} (King, Downie et al. 2005). They confirmed that in healthy controls, S_{cond} was related to the volume of gas trapping at FRC, whereas S_{acin} was not.

The very earliest effects on gas mixing appear to occur in the conducting airways, and S_{cond} was elevated in almost all children with CF, including those with LCI well within the normal range. This corresponds with our understanding about the site of earliest pathology in CF (Brownlee 2006). Increasing ventilation heterogeneity however is not reflected as a worsening of S_{cond}, even though convective flow differences between larger lung units might be anticipated. Indeed, it has been shown by Brown et al. that aerosol bolus dispersion, a phenomenon of convective gas mixing, continues to deteriorate in CF with increasing overall ventilation heterogeneity measured by ^{133}Xe washout (Brown, Gerrity et al. 1998). Furthermore, Curv analysis, also considered a measure of convective gas mixing (Verbanck, Schuermans et al. 2008), continued to increase with LCI (see Figure 5.16). It appears unlikely therefore that convective flow heterogeneity reaches an early maximum and shows so little progression in those with far more severe lung involvement. The failure of S_{cond} to increase in this study may partly be related to the low tidal flows employed. Faster breathing, or greater breath volumes, might reveal dynamic differences in flow-resistance between lung units that would increase measurements of convective gas mixing heterogeneity beyond those seen at tidal breathing. However, this would still fail to explain why S_{acin}, rather than S_{cond}, is so strongly correlated with gas trapping.

Although the principles of phase III slope analysis were first described over 10 years ago, the analysis has largely remained restricted to a single group and their collaborators. Only recently have other investigators begun to apply this analysis, most notably in children with CF (Gustafsson 2007). Phase III slope analysis is based upon persuasive experimental data and modelling, and now supported by a growing number of clinical studies (Verbanck, Schuermans et al. 1997; Verbanck, Schuermans et al. 1998; Verbanck, Schuermans et al. 1999; Verbanck, Schuermans et al. 2003; King, Downie et al. 2005; Verbanck, Schuermans et al. 2006; Downie, Salome et al. 2007). However, these assumptions and modelling were derived from histological data on normal lungs (Paiva and Engel 1984; Crawford, Makowska et al. 1985). Furthermore, all the studies that have reported the use of S_{cond} and S_{acin} have been in those with mild airways disease; either

normal subjects (Crawford, Makowska et al. 1985), smokers (Verbanck, Schuermans et al. 2004; Verbanck, Schuermans et al. 2006), those with asthma or bronchial hyperreactivity (Verbanck, Schuermans et al. 1997; Verbanck, Schuermans et al. 1999; Verbanck, Schuermans et al. 2003; King, Downie et al. 2005; Downie, Salome et al. 2007), and those with mild-moderate COPD (Verbanck, Schuermans et al. 1998).

The pathology in CF however is quite different from any of these groups. Adult CF patients in particular may have marked suppurative lung disease, with radiological evidence of bronchiectasis, regional lung collapse, bullous lung disease, and small airways obstruction (Helbich, Heinz-Peer et al. 1999). Even in early disease, bronchiectasis, mucus plugging and gas trapping are commonly described features (Helbich, Heinz-Peer et al. 1999). It is not clear what effect these fundamental differences will have on the assumptions behind the phase III slope analysis but it is of note that S_{cond} shows no correlation in CF patients with any other measures of lung function, including those such as R_{aw} that might have been anticipated to reflect similar processes (King, Downie et al. 2005).

It is possible that a combination of increasing airway dead space and falling tidal volume in CF patients with more severe lung disease might affect gas mixing. This has been investigated and shown not to be the case. There was no significant difference between dead space: tidal volume ratios in CF and controls, and the values were lower than those previously shown to have an effect on LCI (Schmalisch, Proquitte et al. 2006). Although there was a statistically significant correlation between $S_{condVTc}$ and V_D/V_T, this was weak ($r=-0.42$, $p=0.016$), unconvincing, and in the opposite direction to that which would be expected if this was a true effect.

There are two possible interpretations of these findings. The first is that, compared to other airways diseases (including COPD and asthma), CF represents the severe end of a spectrum. The limitations seen on S_{cond} are thus applicable in all diseases, but are apparent much earlier in disease progression in CF. This also would explain why other authors have so far failed to describe S_{cond} values greater than 0.150. Indeed such a limitation was described by Paiva (Paiva 1975) more than 30 years ago in a two-compartment lung model. He showed that SnIII would increase sequentially during a washout, and that this increase would be steeper with increasing difference in ventilation distribution. However, he also showed that this increase in SnIII would reach an asymptote, and that this would

occur earlier in the washout with more pronounced ventilation heterogeneity. This is precisely what has been described here in patients with CF. It might be possible to make inferences about convective ventilation from the level of this asymptote. Unfortunately however real life data do not perform as neatly as lung models and the reality is that the progression of SnIII tails off rather than coming to an abrupt end, making it hard to determine with precision the level of the asymptote or more particularly the breath at which it is reached.

As an adjunct to this, rather than necessarily an alternative, the derivation of S_{cond} may be particularly susceptible to the nature of airways disease in CF. The effects on ventilation of small airway obstruction due to mucus plugging may be what makes LCI such a sensitive measure of early disease in CF. It is also possible that, as disease progresses, these same pathological processes invalidate some of the assumptions behind phase III slope analysis. Complete obliteration or obstruction of small airways means that they will not contribute to convective ventilation (S_{cond}). Furthermore, as larger airways become obstructed the diffusion front is moved proximally (i.e. towards the mouth). The resulting regional differences in gas distribution therefore occur at very low or zero gas flows and are present at the start of the washout. They would thus be measured by the S_{acin} component of phase III slope analysis. This would also explain the association between hyper-inflation (a result of small airways obstruction), S_{acin}, and LCI. This would also explain why S_{acin} correlates with measures of conducting airway obstruction, such as R_{aw} and FEV_1. This hypothetical model is illustrated in Figure 5.17. More information could be obtained about the contributions of the different gas mixing processes by performing washouts of two different gas species simultaneously, as has been done with SF_6 and helium for single breath washouts. Unfortunately, this is beyond the scope of the current study.

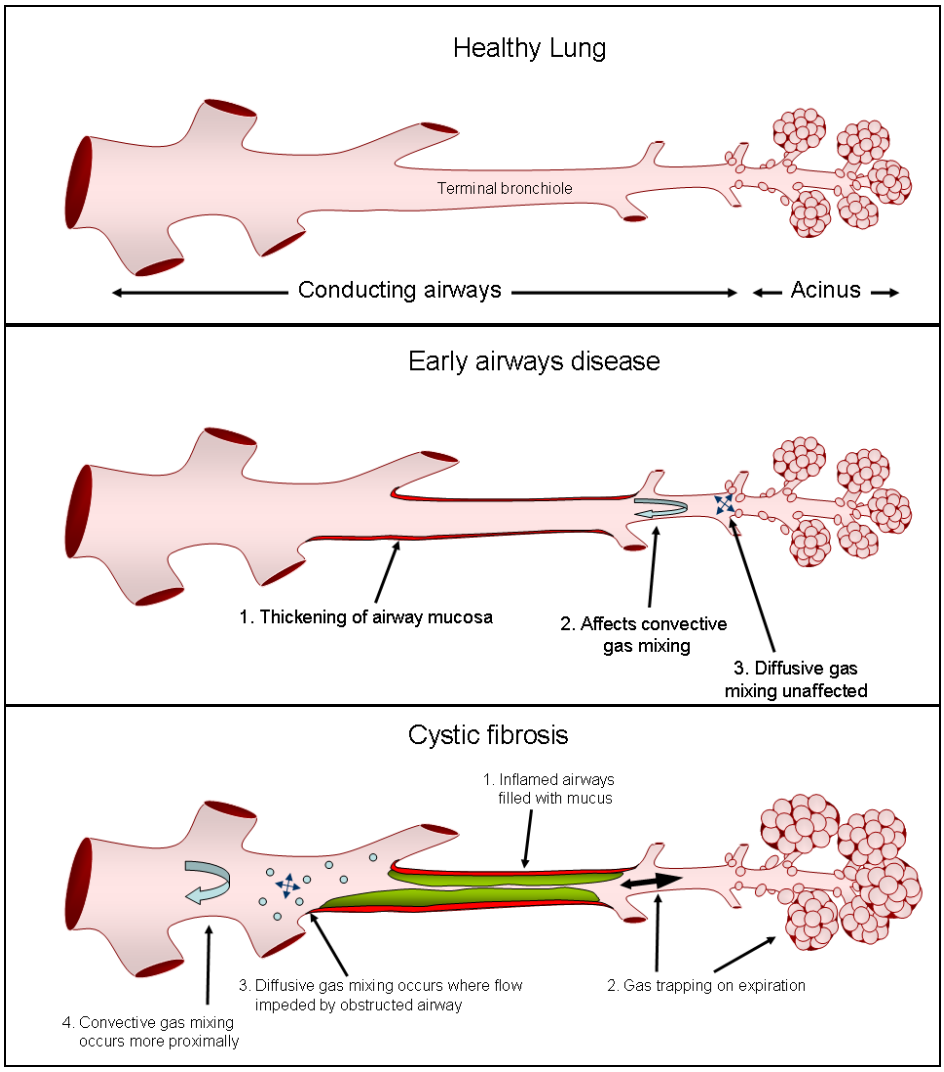


Figure 5.17: Hypothetical model of effects of deteriorating lung function in CF on S_{cond} and S_{acin}. In the healthy lung (top) S_{cond} measures inhomogeneity of gas mixing in the conducting airways and S_{acin} measures diffusion-conduction inhomogeneity in the acinus. In early airways disease (middle) there is inflammation of the small airways which causes increased convection dependent ventilation inhomogeneity (S_{cond}) but acinar function and gas diffusion (measured by S_{acin}) are unaffected. In patients with significant CF related lung disease (bottom) there is inflammation of the small airways which become blocked with mucus. This causes gas trapping on expiration but also results in a proximal movement of the diffusion front. Thus S_{acin} is elevated, whilst the region measure by S_{cond} is diminished since the small airways are no longer a site of bulk convective flow. This also explains the correlation of S_{acin} with gas trapping. The preserved alveolar integrity means that measures of gas transfer are not altered.

Methodological differences between this and previous studies

Since the original description of phase III slope analysis, and the majority of the published clinical observations, come from the group headed by Sylvia Verbanck, it is necessary to consider how this study differs from her work. In Verbanck's studies, very precise expiratory volume targeting of one litre has been employed (Crawford, Makowska et al. 1985; Verbanck, Schuermans et al. 1997). CF patients however, especially those with poorer lung function, found it hard to maintain such large tidal volumes. Even in healthy controls it can be uncomfortable to breathe large volumes of dry gas for long periods, and this is exacerbated in CF patients in whom this can induce the sensation of wanting to cough. In this study, therefore, patients were encouraged to maintain a tidal volume between 500-1000ml, and expiratory volume feedback was used to assist rather than tightly control this. Used in this way, expiratory volume feedback is very effective in ensuring uniformity of breathing pattern and volume. Since the strict 1L breath volume control is an important feature of the work by Verbanck et al., expiratory volume correction has been applied to Sn_{III}, as described by Aurora et al. (Aurora, Kozlowska et al. 2005), in order to allow comparison with these studies. However, the data from the two analysis protocols show strong correlation with each other and conclusions from the uncorrected data were the same as those presented here.

Because of the wider range of V_T permitted in this study, and the heterogeneity of subjects, the range of V_T/FRC ratios (see Figure 5.8) are much higher than those that can be inferred from previous studies (Verbanck, Schuermans et al. 2004). This could influence the relationship between S_{acin} and LCI if one or the other were more susceptible to changes in V_T. However, no statistically significant correlation is seen between any measure of tidal volume and any measure of ventilation heterogeneity.

For S_{cond}, the picture is more complex. A tidal volume that is too small may be insufficient to generate a true alveolar slope - for this reason breaths of less than 400ml were not included in the calculation of S_{cond}. On the other had, particularly in CF patients, a phase IV slope may be seen with larger breaths. In order to address the objection that insufficient breath volumes were included, a number of further analyses were completed. Recalculation of S_{cond} using a minimum breath volume of 850ml actually reduced S_{cond},

and made no substantial change to the shape of the S_{cond} versus LCI graph (Figure 5.9). Secondly, when washouts were selected based upon strict criteria (with a mean tidal volume, and within-washout coefficient of variation of breath volume, very similar to that described by Verbanck (Verbanck, Schuermans et al. 1998)), the conclusions remained unchanged (Figure 5.10). It would therefore appear that, regardless of analysis protocol, S_{cond} does not increase with increasing disease severity, but S_{acin} does.

Effect of tracer gas

Most previously published studies of phase III slope analysis have been performed on nitrogen washouts. SF_6 is denser than nitrogen, and its diffusivity is considerably less. SF_6 may therefore accentuate the diffusive differences in the lungs and lead to differences in S_{cond} and particularly in S_{acin} compared to other, lighter, gases. Gronkvist et al. showed a significant increase in S_{acin} of 30% when measured with SF_6 compared to He (Gronkvist, Bergsten et al. 2002). The difference between SF_6 and nitrogen however is likely to be less than this and cannot explain the findings entirely. The values of S_{acin} obtained in CF are higher than those in patients with asthma (mean values range from 0.113 to 0.264) (Verbanck, Schuermans et al. 1997; Verbanck, Schuermans et al. 1999; Verbanck, Schuermans et al. 2003) but they are similar to S_{acin} values derived from nitrogen washouts in CF patients (0.307) (Gustafsson 2007) and COPD patients (0.48) (Verbanck, Schuermans et al. 1998). Furthermore, S_{acin} in healthy controls reported here is similar to that previously reported in control groups (Verbanck, Schuermans et al. 1997). Finally, Gustafsson reported on nitrogen washouts in children with asthma and CF, and a similar degree of impairment in FEV_1 (Gustafsson 2007). He showed that despite LCI and S_{acin} being significantly greater in the CF patients, there was no statistically significant difference in S_{cond}.

The values for S_{cond} in controls presented here were lower than those reported in nitrogen washouts in healthy adults (Verbanck, Schuermans et al. 2004) and are similar to values reported by Aurora et al. in healthy children up to 18 yrs (Aurora, Kozlowska et al. 2005). In a cohort of 4 subjects, Prisk et al. measured S_{cond} from both nitrogen washout and SF_6 wash-in simultaneously, and found a non-significant reduction in mean S_{cond} calculated from SF_6 as opposed to nitrogen (Prisk, Elliott et al. 1998). They also showed a steadily rising plot of SnIII vs lung TO in three of the four subjects. In one subject

however, SnIII appeared not to change with increasing breath number for both SF_6 and He wash-ins (raw data of nitrogen washout not shown). Although these were trained subjects, performing SF_6 wash-ins, with large tidal volumes (1250ml), the data are consistent with the current study. The phase III slope of healthy subjects reported here was usually around zero (i.e. near horizontal) and changed very little during the course of a washout. This made S_{cond} difficult to calculate in healthy subjects, and more susceptible to the effects of outlier values of Sn_{III}. It also partly explains the poor repeatability of the measurement in control subjects, since small absolute changes in S_{cond} between washouts translate into substantial percent changes. Reproducibility of $S_{condVTc}$ in CF patients is far better than that seen in healthy volunteers, but it is clear that, even in those subjects where S_{cond} can be generated with reasonable certainty, reproducibility is poor compared to LCI. No data on these variables have been published by previous researchers to allow comparison with phase III slope indices derived from nitrogen washouts.

Another criticism of the data presented here relates to the calculation of S_{cond} in controls. When these data were reviewed, the graph of Sn_{III} vs lung TO for the healthy volunteer shown in Figure 3a was not felt to show a neat slope. This graph is representative of those obtained from healthy volunteers, but there are differences in washout analysis that may explain these findings. When washouts are analysed by Verbanck's method, the Sn_{III} data are first averaged for the washout repeats, a method that is made possible by the stricter breath volume control (such that every breath of the washout has the same TO number). The same method is more complicated with the washouts performed for this study, and when this was attempted did not produce smoother plots. Another factor that may partially explain the differences is the contribution of nitrogen in the blood coming out of solution during a nitrogen washout. Although this would only involve very small amounts of nitrogen, this may be enough to affect the slope of the alveolar plateau, and is a phenomenon that does not occur with the use of an exogenous insoluble marker gas.

Summary

This chapter presents novel data on phase III slope analysis in both children and in adults with CF. Although S_{cond} is sensitive to early changes in airway physiology, it does not correlate with other measures of gas mixing or airway function due to an early ceiling value that is reached even in those with apparently mild disease. Since S_{cond} changes little with increasing disease severity, it may not be a useful physiological measurement in CF adults. Phase III slope analysis is complex to perform, and is reliant on compliant subjects. It thus suffers from being difficult to carry out in patients with CF, and in adults with moderate to severe disease the underlying modelling assumptions may be incorrect. In these patients the analysis appears to offer few advantages over LCI. Despite differences between this study and those of Verbanck et al. in the performance of the washout test and analysis, the present findings appear to be robust and are not merely an artefact of less rigorous tidal volume control.

There are a number of questions posed by this work. In particular the validity of volume correction of phase III slope analysis is not universally accepted, although no effect of this on the final conclusions of this study has been shown. In addition, other studies have shown convincing progression of SnIII in healthy controls, and have not reported negative values of S_{cond} in adults. Whether this is an artefact of less intense breath volume control, or an effect of using SF_6 as the tracer gas, remains unclear. It would require a mass spectrometer to simultaneously perform nitrogen and SF_6 washouts, something that is beyond the scope of the present work.

None of this negates the use of this phase III slope analysis in subjects with mild non-CF airways disease. However, fundamental differences in airway pathology exist between asthma and CF and caution should be applied in extending observations from patients with mild airways disease into patients with more severely disordered regional ventilation.

Chapter 6 - Short term effects of physiotherapy on spirometry and multiple breath washout in the CF lung

Introduction

Physiotherapy is a recognised treatment in cystic fibrosis, and is widely used to aid clearance of secretions. Despite having been an accepted therapy for many years, there is actually little hard evidence of benefit in terms of effect on lung function (Bradley, Moran et al. 2006). Although there are numerous small studies looking at the effects of different physiotherapy techniques, previous attempts to assess the effects of physiotherapy on routine lung function measurements have failed to show an improvement over no physiotherapy (van der Schans, Prasad et al. 2000). Since LCI appears to be more sensitive to airway physiology than spirometry, it was hypothesised that it may be more sensitive to the effects of physiotherapy as a short term intervention. In particular, it was hypothesised that improved clearance of secretions, such as have been demonstrated following chest physiotherapy (van der Schans, Prasad et al. 2000), would improve lung gas mixing and lead to a reduction in LCI.

Highly heterogeneous gas mixing would also lead to uneven distribution of nebulised therapies, including gene therapy. It was therefore recognised that this study would also provide important information about the best methods of preparing the lungs of CF patients for nebulised therapies. If a benefit, in terms of LCI, could be shown with physiotherapy, this would support its use prior to gene therapy as a method of reducing the amount of mucus in the lungs and reducing unevenness of distribution of therapy. The original hypothesis was that successful airway clearance would improve gas mixing and lead to a reduction in LCI.

The aims of this study were to:

1. To assess the effect on LCI of 30 minutes of active cycle breathing physiotherapy compared to a control group who receive no physiotherapy.
2. To compare this to the effect on spirometry.

Methods

Study design

This was an open-label cohort study, which involved 14 patients with cystic fibrosis, recruited from the Scottish Adult CF Service. Patients underwent assessment of lung clearance index (LCI) with three washout manoeuvres, according to the standard protocol (as described in Chapter 3), followed by spirometry. Subjects were then assigned to receive either no physiotherapy or 30 minutes of active cycle of breathing technique (ACBT) (described below), administered by a senior respiratory physiotherapist. Subjects were assigned on the basis of availability of the physiotherapist to perform this. Subjects who were not assigned to physio remained seated for 30 minutes, and were allowed, but not encouraged, to expectorate. All sputum expectorated between LCI measurements was collected in a pre-weighed pot. Immediately after the physiotherapy or rest period, subjects repeated three LCI measurements, again followed by spirometry. Patients in the control (no physiotherapy) group were then offered the same 30 minute period of physiotherapist-assisted ACBT chest physiotherapy. Figure 6.1 shows a summary of the study design.

The original study design had an additional 10 minutes for description of ACBT built into the physiotherapy period. This turned out to be unnecessary, since all subjects were familiar with the technique, and a much briefer practical summary was provided by the physiotherapist instead, with the treatment period reduced to a total of 30 minutes.

Inclusion criteria

1. Cystic Fibrosis patients under the care of the Scottish Adult CF Service
2. Chronic sputum producers
3. Usual $FEV_1 \geq 40\%$ predicted
4. Whether carrying out regular physiotherapy or not.

Exclusion criteria

1. Usual $FEV_1 < 40\%$ predicted.
2. Regular / pre-physio short acting bronchodilators.
3. Patients whose airways were colonised by Burkholderia sp. or MRSA.

Patients were not excluded if they were prescribed and taking "as required" short acting bronchodilators, regular long acting bronchodilators or inhaled corticosteroids. Patients were also not excluded if they were on nebulised antibiotics or DNase.

All subjects provided written, informed consent, and this study was approved by the Lothian Research and Ethics Committee.

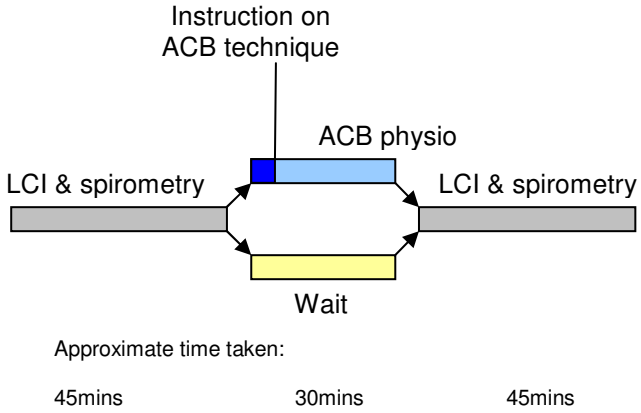


Figure 6.1: Study protocol design. Subjects completed triplicate MBW measurements, followed by spirometry, followed by 30 minutes of active cycle breathing (ACB) physiotherapy or a rest of 30 minutes. MBW and spirometry were then repeated immediately after this.

Lung function assessments

Lung clearance index

Lung clearance index (LCI) was measured by multiple breath inert gas washout, using the modified Innocor gas analyser (Innovision, Odense, Denmark) and 0.2% SF_6 as the tracer gas, as described in Chapter 3.

Spirometry

FEV_1 and FVC are quoted as the highest of three repeat manoeuvres (Miller, Hankinson et al. 2005). Predicted values for FEV_1 are those provided by the European Community for Coal and Steel (adults≥17 yrs) (Quanjer, Tammeling et al. 1993). Lung function testing was performed immediately after LCI measurement.

Chest physiotherapy

Active cycle of breathing physiotherapy was selected as the physiotherapy intervention. This technique is familiar to the majority of patients, and consists of a series of forced expirations (huffs), interspersed with thoracic expansion exercises, relaxation and breathing control (Prasad 1993). Huffs performed from mid to low lung volumes are considered to allow mobilisation of more peripheral secretions. Although chest percussion, positive expiratory pressure devices, or autogenic drainage can be used as adjuncts to this technique, they were not employed in this study and subjects performed physiotherapy sitting in a chair. All expectorated sputum was collected in a pre-weighed container.

Patients were asked to withhold their usual physio on the day of assessment.

Statistical analysis

Data were analysed using Prism (GraphPad Software Inc, CA, USA). Normal distribution was assessed using the D'Agostino and Pearson omnibus normality test. Parametric data are quoted as mean (SD), unless otherwise stated, and were compared using t-tests. Non-parametric data are quoted as median (inter-quartile range), and compared using the Mann-Whitney U test. Correlations were analysed using Spearman's rank correlation.

Results

Fourteen patients were recruited and completed the study. Nine subjects underwent physiotherapy and five subjects completed the control arm. Summary demographics are presented in Table 6.1 and a summary of the individual subjects, including potential confounding factors, is presented in Table 6.2. Although subjects with a usual baseline FEV_1 of less than 40% were not approached, three subjects failed to achieve an FEV_1 of $\geq$40% on the day of assessment, including one subject with an FEV_1 of 21% predicted. These subjects nonetheless completed the study. The reason for not approaching those with FEV_1<40% was that these subjects tend to take longer to complete washouts, and it was felt that the study would be excessively prolonged in those with the most severe lung disease. There were no differences between groups in age or baseline percent predicted FEV_1. Baseline LCI was significantly higher in the control group (p=0.046). However this difference only just achieved statistical significance and should be interpreted with caution given the small numbers of patients involved.

From Table 6.2, it can be seen that some patients attended for assessment having already completed their physiotherapy that day. In addition, three subjects had taken short acting bronchodilators within the preceding four hours. It was impractical to delay the study for those who had already completed chest physiotherapy or taken bronchodilators.

	Physiotherapy	Control	Difference (95% CI)	p value
n	9	5		
M:F	7 : 2	3 : 2		
Median age (range) yrs	21 (17-44)	34 (18-41)	-6.5 (-16.9 to 4.0)	0.20
Median baseline FEV_1 % predicted [range]	53.6 [39-99]	41.5 [21-98]	14.4 (-13.6 to 42.3)	0.28
Median baseline LCI [range]	13.2 [10.6 – 14.6]	17.0 [10.2 – 17.8]	-2.7 (-5.3 to -0.1)	0.05

Table 6.1: Summary demographics of patients.

Subject code	Age Gender	Baseline FEV_1% predicted	Usual sputum production (mls/d)	Usual physio regime	Last chest physio	Nebulised therapies	Notes
Physio 1	27 M	51.0	10 to 20	ACB	4hrs	nil	Took salbutamol <4hrs ago
Physio 2	21 M	65.5	15	Accapella	< 2hrs	DNase Colomycin	Physio <2hrs previously, incl. 1.25mg salbutamol with colomycin
Physio 3	27 M	38.6	150	AD & flutter	3hrs	DNase Colomycin	Completed physio < 3hrs previously
Physio 4	44 F	49.7	0 to 30	ACB & percussion	3d	nil	
Physio 5	17 M	53.6	30 to 50	ACB	12hrs	DNase	
Physio 6	23 F	83.9	Swallows all her sputum	AD & percussion	days-wks	nil	
Physio 7	21 M	52.6	10	AD	weeks	nil	
Physio 8	20 M	99.4	10	Accapella	>12hrs	nil	
Physio 9	19 M	70.3	15	ACB	12hrs	Colomycin	
Control 1	37 F	21.4	50	ACB	2d	DNase Colomycin	
Control 2	24 M	33.5	15 to 30	AD	>12hrs	nil	
Control 3	34 M	41.5	0 to 100ml	Mix of ACB, Flutter, PEP	>12hrs	nil	
Control 4	18 M	47.0	up to 50	AD	1.5hrs	DNase	Took Bricanyl < 1hr before
Control 5	41 F	97.6	up to 60	ACB + AD	18hrs	Colomycin	

Table 6.2: Individual patient summary.

ACB = Active cycle of breathing, AD = Autogenic drainage, PEP = positive expiratory pressure device

Effects of physiotherapy on LCI and Spirometry

There were no significant changes in group mean FEV_1, FVC, LCI or FRC after either intervention (physio or control). Mean % change in FRC and LCI in the physio group was non-significantly higher than in controls (5.73 vs 1.92%, p=0.06, and 4.07 vs 2.55%, p=0.15, respectively). There was a wider variability in % change in both of the MBW variables after physiotherapy than in spirometry or in the control group (see Figures 6.2 to 6.4). Maximum change in LCI in controls was 7%. In contrast 4/9 of the physiotherapy group had a change in LCI of greater than +/- 7%, and overall maximum change was 15%. Similarly maximum change in FRC after physiotherapy was 21% vs 7% in controls. There was no consistent pattern of response, and no correlation between change in the individual markers of lung function (FEV_1, FRC and LCI).

Median wet weight of sputum expectorated after physiotherapy was 3.33g. This was not significantly different to that expectorated in control subjects (3.29g), though once again there was a greater range of values in the physio group (Figure 6.5).

When sputum weight was plotted against lung function, the only significant correlation was with absolute percent change in LCI (Spearman r=0.62, p=0.018) (see Figure 6.6).

		Physio	Control
FEV_1 (L)	Start	2.45 (2.28-3.00)	1.75 (1.14 – 2.31)
	End	2.48 (2.17 – 2.88)	1.70 (1.12 – 2.37)
	Median % change (range)	2.36 (-9.14 – 11.01)	0 (-4.57 – 4.64)
FVC (L)	Start	4.06 (3.16 – 4.12)	3.53 (2.16 – 3.83)
	End	3.92 (3.66 – 4.51)	3.56 (2.27 – 3.85)
	Median % change (range)	-3.62 (-6.61 – 22.11)	0.54 (-4.17 – 8.68)
LCI	Start	13.2 (11.4 – 14.3)	17.0 (12.8 – 17.6)
	End	13.6 (11.5 – 15.1)	17.0 (12.9 – 18.5)
	Median % change (range)	4.07 (-7.65 – 14.8)	2.99 (-1.29 – 7.04)
FRC (L)	Start	2.89 (2.26 – 3.21)	3.34 (1.67 – 4.52)
	End	2.92 (2.41 – 3.36)	3.41 (1.69 – 4.61)
	Median % change (range)	5.18 (-2.23 – 20.54)	1.90 (-3.98 – 7.03)
Sputum wet weight (g)		3.33 (1.39 – 5.43)	3.29 (0.45 – 6.36)

Table 6.3: Summary of lung function, pre and post intervention, in physio and control groups. Data are presented as median (interquartile range), except the data on median % change, where the entire range is given. "Median % change" refers to the intra-subject percentage change in the parameter described, rather than change in group median.

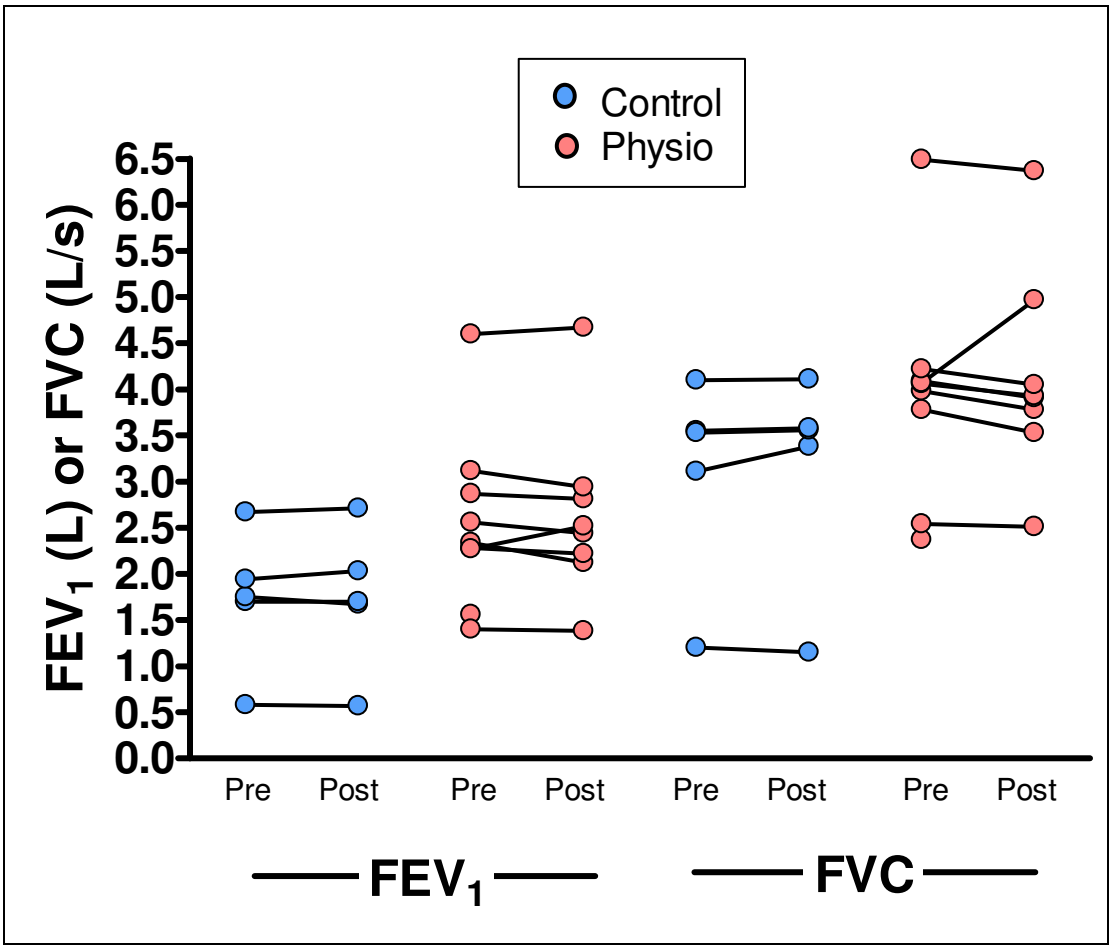


Figure 6.2: Change in spirometric indices pre and post-intervention for the physiotherapy and control groups. Each pair of points joined by a single line represents paired measurements on a single subject.

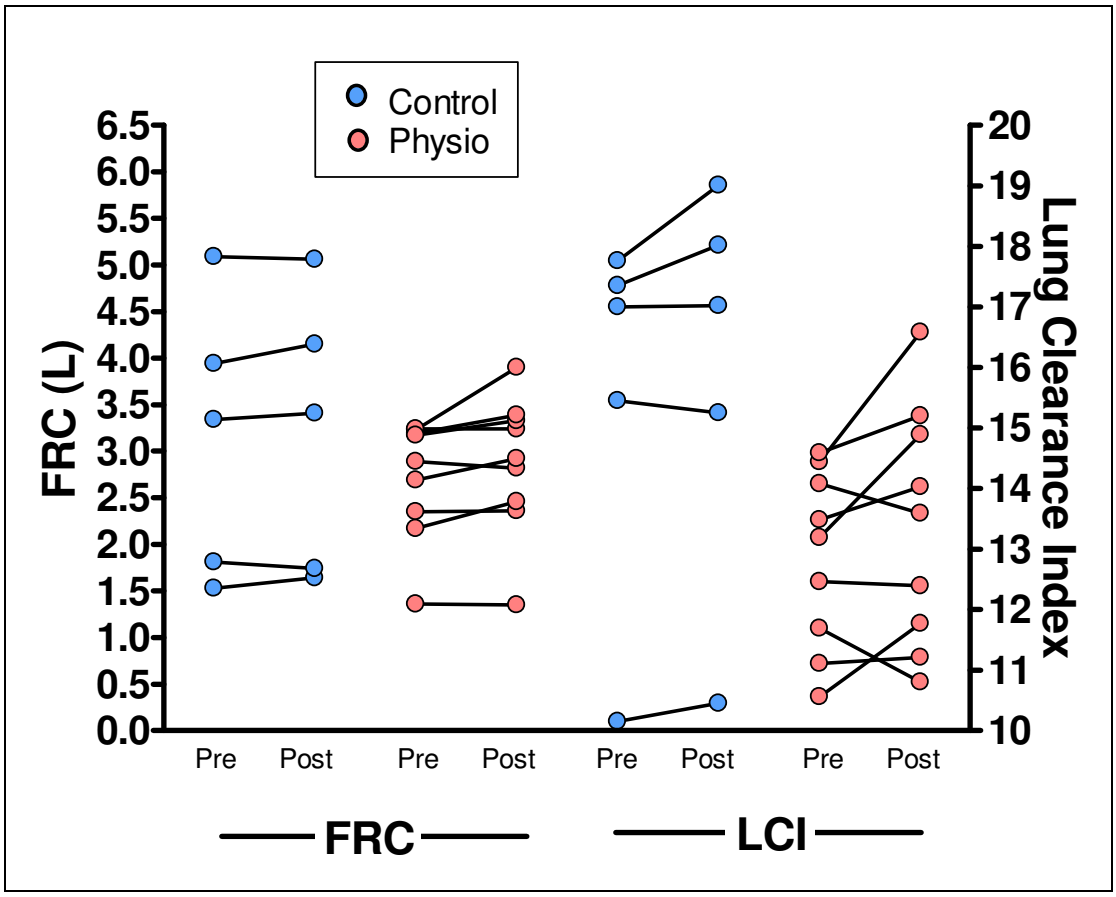


Figure 6.3: Change in MBW indices pre and post-intervention for the physiotherapy and control groups. Each pair of points joined by a single line represents paired measurements on a single subject.

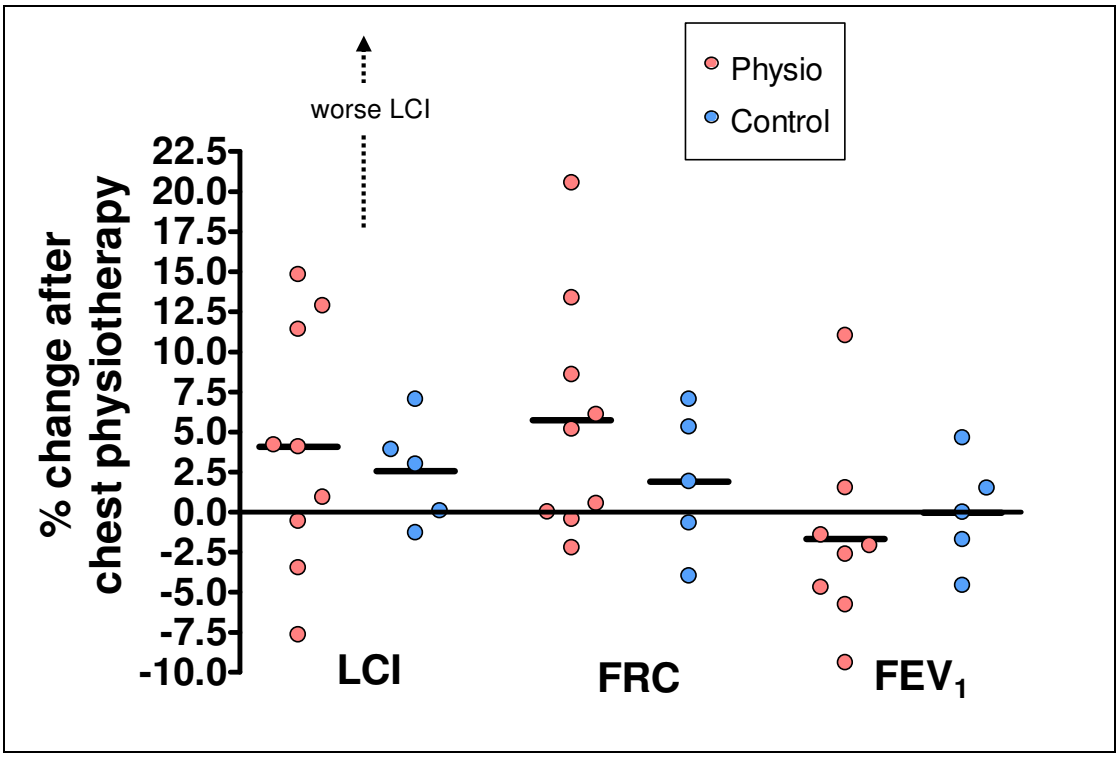


Figure 6.4: Summary of percent change in the different lung function variable in the two groups before and after intervention. Each point represents the percent change in a single individual. Horizontal bars represent group mean.

Increase in LCI indicates increased ventilation heterogeneity. Increase in FRC suggests opening up of previously unventilated lung.

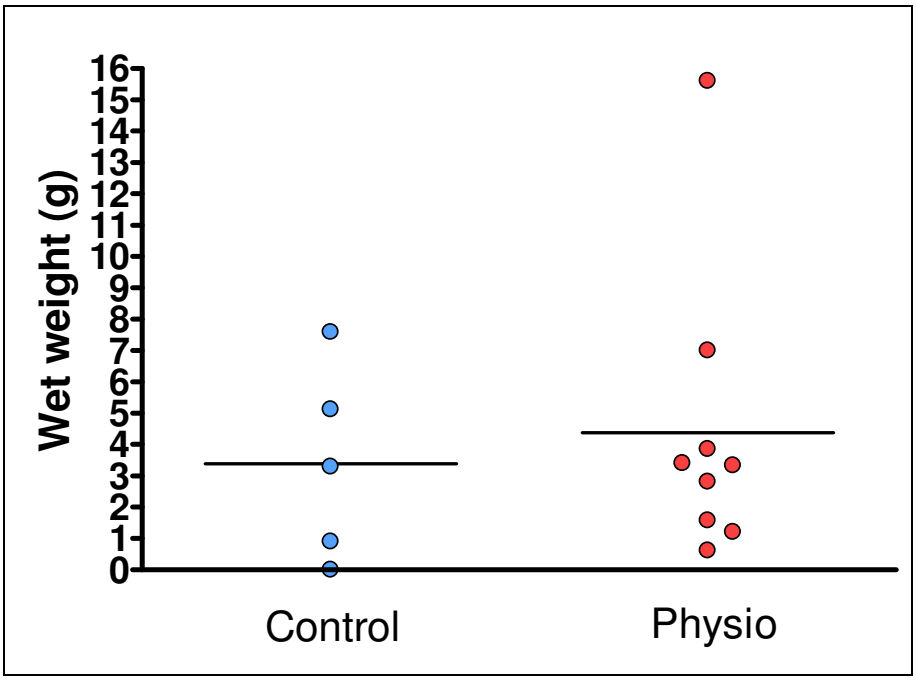


Figure 6.5: Comparison of wet weight of sputum expectorated in each group between the two MBW assessments. Each point represents a single individual, horizontal bars represent group mean.

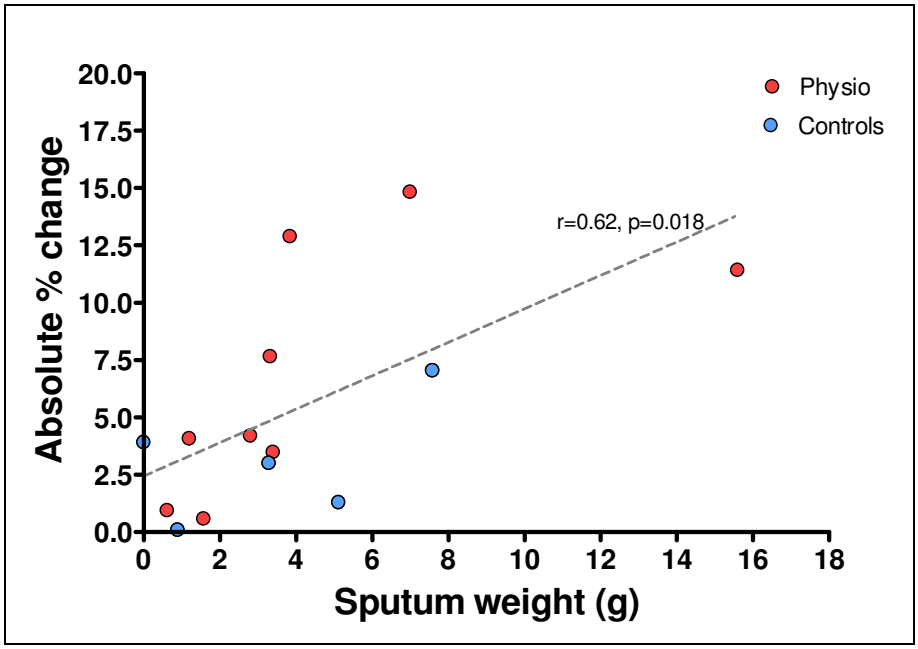


Figure 6.6: Correlation between wet weight of sputum expectorated, for all subjects, during intervention period and absolute percent change in LCI. The non-parametric regression line is shown as a dotted line.

Discussion

When this study was originally conceived, it was anticipated that physiotherapy, by aiding the clearance of mucus from the lungs, would improve gas mixing and lead to a reduction in LCI. This is not what was found. Although none of the lung function parameters achieved a statistically significant change after physiotherapy, the trend was for an increase in LCI, and in FRC.

There are a number of methodological issues with this study that need to be considered (see below), not least the small numbers involved, which make it hard to arrive at any firm conclusions. However, it would appear from Figures 6.2 to 6.4, that physiotherapy has complex effects on MBW measurements. Whilst the paired spirometry measurements, and the paired control group MBW measurements, changed relatively little, there was a wide spread of % change in LCI and FRC in the physiotherapy group. However, there was no consistent pattern of change, and no correlation between the change in the different lung function variables.

Since this study was commenced, other evidence has come to light which supports and helps to explain this observation. The first concerns two abstracts from the paediatric physiology research group at Great Ormond Street Hospital, who have been using LCI to follow longitudinal changes in two groups of children assigned to different physiotherapy positive expiratory pressure (PEP) devices (Main, Tannenbaum et al. 2004; Main, Tannenbaum et al. 2006). In the first of these abstracts, Main et al. reported on a cross-sectional analysis of 11 children (median age 12.9 yrs) before and after physiotherapy with either the PEP mask or the Cornet device (an oscillatory expiratory pressure device). There was no control group for this study, but they showed a non-significant elevation in mean LCI, and 4 of the subjects showed a significant elevation in LCI after physiotherapy (Main, Tannenbaum et al. 2004). This is concordant with the conclusions from this study, that physiotherapy can cause an elevation in LCI.

The second piece of work, which helps to explain some of these observations, relates to hyperpolarised helium MRI scanning in CF patients before and after chest physiotherapy. Hyperpolarised helium MRI scanning involves the subject inspiring a single vital capacity breath containing helium 3. This is first hyperpolarized to align the spins of the helium nuclei, which improves resolution of MRI and allows an image to be

obtained of the distribution of that breath in the lungs (van Beek and Wild 2005). Mentore et al. reported on the changes in ventilation distribution images in 15 CF adults (Mentore, Froh et al. 2005). In eight of the patients they also recorded MRI before and after salbutamol, and then again after nebulised DNase followed by 30 minutes of chest physiotherapy. There was evidence of ventilation heterogeneity in CF patients with normal FEV_1 using MRI, a finding which is consistent with those described in this thesis with LCI (Chapter 3). In the eight subjects who underwent further investigation, there was a significant improvement in ventilation heterogeneity after salbutamol, despite no change in FEV_1. After physiotherapy, there was an increase in ventilation heterogeneity in 6 of the 8 subjects. More importantly, this increase in ventilation heterogeneity was unpredictable, with some areas becoming ventilated that were not previously ventilated, and some becoming less well ventilated that were previously well ventilated. An image taken from this paper is reproduced in Figure 6.7. These observations were repeated by Woodhouse et al., who reported on 9 children with CF before and after physiotherapy, and showed a similar unpredictable change in ventilation distribution (Woodhouse, Wild et al. 2007).

Both of these reports support the findings of this study, namely that physiotherapy causes alterations in lung gas mixing which appear to be greater than those of control subjects who have not undergone physiotherapy. It does not appear to be possible to predict which factors determine how LCI will change, certainly not from the small numbers involved in this study. It is likely that similar processes to those discussed in Figure 4.33 are occurring with physiotherapy.

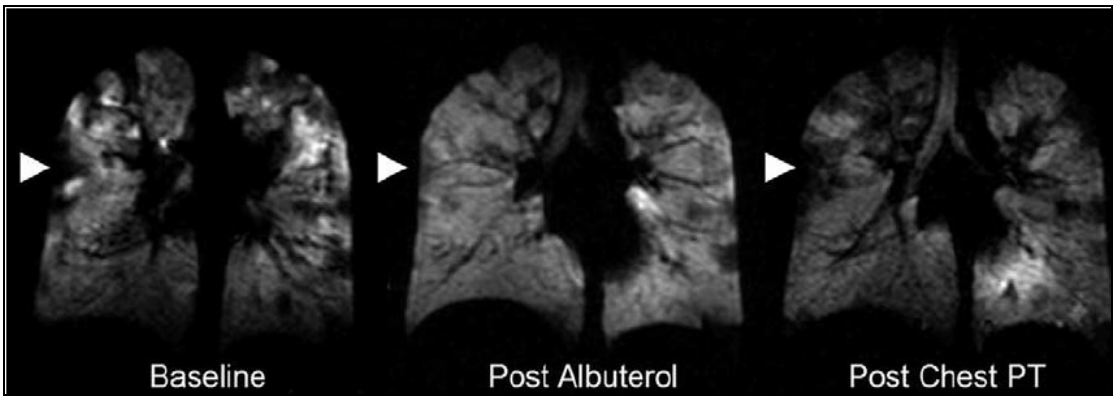


Figure 6.7: Taken from Mentore et al. (Mentore, Froh et al. 2005). Coronal hyperpolarised helium MRI images of a representative subject with CF at baseline, after nebulised salbutamol (albuterol) and following treatment with nebulised DNase and chest physiotherapy. Ventilation is shown in light grey, with dark areas representing areas of regional hypoventilation. The number of ventilation defects decreased after salbutamol (middle image), and then increased again after DNase and physiotherapy (e.g. area indicated by arrowhead).

Limitations of the study

There are a number of methodological limitations with this study that require further consideration. The study design was compromised by practical and logistical limitations, and it would have been preferable to have repeated measurements at least once more, after a short rest period (e.g. 1 hour after physiotherapy). It is known that sputum loosened at physiotherapy is often not expectorated until 1-2 hours later (Van Ginderdeuren, Verbanck et al. 2007), and it may be that any beneficial effect of physiotherapy on LCI would not be seen until this time. However, the two assessments and intervention already took around 2 hours for the majority of patients, and even this was an unpalatably long study for many. For the same reason, it was not possible to use a crossover study design, in what is already a heavily studied CF population. Patients with more severe lung disease (i.e. FEV_1<40% predicted) were not approached, as they were likely to require even longer to complete the study, but it is possible that greatest change in gas mixing would have been seen in those with the most abnormal gas mixing, and most copious sputum production, at the start.

It is unfortunate that some patients arrived having already recently completed physiotherapy or taken bronchodilators. Because of the small numbers, no subgroup analysis is possible on these subjects, but this may have reduced the effects of further treatment in the two patients who were in the physiotherapy group but had already performed physiotherapy on themselves that morning. Patients had been asked to forego morning physiotherapy if possible, but it was recognised that if patients felt that they required physiotherapy they should not withhold it for the purposes of this investigation.

Although there were no statistically significant differences between the two cohorts in terms of baseline variables, this also reflects the small numbers involved. It can be seen from Figure 6.2 and 6.3 that the subjects in the physio group tended to be less unwell than those in the control group, with higher mean FEV_1 and lower mean LCI. In addition, from Table 6.2 it would appear that those in the physio group were less heavy producers of sputum than those in the control group, although this can be difficult to quantify from subjective recall. With hindsight, it would have been useful to have provided the patients with a sputum pot to collect all sputum expectorated in the preceding 24 hours.

The final, and possibly most important, limitation of this study concerns the adequacy of the control group. All subjects, including those in the control group, completed spirometry after the first set of LCI measurements. Unfortunately spirometry - being a forced expiratory manoeuvre - is very similar to the manoeuvres used in physiotherapy, and often induces coughing and expectoration in patients. This can be seen from the weight of sputum expectorated by the control group during the 30 minute period of resting, which was very similar to the amount expectorated by the physiotherapy group. If these subjects expectorated at this rate all day, they would produce considerably more sputum than they described. This may help to explain some of the variability seen in LCI and FRC in the control group, and makes comparisons between the control and active intervention groups less reliable.

Implications for future research

This study highlights some of the difficulties with clinical studies, particularly in cystic fibrosis patients. Patients with CF are very heterogeneous in terms of disease severity, have an already considerable treatment burden and, particularly in Edinburgh, are a heavily studied group. In order to study patients in whom change was likely, the requirement for this study was that patients be sufficiently affected by CF to be chronic producers of sputum, whilst avoiding those with the most severely affected lungs. This places significant restrictions on the numbers of subjects available to be recruited to the study.

The study also highlights the need for balanced groups. Although there were no statistically significant differences between the control and intervention groups in terms of baseline physiology and demographics, this is likely to be an artefact of the small numbers involved. Inspection of the raw data, summarised in Table 6.1, reveals convincing evidence that the control group were less well than the intervention group, with lower median FEV_1 and higher median LCI. If the groups are not comparable, particularly likely when small numbers of participants are involved, this can distort the conclusions. Strategies for balancing the groups include stratifying the group allocation based upon key clinical indicators, such as baseline FEV_1 or sputum production.

Another learning point from this study is the importance of controlling conditions. This is particularly relevant in terms of those subjects who completed physiotherapy, or

who took bronchodilators shortly before attending for the study. It was impractical to require them to attend on a later occasion, but this may have affected the amount of sputum they were able to expectorate.

Finally, there is the issue of the spirometry, one of comparative measurements, affecting the primary outcome, LCI. This was a failing of study design, and was one of the key learning points of this study – a lesson that has been incorporated into subsequent study design. It had been appreciated prior to this that spirometry might interfere with LCI, and this is why spirometry has always been performed after LCI. The extent of the effect was however underestimated.

If this study were to be repeated, the design would be amended to a randomised crossover study, notwithstanding the difficulties this might present in terms of recruitment. The restrictions on patient eligibility would be relaxed to include those who produced any sputum (at one end of the spectrum) to those with FEV_1>30% predicted (at the other end). The difficulty of assessing LCI in those with the most severe lung disease remain, and it would be inappropriate to approach those with FEV_1 below 30% predicted. Tests would only be performed in the morning, in those able to withhold morning physiotherapy, and inability to do this would be an exclusion criterion for the study. This should maximise the volume of sputum produced and hence the chance of detecting a measurable change in LCI. All short-acting bronchodilators and other nebulised therapies would be omitted pre-physiotherapy, and patients unable to complete physiotherapy without these would not be included. Patients would also be asked to provide a 24 hour sputum collection for the preceding 24 hours, on each study day.

The protocol would be amended to include "baseline" spirometry on day 1, followed by 24 hour sputum collection, followed by assessment 1 on day 2, and assessment 2 on day 3. Each assessment would consist of LCI, followed by intervention, followed by LCI immediately, and again at 90 minutes post intervention (see Figure 6.7 for amended study proposal). All sputum would be collected. Spirometry would only be performed after the third LCI assessment (i.e. at the end of the assessment), so as not to interfere with sputum production or LCI. This design would mean that FEV_1 could not be used as a comparative outcome measure since it is not measured immediately pre-physiotherapy, but this is an acceptable limitation in order to assess the effects on LCI properly.

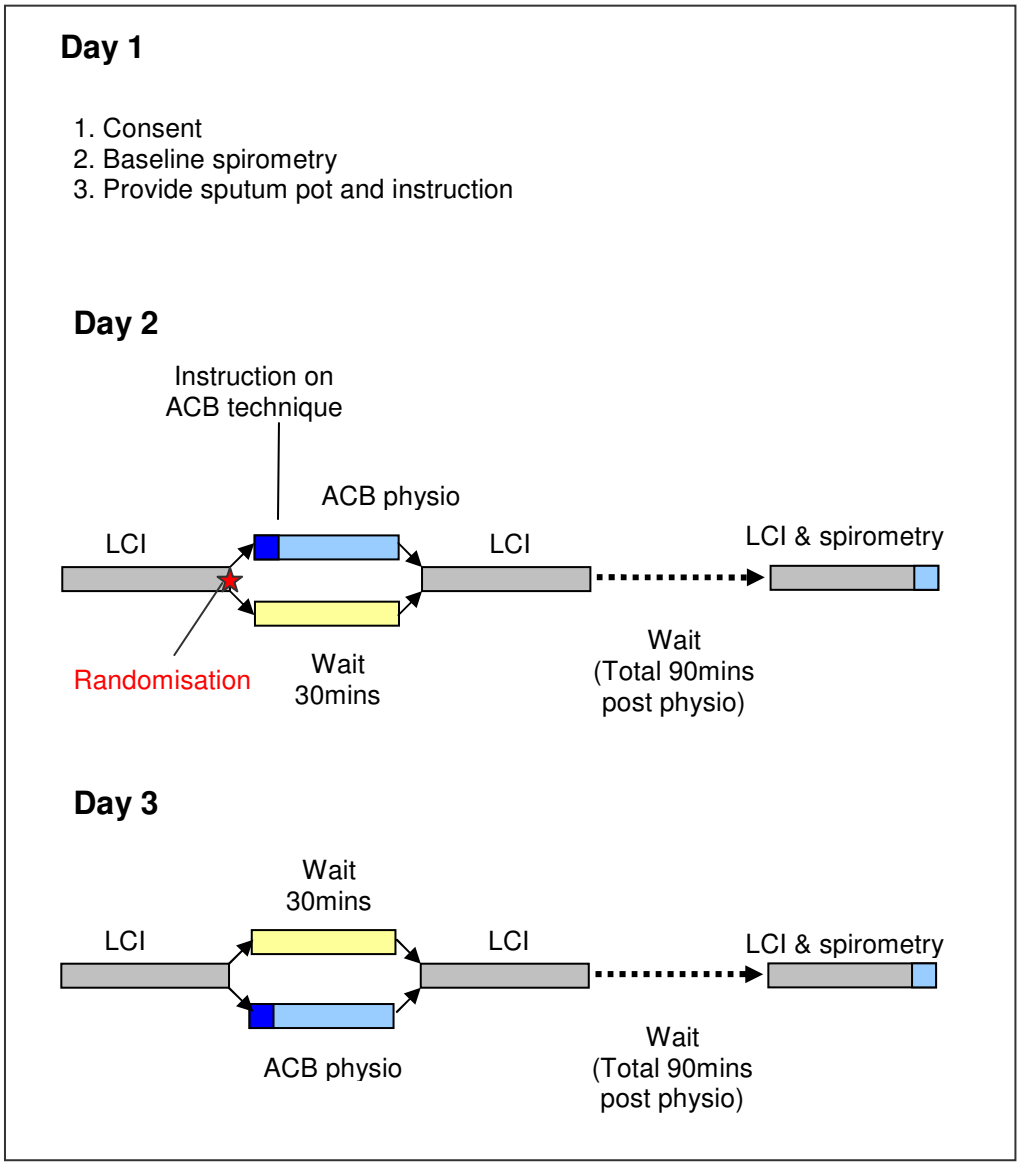


Figure 6.8: Proposed amended study protocol, based upon experience gained from the study described in this chapter. The study is now a randomised crossover design, and requires subjects to attend on three occasions. Subjects are randomised at day 2, and receive the alternative intervention at day 3. Each of the two LCI assessment days is now longer, with a third assessment at 90 minutes post intervention. The merits and difficulties with this study design are discussed in the text.

The use of a crossover study design avoids the need for complex group stratification protocols. This would considerably increase the burden of the study on subjects, and is likely to affect recruitment negatively. However, it would offer the best chance of detecting a consistent change in LCI with physiotherapy, something that has not been possible with the current study design. At the moment, such a study is unfortunately not possible in Edinburgh because the ongoing CF research precludes additional recruitment of subjects to such a demanding schedule.

Summary

Despite the limitations of this study, chest physiotherapy does appear to cause changes in LCI and FRC in patients with CF, despite unpredictable direction of change and little effect on spirometry. This contradicts the original hypothesis – that physiotherapy would improve lung gas mixing – and may stem from the movement of secretions in the small and medium sized airways.

It is not known what happens subsequently though, and it may that, if assayed a few hours later, LCI would improve or return to baseline. That LCI changes at all with physiotherapy however, supports its role as a sensitive marker of airways function in these patients. It is less clear what this means for gene therapy delivery. It would appear to make sense to precede therapy with physiotherapy in order to encourage expectoration of secretions. However, this may actually worsen ventilation heterogeneity in the short term, and perhaps nebulised therapies should be delayed for an hour or so afterwards in order to let this settle, though there is a lack of hard evidence to support this suggestion.

Important lessons have been learned from this study about the sensitivity of LCI to respiratory interventions, and the importance of controlling timing of these. A proposal for an improved study design is included, which would address the majority of these issues, albeit at the expense of a more demanding study schedule.

Chapter 7: Discussion

Both within the CF Gene Therapy Consortium and in the larger CF research community, there is a need for sensitive, repeatable, and practical measures of airways disease (Rosenfeld 2007). Neonatal screening offers the opportunity to detect those with milder mutations early on, before lung disease becomes well established. Longitudinal assessment of these subjects will therefore require better physiological tests than are routinely available. From a gene therapy perspective, sensitive measures that reflect disease in the airways of interest are necessary if any physiological change in response to therapy is to be demonstrated without requiring very large numbers of subjects (Que, Cullinan et al. 2006).

In all of these respects MBW tests, and in particular LCI, offer distinct advantages over conventional lung function assessments. Prior to the studies presented here, LCI was already recognised to be a more sensitive measure of airways dysfunction in children, with a normal range that was stable throughout childhood (Gustafsson, Aurora et al. 2003; Aurora, Gustafsson et al. 2004; Aurora, Bush et al. 2005). These assessments were performed using a mass spectrometer, which is impractical for the multi-centre studies planned by the CF Gene Therapy Consortium.

The first part of this thesis deals with the technical capabilities of an alternative gas analyser, one that was originally developed and marketed for an entirely different role. On paper the Innocor device presents an attractive option for use in multiple breath washouts, as it is both compact and robust, and already contains the two important elements of a MBW apparatus – a gas analyser and a pressure transducer for a flow meter. However, the device has not been designed for the purpose of MBW assessment, and requires both physical modification and the use of custom-prepared software to interpret the data.

The result of the modifications described in Chapter 2 is a system that is practical for use in multi-centre studies with a high patient throughput. In order to achieve this, compromises have been necessary. For example, the calculation of flow-gas delay used is that generated by the Innocor device from a series of fast inspirations. Although the reproducibility of this is good, the calculations are not open to close inspection by the operator, and also require the inclusion of the on-board oxygen analyser. This is in series with the photoacoustic gas analyser and is known to adversely affect rise time. Alternative

methods of assessing flow-gas delay are possible, which could reduce variability in how the manoeuvre is performed, but would require additional hardware and software and are outwith the scope of the work presented in this thesis.

The second part of this thesis deals with the use of the modified Innocor device in clinical assessments of healthy subjects and patients with cystic fibrosis. It has been shown that washouts can be performed in the majority of controls and patients, and that this assessment can usually be completed within 10 minutes in patients with CF. The number of washouts that failed to meet quality control criteria was small, consisting of less than 2% of all washout repeats in adults. In healthy volunteers and those with mild CF, intra-visit reproducibility of LCI was good. In those with more severe disease however, the reproducibility of repeat measurements was seen to deteriorate, and this reflects the clinical experience that these subjects tend to find the washout harder to complete and often have less comfortable and relaxed breathing patterns. The reasons for this may include the effects of dry gas mix on the mouth and lungs, mouthpiece discomfort and accumulation of saliva around the mouthpiece. In these subjects, with very abnormal gas mixing, LCI is a less useful physiological assessment.

In healthy subjects, the normal range of LCI is remarkably stable over a wide age range, in contrast to standard measures of lung function. Normal LCI is also reproducible between different centres, and the normal ranges reported in this thesis are almost identical to those previously published in paediatric populations (Gustafsson, Aurora et al. 2003; Aurora, Gustafsson et al. 2004). This is a major advantage of LCI over spirometry, and makes it particularly suitable for longitudinal assessments and in adolescents. In adults, the sensitivity of LCI to disease status means that it is elevated even in CF subjects with normal spirometry and no symptoms. Since an abnormality can be measured, the effect of therapeutic interventions on lung physiology can now be assessed in these subjects. They form an ideal cohort in which to assess the effects of gene therapy, not least since the condition of their lungs most closely mimics that of children, in whom it is not currently possible to trial gene therapy.

In patients, as might be expected, there is a significant correlation between FEV_1 percent predicted and LCI. Although the relationship between disease severity and genotype is complex, LCI is more abnormal in those with pancreatic insufficiency and

shows a trend to greater abnormality in those with chronic colonisation of the lungs with *Pseudomonas aeruginosa.*.

The third part of this thesis concerns the effects of interventions on LCI. Two interventions have been reported on: intravenous antibiotics in CF patients undergoing an exacerbation, and lung physiotherapy in chronic sputum producers. In the first of these studies, there was a small fall in LCI in those treated with antibiotics. Although this fall, relative to baseline, was no greater than that in FEV_1, there was considerable heterogeneity in the response to antibiotics with some subjects showing an elevation in LCI. This may reflect the opening up of previously poorly ventilated lung regions by treatment. In some of these subjects, with already very abnormal gas mixing and significant structural lung disease, LCI may not be ideal as a clinical outcome. This was also seen when the effects of physiotherapy on LCI were investigated. Although formal physiotherapy appeared to have a greater effect on LCI and FRC than spirometry alone, the effect was neither consistent nor predictable, and was as likely to lead to a deterioration in LCI as an improvement. However, the fact that an improvement in LCI with antibiotic therapy could be shown, even in those with the lowest initial LCI, is reassuring for the use of LCI as an outcome measure.

Neither of the two interventions described is an ideal surrogate to predict the usefulness of LCI in measuring the effects of gene therapy. Both preferentially involve patients with more marked lung disease, either because these are more likely to experience exacerbations or to be chronic sputum producers, and these are not the ideal candidates for nebulised gene therapy. The CT data described in Chapter 4 show clearly that the majority of the patients had substantial structural lung disease that did not improve with antibiotics, and would result in impaired gas mixing efficiency even if the acute inflammation was entirely resolved. Furthermore, in both cases there is likely to be widespread disease of both small and large airways, as confirmed by the CT scans performed after antibiotics. Since the advantages of LCI lie in measuring early airways disease, assumed to be occurring largely in the small airways, it may not be the best outcome measure for the studies described here. Alternative studies that LCI may be a more appropriate and sensitive outcome measure for include the effects of nebulised hypertonic saline, DNase or azithromycin. The effect of these interventions on LCI in subjects with mild disease and normal spirometry could be the subject of future research.

The experience of assessing LCI before and after physiotherapy, although difficult to interpret, has provided important insights into the effects of spirometry on lung gas mixing which would assist in the design of future short-term interventional studies.

Although LCI is the most commonly reported MBW analysis, there has been increasing interest in the use of phase III slope analysis of MBW in order to separate the effects of ventilation heterogeneity in the conducting airways and at the diffusion front (Cosio 2006). In CF however, this does not appear to be a useful analysis perform. S_{cond}, proposed as a measure of convective ventilation heterogeneity in normal subjects and those with mild airways disease (Verbanck, Schuermans et al. 1997), is elevated very early in the course of CF, including in children and those with normal LCI. However, it fails to reflect worsening convective gas mixing with increased disease severity. This appears to be due to theoretical and practical limitations of the analysis, rather than to convective gas mixing reaching a true maximum. Phase III slope analysis therefore adds little to MBW analysis in patients with CF, and it is not proposed that it be used in future analyses in this patient group.

Proposals for future research

Much of the impetus to develop MBW techniques has come from paediatricians, driven by the requirement for simple non-invasive and sensitive measures of early airways disease (Gustafsson 2005). Most notably this has been in the field of CF research, but the benefits of MBW analysis in other disease groups in children has recently also been recognised (Macleod, Horsley et al. 2008). Although the Innocor gas analyzer has been used in children down to the age of 5 yrs, it would not, in its' current format, be suitable for use in children and infants younger than this. The two aspects of its' function that are likely to restrict use in younger subjects are the gas analyzer response time (160 ms) and the gas sample flow rate (120 ml/min). In order to permit use in pre-school children, these would either have to be substantially altered to fit with current recommendations (Beydon, Davis et al. 2007), or further validation would be required, using appropriate in vitro models, to demonstrate that the system was accurate at high respiratory rates and low tidal volumes. The technical demands of performing MBW tests in infants mean that

both current response time and gas sample flow rate are excessive for use in these subjects.

There are a number of techniques by which the Innocor performance could be improved. Firstly, removing the oxygen analyser affords some improvement in analyser response time. Alterations to the gas sample line and gas sample flow rate could also affect gas signal rise time (Schena, Thompson et al. 1984). In order to make the Innocor device a more straightforward system for MBW tests, the on-board software would need to be altered to allow better display of real time flow and gas signals, as well as breath volume feedback. Both of these aspects can only be pursued with the assistance of the manufacturers.

In CF, there is a need for more work on the longitudinal changes in LCI. As discussed in the previous section, there is also a need for intervention studies in subjects with milder disease both to assess the short term effects of interventions on airways function, as well as the longer term effects. LCI would appear to be an ideal tool for these sorts of studies.

In adults, much of the previously published work on washouts has focussed on S_{cond} and S_{acin}, products of phase III slope analysis. These studies have largely focussed on how S_{cond} and S_{acin} relate to the underlying diseases, but have also shown the value of washouts in adults with mild disease. In particular, there is increased ventilation heterogeneity in smokers with no other evidence of lung function impairment (Verbanck, Schuermans et al. 2004), and in ventilation heterogeneity in response to inhaled corticosteroids (Downie, Salome et al. 2007). In adult studies, despite the availability of a range of proposed small airway assays (Cohen, Douma et al. 2008), there remains a need for sensitive and repeatable measures that can reliably reflect small airways function. Areas where there is a particular need for these include the early, non-invasive, identification of bronchiolitis obliterans in lung transplant patients. At present, this is identified initially by changes in the mid expiratory flows, but these tests are poorly reproducible and not reliable in those with airflow obstruction due to narrowing around the anastomosis. Changes in single breath washout tests have been shown to predate those of FEF_{25-75} (Estenne, Van Muylem et al. 2000), and there is good reason to expect LCI to do the same .

MBW analyses may also be able to identify early airways disease in healthy smokers with normal spirometry, or to detect response to treatment in asthmatics. At present, the use of MBW tests remain under-exploited in adult studies.

The washout curves contain considerably more data than that represented by LCI alone, and a number of alternative analyses are possible. Data presented in this thesis, as well as additional analyses not described here, have failed to identify a superior measure of ventilation heterogeneity in terms of sensitivity or robustness. However, it is possible that different measures may be more suited to different patient groups. It is also possible that wash-in curves could provide much of the same information, whilst halving the time required for the investigation, and therefore greatly improving patient acceptability.

In summary, the following future studies are proposed:

1. Improving Innocor performance to facilitate use in younger subjects*
2. Improving Innocor operating system
3. Longitudinal assessments of gas mixing in CF*
4. Long term response to interventions likely to affect small airways in CF, e.g. DNase, hypertonic saline, azithromycin.
5. Use in bronchiolitis obliterans*
6. Use as an endpoint in asthma intervention studies
7. Detection of early airways disease in smokers*
8. Analysis of wash-in curves*

* Currently under investigation with the assistance of other researchers.

References

(1994). "Guidelines for the measurement of respiratory function. Recommendations of the British Thoracic Society and the Association of Respiratory Technicians and Physiologists." Respir Med **88**(3): 165-94.

Abman, S. H., J. W. Ogle, et al. (1988). "Role of respiratory syncytial virus in early hospitalizations for respiratory distress of young infants with cystic fibrosis." J Pediatr **113**(5): 826-30.

Agostoni, P., G. Cattadori, et al. (2005). "Noninvasive measurement of cardiac output during exercise by inert gas rebreathing technique: a new tool for heart failure evaluation." J Am Coll Cardiol **46**(9): 1779-81.

Aldallal, N., E. E. McNaughton, et al. (2002). "Inflammatory response in airway epithelial cells isolated from patients with cystic fibrosis." Am J Respir Crit Care Med **166**(9): 1248-56.

Alton, E. W. (2004). "Use of nonviral vectors for cystic fibrosis gene therapy." Proc Am Thorac Soc **1**(4): 296-301.

Alton, E. W., M. Stern, et al. (1999). "Cationic lipid-mediated CFTR gene transfer to the lungs and nose of patients with cystic fibrosis: a double-blind placebo-controlled trial." Lancet **353**(9157): 947-54.

Arieli, R. and H. D. Van Liew (1981). "Corrections for the response time and delay of mass spectrometers." J Appl Physiol **51**(6): 1417-22.

Armstrong, D. S., K. Grimwood, et al. (1997). "Lower airway inflammation in infants and young children with cystic fibrosis." Am J Respir Crit Care Med **156**(4 Pt 1): 1197-204.

Aurora, P., A. Bush, et al. (2005). "Multiple-breath washout as a marker of lung disease in preschool children with cystic fibrosis." Am J Respir Crit Care Med **171**(3): 249-56.

Aurora, P., P. Gustafsson, et al. (2004). "Multiple breath inert gas washout as a measure of ventilation distribution in children with cystic fibrosis." Thorax **59**(12): 1068-73.

Aurora, P., W. Kozlowska, et al. (2005). "Gas mixing efficiency from birth to adulthood measured by multiple-breath washout." Respir Physiol Neurobiol **148**(1-2): 125-39.

Aziz, Z. A., J. C. Davies, et al. (2007). "Computed tomography and cystic fibrosis: promises and problems." Thorax **62**(2): 181-6.

Aziz, Z. A., A. U. Wells, et al. (2007). "Computed tomography in infective exacerbations of cystic fibrosis: serial change and observer agreement. ." Thorax **62**(Supplement III): A30.

Balough, K., M. McCubbin, et al. (1995). "The relationship between infection and inflammation in the early stages of lung disease from cystic fibrosis." Pediatr Pulmonol **20**(2): 63-70.

Baltimore, R. S., C. D. Christie, et al. (1989). "Immunohistopathologic localization of Pseudomonas aeruginosa in lungs from patients with cystic fibrosis. Implications for the pathogenesis of progressive lung deterioration." Am Rev Respir Dis **140**(6): 1650-61.

Bates, J. H., G. K. Prisk, et al. (1983). "Correcting for the dynamic response of a respiratory mass spectrometer." J Appl Physiol **55**(3): 1015-22.
Becker, M. N., M. S. Sauer, et al. (2004). "Cytokine secretion by cystic fibrosis airway epithelial cells." Am J Respir Crit Care Med **169**(5): 645-53.
Becklake, M. R. (1952). "A new index of the intrapulmonary mixture of inspired air." Thorax **7**(1): 111-6.
Bedrossian, C. W., S. D. Greenberg, et al. (1976). "The lung in cystic fibrosis. A quantitative study including prevalence of pathologic findings among different age groups." Hum Pathol **7**(2): 195-204.
Bell, S. C., A. M. Bowerman, et al. (2000). "Metabolic and inflammatory responses to pulmonary exacerbation in adults with cystic fibrosis." Eur J Clin Invest **30**(6): 553-9.
Beydon, N., S. D. Davis, et al. (2007). "An official American Thoracic Society/European Respiratory Society statement: pulmonary function testing in preschool children." Am J Respir Crit Care Med **175**(12): 1304-45.
Bland, J. M. and D. G. Altman (1986). "Statistical methods for assessing agreement between two methods of clinical measurement." Lancet **1**(8476): 307-10.
Bobadilla, J. L., M. Macek, Jr., et al. (2002). "Cystic fibrosis: a worldwide analysis of CFTR mutations--correlation with incidence data and application to screening." Hum Mutat **19**(6): 575-606.
Boucher, R. C. (2007). "Airway surface dehydration in cystic fibrosis: pathogenesis and therapy." Annu Rev Med **58**: 157-70.
Bouhuys, A. (1963). "Pulmonary nitrogen clearance in relation to age in healthy males." J Appl Physiol **18**: 297-300.
Bouhuys, A., S. Lichtneckert, et al. (1961). "Voluntary changes in breathing pattern and N2 clearance from lungs." J Appl Physiol **16**: 1039-42.
Bradley, J. M., F. M. Moran, et al. (2006). "Evidence for physical therapies (airway clearance and physical training) in cystic fibrosis: an overview of five Cochrane systematic reviews." Respir Med **100**(2): 191-201.
Brody, A. S., J. S. Klein, et al. (2004). "High-resolution computed tomography in young patients with cystic fibrosis: distribution of abnormalities and correlation with pulmonary function tests." J Pediatr **145**(1): 32-8.
Brody, A. S., H. Sucharew, et al. (2005). "Computed tomography correlates with pulmonary exacerbations in children with cystic fibrosis." Am J Respir Crit Care Med **172**(9): 1128-32.
Brown, J. S., T. R. Gerrity, et al. (1998). "Effect of ventilation distribution on aerosol bolus dispersion and recovery." J Appl Physiol **85**(6): 2112-7.
Brown, R., A. J. Woolcock, et al. (1969). "Physiological effects of experimental airway obstruction with beads." J Appl Physiol **27**(3): 328-35.
Brownlee, K. G. (2006). "Small airways disease in cystic fibrosis." European Respiratory Momgraph **35**: 21-37.
Brunner, J. X., G. Wolff, et al. (1985). "Accurate measurement of N2 volumes during N2 washout requires dynamic adjustment of delay time." J Appl Physiol **59**(3): 1008-12.
Buess, C., P. Pietsch, et al. (1986). "A pulsed diagonal-beam ultrasonic airflow meter." J Appl Physiol **61**(3): 1195-9.

Burgel, P. R., D. Montani, et al. (2007). "A morphometric study of mucins and small airway plugging in cystic fibrosis." Thorax **62**(2): 153-61.
Caplen, N. J., E. W. Alton, et al. (1995). "Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis." Nat Med **1**(1): 39-46.
Chadwick, S. L., H. D. Kingston, et al. (1997). "Safety of a single aerosol administration of escalating doses of the cationic lipid GL-67/DOPE/DMPE-PEG5000 formulation to the lungs of normal volunteers." Gene Ther **4**(9): 937-42.
Cheng, K., R. L. Smyth, et al. (1996). "Spread of beta-lactam-resistant Pseudomonas aeruginosa in a cystic fibrosis clinic." Lancet **348**(9028): 639-42.
Chirmule, N., K. Propert, et al. (1999). "Immune responses to adenovirus and adeno-associated virus in humans." Gene Ther **6**(9): 1574-83.
Chow, C. W., L. I. Landau, et al. (1982). "Bronchial mucous glands in the newborn with cystic fibrosis." Eur J Pediatr **139**(4): 240-3.
Clancy, J. P., S. M. Rowe, et al. (2007). "No detectable improvements in cystic fibrosis transmembrane conductance regulator by nasal aminoglycosides in patients with cystic fibrosis with stop mutations." Am J Respir Cell Mol Biol **37**(1): 57-66.
Cohen, J., W. R. Douma, et al. (2008). "Ciclesonide improves measures of small airway involvement in asthma." Eur Respir J **31**(6): 1213-20.
Cosio, M., H. Ghezzo, et al. (1978). "The relations between structural changes in small airways and pulmonary-function tests." N Engl J Med **298**(23): 1277-81.
Cosio, M. G. (2006). "Looking at the acinus with function tests: can you believe it?" Am J Respir Crit Care Med **174**(8): 847-8.
Couriel, J. M., M. Schier, et al. (1985). "Distribution of ventilation in young children with cystic fibrosis." Pediatr Pulmonol **1**(6): 314-8.
Crawford, A. B., M. Makowska, et al. (1985). "Convection- and diffusion-dependent ventilation maldistribution in normal subjects." J Appl Physiol **59**(3): 838-46.
Cunningham, S., J. R. McColm, et al. (2003). "Duration of effect of intravenous antibiotics on spirometry and sputum cytokines in children with cystic fibrosis." Pediatr Pulmonol **36**(1): 43-8.
Cystic-Fibrosis-Foundation (2005). Patient Registry Annual Report 2005. Bethseda, Maryland.
Dakin, C. J., A. H. Numa, et al. (2002). "Inflammation, infection, and pulmonary function in infants and young children with cystic fibrosis." Am J Respir Crit Care Med **165**(7): 904-10.
Davies, D. G., M. R. Parsek, et al. (1998). "The involvement of cell-to-cell signals in the development of a bacterial biofilm." Science **280**(5361): 295-8.
Davis, P. B. (2006). "Cystic fibrosis since 1938." Am J Respir Crit Care Med **173**(5): 475-82.
Davis, P. B., P. J. Byard, et al. (1997). "Identifying treatments that halt progression of pulmonary disease in cystic fibrosis." Pediatr Res **41**(2): 161-5.
Davis, S. D., L. A. Fordham, et al. (2007). "Computed tomography reflects lower airway inflammation and tracks changes in early cystic fibrosis." Am J Respir Crit Care Med **175**(9): 943-50.
Dawson, M., D. Wirtz, et al. (2003). "Enhanced viscoelasticity of human cystic fibrotic sputum correlates with increasing microheterogeneity in particle transport." J Biol Chem **278**(50): 50393-401.

de Gracia, J., F. Mata, et al. (2005). "Genotype-phenotype correlation for pulmonary function in cystic fibrosis." Thorax **60**(7): 558-63.
de Jong, P. A., Y. Nakano, et al. (2005). "Changes in airway dimensions on computed tomography scans of children with cystic fibrosis." Am J Respir Crit Care Med **172**(2): 218-24.
de Jong, P. A., Y. Nakano, et al. (2004). "Progressive damage on high resolution computed tomography despite stable lung function in cystic fibrosis." Eur Respir J **23**(1): 93-7.
Demirkazik, F. B., O. M. Ariyurek, et al. (2001). "High resolution CT in children with cystic fibrosis: correlation with pulmonary functions and radiographic scores." Eur J Radiol **37**(1): 54-9.
Dodge, J. A., P. A. Lewis, et al. (2007). "Cystic fibrosis mortality and survival in the UK: 1947-2003." Eur Respir J **29**(3): 522-6.
Dodge, J. A. and D. Turck (2006). "Cystic fibrosis: nutritional consequences and management." Best Pract Res Clin Gastroenterol **20**(3): 531-46.
Donaldson, S. H., W. D. Bennett, et al. (2006). "Mucus clearance and lung function in cystic fibrosis with hypertonic saline." N Engl J Med **354**(3): 241-50.
Downey, D. G., S. Brockbank, et al. (2007). "The effect of treatment of cystic fibrosis pulmonary exacerbations on airways and systemic inflammation." Pediatr Pulmonol **42**(8): 729-35.
Downie, S. R., C. M. Salome, et al. (2007). "Ventilation heterogeneity is a major determinant of airway hyperresponsiveness in asthma, independent of airway inflammation." Thorax **62**(8): 684-9.
Downie, S. R., C. M. Salome, et al. (2007). "Ventilation heterogeneity is a major determinant of airway hyperresponsiveness in asthma, independent of airway inflammation." Thorax.
Dutrieue, B., F. Vanholsbeeck, et al. (2000). "A human acinar structure for simulation of realistic alveolar plateau slopes." J Appl Physiol **89**(5): 1859-67.
Elkins, M. R., M. Robinson, et al. (2006). "A controlled trial of long-term inhaled hypertonic saline in patients with cystic fibrosis." N Engl J Med **354**(3): 229-40.
Emmanuel, G., W. A. Briscoe, et al. (1961). "A method for the determination of the volume of air in the lungs: measurements in chronic pulmonary emphysema." J Clin Invest **40**: 329-37.
Engelhardt, J. F., M. Zepeda, et al. (1994). "Expression of the cystic fibrosis gene in adult human lung." J Clin Invest **93**(2): 737-49.
Espiritu, J. D., G. Ruppel, et al. (2003). "The diffusing capacity in adult cystic fibrosis." Respir Med **97**(6): 606-11.
Estenne, M., A. Van Muylem, et al. (2000). "Detection of obliterative bronchiolitis after lung transplantation by indexes of ventilation distribution." Am J Respir Crit Care Med **162**(3 Pt 1): 1047-51.
Fleming, G. M., E. H. Chester, et al. (1980). "Ventilation inhomogeneity using multibreath nitrogen washout: comparison of moment ratios and other indexes." Am Rev Respir Dis **121**(5): 789-94.
Forkert, L., S. Dhingra, et al. (1979). "Airway closure and closing volume." J Appl Physiol **46**(1): 24-30.
Fowler, R. W., R. A. Pluck, et al. (1987). "Maximal expiratory flow-volume curves in Londoners aged 60 years and over." Thorax **42**(3): 173-82.

Fowler, W. S. (1948). "Lung function studies; the respiratory dead space." Am J Physiol **154**(3): 405-16.
Fowler, W. S. (1949). "Lung function studies. III. Uneven pulmonary ventilation in normal subjects and in patients with pulmonary disease." Journal of Applied Physiology **2**(6): 283-299.
Fuchs, H. J., D. S. Borowitz, et al. (1994). "Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. The Pulmozyme Study Group." N Engl J Med **331**(10): 637-42.
Fuchs, S. I., C. Buess, et al. (2006). "Multiple breath washout with a sidestream ultrasonic flow sensor and mass spectrometry: a comparative study." Pediatr Pulmonol **41**(12): 1218-25.
Gadsby, D. C., P. Vergani, et al. (2006). "The ABC protein turned chloride channel whose failure causes cystic fibrosis." Nature **440**(7083): 477-83.
Ghofrani, H. A., F. Reichenberger, et al. (2004). "Sildenafil increased exercise capacity during hypoxia at low altitudes and at Mount Everest base camp: a randomized, double-blind, placebo-controlled crossover trial." Ann Intern Med **141**(3): 169-77.
Gill, D. R., K. W. Southern, et al. (1997). "A placebo-controlled study of liposome-mediated gene transfer to the nasal epithelium of patients with cystic fibrosis." Gene Ther **4**(3): 199-209.
Gimeno, F., L. T. van der Weele, et al. (1993). "Variability of forced oscillation (Siemens Siregnost FD 5) measurements of total respiratory resistance in patients and healthy subjects." Ann Allergy **71**(1): 56-60.
Goldman, M. J., G. M. Anderson, et al. (1997). "Human beta-defensin-1 is a salt-sensitive antibiotic in lung that is inactivated in cystic fibrosis." Cell **88**(4): 553-60.
Gronkvist, M., E. Bergsten, et al. (2002). "Effects of body posture and tidal volume on inter- and intraregional ventilation distribution in healthy men." J Appl Physiol **92**(2): 634-42.
Gronkvist, M. J., M. J. Emery, et al. (2002). "Mechanisms of ventilation inhomogeneity during vital capacity breaths standing and supine." Respir Physiol **129**(3): 345-55.
Gustafsson, P. M. (2005). "Inert gas washout in preschool children." Paediatr Respir Rev **6**(4): 239-45.
Gustafsson, P. M. (2007). "Peripheral airway involvement in CF and asthma compared by inert gas washout." Pediatr Pulmonol **42**(2): 168-76.
Gustafsson, P. M., P. Aurora, et al. (2003). "Evaluation of ventilation maldistribution as an early indicator of lung disease in children with cystic fibrosis." Eur Respir J **22**(6): 972-9.
Gustafsson, P. M., P. A. de Jong, et al. (2007). "Multiple-Breath Inert Gas Washout And Spirometry Vs. Structural Lung Disease In Cystic Fibrosis." Thorax.
Gustafsson, P. M., P. A. De Jong, et al. (2008). "Multiple-breath inert gas washout and spirometry versus structural lung disease in cystic fibrosis." Thorax **63**(2): 129-34.
Habib, R. H. and K. R. Lutchen (1991). "Moment analysis of a multibreath nitrogen washout based on an alveolar gas dilution number." Am Rev Respir Dis **144**(3 Pt 1): 513-9.
Haeusler, G., H. Frisch, et al. (1994). "Perspectives of longitudinal growth in cystic fibrosis from birth to adult age." Eur J Pediatr **153**(3): 158-63.

Hamutcu, R., J. M. Rowland, et al. (2002). "Clinical findings and lung pathology in children with cystic fibrosis." Am J Respir Crit Care Med **165**(8): 1172-5.
Hankinson, J. L., J. Stocks, et al. (1998). "Reproducibility of lung volume measurements." Eur Respir J **11**(3): 787-90.
Hansell, D. M. (2001). "Small airways diseases: detection and insights with computed tomography." Eur Respir J **17**(6): 1294-313.
Hansell, D. M., M. B. Rubens, et al. (1997). "Obliterative bronchiolitis: individual CT signs of small airways disease and functional correlation." Radiology **203**(3): 721-6.
Harris, A. (1997). "Towards an ovine model of cystic fibrosis." Hum Mol Genet **6**(13): 2191-4.
Hart, M. C., M. M. Orzalesi, et al. (1963). "Realtionship between anatomic dead space and body size and lung volume." Journal of Applied Physiology **18**(3): 519-522.
Helbich, T. H., G. Heinz-Peer, et al. (1999). "Cystic fibrosis: CT assessment of lung involvement in children and adults." Radiology **213**(2): 537-44.
Helbich, T. H., G. Heinz-Peer, et al. (1999). "Evolution of CT findings in patients with cystic fibrosis." AJR Am J Roentgenol **173**(1): 81-8.
Horsley, A., K. Macleod, et al. (2007). "Lung clearance index is superior to moment ratios in the analysis of inert gas washouts in cystic fibrosis." European Respiratory Journal **30**(Supplement 51): 339s.
Horsley, A. R., P. M. Gustafsson, et al. (2008). "Lung clearance index is a sensitive, repeatable and practical measure of airways disease in adults with cystic fibrosis." Thorax **63**(2): 135-40.
Hyde, S. C., K. W. Southern, et al. (2000). "Repeat administration of DNA/liposomes to the nasal epithelium of patients with cystic fibrosis." Gene Ther **7**(13): 1156-65.
Johnson, L. G., J. C. Olsen, et al. (1992). "Efficiency of gene transfer for restoration of normal airway epithelial function in cystic fibrosis." Nat Genet **2**(1): 21-5.
Joris, L., I. Dab, et al. (1993). "Elemental composition of human airway surface fluid in healthy and diseased airways." Am Rev Respir Dis **148**(6 Pt 1): 1633-7.
Khan, T. Z., J. S. Wagener, et al. (1995). "Early pulmonary inflammation in infants with cystic fibrosis." Am J Respir Crit Care Med **151**(4): 1075-82.
King, G. G., S. R. Downie, et al. (2005). "Effects of methacholine on small airway function measured by forced oscillation technique and multiple breath nitrogen washout in normal subjects." Respir Physiol Neurobiol **148**(1-2): 165-77.
Knowles, M., J. Gatzy, et al. (1981). "Increased bioelectric potential difference across respiratory epithelia in cystic fibrosis." N Engl J Med **305**(25): 1489-95.
Konstan, M. W. and M. Berger (1997). "Current understanding of the inflammatory process in cystic fibrosis: onset and etiology." Pediatr Pulmonol **24**(2): 137-42; discussion 159-61.
Kraemer, R., D. N. Baldwin, et al. (2006). "Progression of pulmonary hyperinflation and trapped gas associated with genetic and environmental factors in children with cystic fibrosis." Respir Res **7**: 138.
Kraemer, R., A. Blum, et al. (2005). "Ventilation inhomogeneities in relation to standard lung function in patients with cystic fibrosis." Am J Respir Crit Care Med **171**(4): 371-8.

Kraemer, R., N. Delosea, et al. (2006). "Effect of allergic bronchopulmonary aspergillosis on lung function in children with cystic fibrosis." Am J Respir Crit Care Med **174**(11): 1211-20.
Kreda, S. M., M. Mall, et al. (2005). "Characterization of wild-type and deltaF508 cystic fibrosis transmembrane regulator in human respiratory epithelia." Mol Biol Cell **16**(5): 2154-67.
Kreda, S. M., R. J. Pickles, et al. (2000). "G-protein-coupled receptors as targets for gene transfer vectors using natural small-molecule ligands." Nat Biotechnol **18**(6): 635-40.
Krowka, M. J., P. L. Enright, et al. (1987). "Effect of effort on measurement of forced expiratory volume in one second." Am Rev Respir Dis **136**(4): 829-33.
Kunzelmann, K., R. Schreiber, et al. (2000). "Control of epithelial Na+ conductance by the cystic fibrosis transmembrane conductance regulator." Pflugers Arch **440**(2): 193-201.
Larsson, A., C. Jonmarker, et al. (1988). "Ventilation inhomogeneity during controlled ventilation. Which index should be used?" J Appl Physiol **65**(5): 2030-9.
Latzin, P., L. Sauteur, et al. (2007). "Optimized temperature and deadspace correction improve analysis of multiple breath washout measurements by ultrasonic flowmeter in infants." Pediatr Pulmonol **42**(10): 888-97.
Lechardeur, D., K. J. Sohn, et al. (1999). "Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer." Gene Ther **6**(4): 482-97.
Ljungberg, H. K. and P. M. Gustafsson (2003). "Peripheral airway function in childhood asthma, assessed by single-breath He and SF6 washout." Pediatr Pulmonol **36**(4): 339-47.
Lum, S., P. Gustafsson, et al. (2007). "Early detection of cystic fibrosis lung disease: multiple-breath washout versus raised volume tests." Thorax **62**(4): 341-7.
Lutchen, K. R., R. H. Habib, et al. (1990). "Respiratory impedance and multibreath N2 washout in healthy, asthmatic, and cystic fibrosis subjects." J Appl Physiol **68**(5): 2139-49.
Ma, T., L. Vetrivel, et al. (2002). "High-affinity activators of cystic fibrosis transmembrane conductance regulator (CFTR) chloride conductance identified by high-throughput screening." J Biol Chem **277**(40): 37235-41.
Macintyre, N., R. O. Crapo, et al. (2005). "Standardisation of the single-breath determination of carbon monoxide uptake in the lung." Eur Respir J **26**(4): 720-35.
Macklem, P. T. and J. Mead (1967). "Resistance of central and peripheral airways measured by a retrograde catheter." J Appl Physiol **22**(3): 395-401.
Macklem, P. T., W. M. Thurlbeck, et al. (1971). "Chronic obstructive disease of small airways." Ann Intern Med **74**(2): 167-77.
Macleod, K., A. R. Horsley, et al. (2007). "Development of standardisation criteria for the measurement of lung clearance index in a multi-centre study. ." European Respiratory Journal **ERS conference, Stockholm 2007**.
Macleod, K. A., A. R. Horsley, et al. (2008). "Ventilation heterogeneity in well controlled asthmatic children, with normal spirometry, indicates residual airways disease." Thorax.
Main, E., E. L. Tannenbaum, et al. (2004). "Evaluation of Lung Clearance Index as an outcome measure for airway clearance intervention studies." Journal of Cystic Fibrosis **3**(Supplement): S89.

Main, E., E. L. Tannenbaum, et al. (2006). "The effects of positive expiratory pressure or oscillatory positive pressure on FEV1 and lung cearance index over a twelve month period in children with cystic fibrosis." Pediatr Pulmonol **41**(Supplement): S351.
Mall, M., B. R. Grubb, et al. (2004). "Increased airway epithelial Na+ absorption produces cystic fibrosis-like lung disease in mice." Nat Med **10**(5): 487-93.
Matsui, H., B. R. Grubb, et al. (1998). "Evidence for periciliary liquid layer depletion, not abnormal ion composition, in the pathogenesis of cystic fibrosis airways disease." Cell **95**(7): 1005-15.
Mayer-Hamblett, N., M. L. Aitken, et al. (2007). "Association between pulmonary function and sputum biomarkers in cystic fibrosis." Am J Respir Crit Care Med **175**(8): 822-8.
McLachlan, G., H. Davidson, et al. (2005). "Ovine lung comparison of non-viral gene transfer agents for CF gene therapy." Pediatr Pulmonol **28 (Suppl)**: A256.
McLachlan, G., B. J. Stevenson, et al. (2000). "Bacterial DNA is implicated in the inflammatory response to delivery of DNA/DOTAP to mouse lungs." Gene Ther **7**(5): 384-92.
McLean, A., P. M. Warren, et al. (1992). "Microscopic and macroscopic measurements of emphysema: relation to carbon monoxide gas transfer." Thorax **47**(3): 144-9.
Mentore, K., D. K. Froh, et al. (2005). "Hyperpolarized HHe 3 MRI of the lung in cystic fibrosis: assessment at baseline and after bronchodilator and airway clearance treatment." Acad Radiol **12**(11): 1423-9.
Merkus, P. J., H. A. Tiddens, et al. (2002). "Annual lung function changes in young patients with chronic lung disease." Eur Respir J **19**(5): 886-91.
Michaelson, E. D., E. D. Grassman, et al. (1975). "Pulmonary mechanics by spectral analysis of forced random noise." J Clin Invest **56**(5): 1210-30.
Middleton, P. and E. W. F. W. Alton (2006). In vivo measurement of airway potential difference to assess CFTR function in man. Cystic Fibrosis in the 21st Century. A. Bush, E. W. F. W. Alton, J. C. Davies, U. Griesenbach and A. Jaffe. Basel, Karger. **34:** 102-108.
Milic-Emili, J., J. A. Henderson, et al. (1966). "Regional distribution of inspired gas in the lung." J Appl Physiol **21**(3): 749-59.
Miller, M. R., J. Hankinson, et al. (2005). "Standardisation of spirometry." Eur Respir J **26**(2): 319-38.
Miller, M. R. and T. Sigsgaard (1994). "Prevention of thermal and condensation errors in pneumotachographic recordings of the maximal forced expiratory manoeuvre." Eur Respir J **7**(1): 198-201.
Montier, T., P. Delepine, et al. (2004). "Non-viral vectors in cystic fibrosis gene therapy: progress and challenges." Trends Biotechnol **22**(11): 586-92.
Mosteller, R. D. (1987). "Simplified calculation of body-surface area." N Engl J Med **317**(17): 1098.
Nasr, S. Z., D. Gordon, et al. (2006). "High resolution computerized tomography of the chest and pulmonary function testing in evaluating the effect of tobramycin solution for inhalation in cystic fibrosis patients." Pediatr Pulmonol **41**(12): 1129-37.
Noble, D. D., J. B. McCafferty, et al. (2007). "Respiratory heat and moisture loss is associated with eosinophilic inflammation in asthma." Eur Respir J **29**(4): 676-81.

Noone, P. G., K. W. Hohneker, et al. (2000). "Safety and biological efficacy of a lipid-CFTR complex for gene transfer in the nasal epithelium of adult patients with cystic fibrosis." Mol Ther **1**(1): 105-14.
O'Riordan, C. R., A. Lachapelle, et al. (1999). "PEGylation of adenovirus with retention of infectivity and protection from neutralizing antibody in vitro and in vivo." Hum Gene Ther **10**(8): 1349-58.
Oostveen, E., D. MacLeod, et al. (2003). "The forced oscillation technique in clinical practice: methodology, recommendations and future developments." Eur Respir J **22**(6): 1026-41.
Orzalesi, M. M., M. C. Hart, et al. (1965). "Distribution Of Ventilation In Normal Subjects From 7 To 45 Years Of Age." J Appl Physiol **20**: 77-8.
Padman, R., S. A. McColley, et al. (2007). "Infant care patterns at epidemiologic study of cystic fibrosis sites that achieve superior childhood lung function." Pediatrics **119**(3): e531-7.
Paiva, M. (1975). "Two new pulmonary functional indexes suggested by a simple mathematical model." Respiration **32**(5): 389-403.
Paiva, M. and L. A. Engel (1984). "Model analysis of gas distribution within human lung acinus." J Appl Physiol **56**(2): 418-25.
Pavord, I. D., M. M. Pizzichini, et al. (1997). "The use of induced sputum to investigate airway inflammation." Thorax **52**(6): 498-501.
Pedemonte, N., G. L. Lukacs, et al. (2005). "Small-molecule correctors of defective DeltaF508-CFTR cellular processing identified by high-throughput screening." J Clin Invest **115**(9): 2564-71.
Perez, A., A. C. Issler, et al. (2007). "CFTR inhibition mimics the cystic fibrosis inflammatory profile." Am J Physiol Lung Cell Mol Physiol **292**(2): L383-95.
Peslin, R., C. Duvivier, et al. (1985). "Respiratory impedance measured with head generator to minimize upper airway shunt." J Appl Physiol **59**(6): 1790-5.
Petty, T. L., G. W. Silvers, et al. (1982). "Small airway dimension and size distribution in human lungs with an increased closing capacity." Am Rev Respir Dis **125**(5): 535-9.
Poets, C. F., G. A. Rau, et al. (1997). "Determinants of lung volume in spontaneously breathing preterm infants." Am J Respir Crit Care Med **155**(2): 649-53.
Porteous, D. J., J. R. Dorin, et al. (1997). "Evidence for safety and efficacy of DOTAP cationic liposome mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis." Gene Ther **4**(3): 210-8.
Prasad, S. A. (1993). "Current concepts in physiotherapy." J R Soc Med **86 Suppl 20**: 23-9.
Prisk, G. K., A. R. Elliott, et al. (1998). "Multiple-breath washin of helium and sulfur hexafluoride in sustained microgravity." J Appl Physiol **84**(1): 244-52.
Prisk, G. K., A. M. Lauzon, et al. (1996). "Anomalous behavior of helium and sulfur hexafluoride during single-breath tests in sustained microgravity." J Appl Physiol **80**(4): 1126-32.
Quanjer, P. H., G. J. Tammeling, et al. (1993). "Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society." Eur Respir J Suppl **16**: 5-40.

Que, C., P. Cullinan, et al. (2006). "Improving rate of decline of FEV1 in young adults with cystic fibrosis." Thorax **61**(2): 155-7.
Quinton, P. M. (1990). "Cystic fibrosis: a disease in electrolyte transport." Faseb J **4**(10): 2709-17.
Ramsey, B. W. and T. F. Boat (1994). "Outcome measures for clinical trials in cystic fibrosis. Summary of a Cystic Fibrosis Foundation consensus conference." J Pediatr **124**(2): 177-92.
Ramsey, B. W., M. S. Pepe, et al. (1999). "Intermittent administration of inhaled tobramycin in patients with cystic fibrosis. Cystic Fibrosis Inhaled Tobramycin Study Group." N Engl J Med **340**(1): 23-30.
Riekert, K. A., S. J. Bartlett, et al. (2007). "The association between depression, lung function, and health-related quality of life among adults with cystic fibrosis." Chest **132**(1): 231-7.
Riordan, J. R., J. M. Rommens, et al. (1989). "Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA." Science **245**(4922): 1066-73.
Robinson, T. E., M. L. Goris, et al. (2005). "Dornase alfa reduces air trapping in children with mild cystic fibrosis lung disease: a quantitative analysis." Chest **128**(4): 2327-35.
Roca, J., F. Burgos, et al. (1998). "References values for forced spirometry. Group of the European Community Respiratory Health Survey." Eur Respir J **11**(6): 1354-62.
Rosenberg, S. M., W. F. Howatt, et al. (1992). "Spirometry and chest roentgenographic appearance in adults with cystic fibrosis." Chest **101**(4): 961-4.
Rosenfeld, M. (2007). "An overview of endpoints for cystic fibrosis clinical trials: one size does not fit all." Proc Am Thorac Soc **4**(4): 299-301.
Rosenfeld, M., R. L. Gibson, et al. (2001). "Early pulmonary infection, inflammation, and clinical outcomes in infants with cystic fibrosis." Pediatr Pulmonol **32**(5): 356-66.
Rosenthal, M., S. H. Bain, et al. (1993). "Lung function in white children aged 4 to 19 years: I--Spirometry." Thorax **48**(8): 794-802.
Rowe, S. M., F. Accurso, et al. (2007). "Detection of cystic fibrosis transmembrane conductance regulator activity in early-phase clinical trials." Proc Am Thorac Soc **4**(4): 387-98.
Rowe, S. M., S. Miller, et al. (2005). "Cystic fibrosis." N Engl J Med **352**(19): 1992-2001.
Sagel, S. D., J. F. Chmiel, et al. (2007). "Sputum biomarkers of inflammation in cystic fibrosis lung disease." Proc Am Thorac Soc **4**(4): 406-17.
Samee, S., T. Altes, et al. (2003). "Imaging the lungs in asthmatic patients by using hyperpolarized helium-3 magnetic resonance: assessment of response to methacholine and exercise challenge." J Allergy Clin Immunol **111**(6): 1205-11.
Saniie, J., G. M. Saidel, et al. (1979). "Real-time moment analysis of pulmonary nitrogen washout." J Appl Physiol **46**(6): 1184-90.
Schechter, M. S., B. J. Shelton, et al. (2001). "The association of socioeconomic status with outcomes in cystic fibrosis patients in the United States." Am J Respir Crit Care Med **163**(6): 1331-7.
Schena, J., J. Thompson, et al. (1984). "Mechanical influences on the capnogram." Crit Care Med **12**(8): 672-4.
Schidlow, D. V. (1994). "Primary ciliary dyskinesia (the immotile cilia syndrome)." Ann Allergy **73**(6): 457-68; quiz 468-70.

Schmalisch, G., H. Proquitte, et al. (2006). "The effect of changing ventilator settings on indices of ventilation inhomogeneity in small ventilated lungs." BMC Pulm Med **6**: 20.
Schwartz, D. A., T. J. Quinn, et al. (1997). "CpG motifs in bacterial DNA cause inflammation in the lower respiratory tract." J Clin Invest **100**(1): 68-73.
Shah, R. M., W. Sexauer, et al. (1997). "High-resolution CT in the acute exacerbation of cystic fibrosis: evaluation of acute findings, reversibility of those findings, and clinical correlation." AJR Am J Roentgenol **169**(2): 375-80.
Shwachman, H. and L. L. Kulczycki (1958). "Long-term study of one hundred five patients with cystic fibrosis; studies made over a five- to fourteen-year period." AMA J Dis Child **96**(1): 6-15.
Simel, D. L., J. P. Mastin, et al. (1984). "Scanning electron microscopic study of the airways in normal children and in patients with cystic fibrosis and other lung diseases." Pediatr Pathol **2**(1): 47-64.
Smith, J. J., S. M. Travis, et al. (1996). "Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid." Cell **85**(2): 229-36.
Sobonya, R. E. and L. M. Taussig (1986). "Quantitative aspects of lung pathology in cystic fibrosis." Am Rev Respir Dis **134**(2): 290-5.
Somia, N. and I. M. Verma (2000). "Gene therapy: trials and tribulations." Nat Rev Genet **1**(2): 91-9.
Southern, K. W., P. M. Barker, et al. (2004). "Macrolide antibiotics for cystic fibrosis." Cochrane Database Syst Rev(2): CD002203.
Stanojevic, S., A. Wade, et al. (2008). "Reference ranges for spirometry across all ages: a new approach." Am J Respir Crit Care Med **177**(3): 253-60.
Tang, Y., M. J. Turner, et al. (2005). "Effects of lung time constant, gas analyser delay and rise time on measurements of respiratory dead-space." Physiol Meas **26**(6): 1103-14.
Tarran, R., B. Button, et al. (2005). "Normal and cystic fibrosis airway surface liquid homeostasis. The effects of phasic shear stress and viral infections." J Biol Chem **280**(42): 35751-9.
Tarran, R., B. R. Grubb, et al. (2001). "The CF salt controversy: in vivo observations and therapeutic approaches." Mol Cell **8**(1): 149-58.
Tiddens, H. A., L. P. Koopman, et al. (2000). "Cartilaginous airway wall dimensions and airway resistance in cystic fibrosis lungs." Eur Respir J **15**(4): 735-42.
Timonen, K. L., J. T. Randell, et al. (1997). "Short-term variations in oscillatory and spirometric lung function indices among school children." Eur Respir J **10**(1): 82-7.
van Beek, E. J. and J. M. Wild (2005). "Hyperpolarized 3-helium magnetic resonance imaging to probe lung function." Proc Am Thorac Soc **2**(6): 528-32, 510.
van der Schans, C., A. Prasad, et al. (2000). "Chest physiotherapy compared to no chest physiotherapy for cystic fibrosis." Cochrane Database Syst Rev(2): CD001401.
Van Ginderdeuren, F., S. Verbanck, et al. (2007). "Chest Physiotherapy in Cystic Fibrosis: Short-Term Effects of Autogenic Drainage Preceded by Wet Inhalation of Saline versus Autogenic Drainage Preceded by Intrapulmonary Percussive Ventilation with Saline." Respiration.
Venegas, J. (2007). "Linking ventilation heterogeneity and airway hyperresponsiveness in asthma." Thorax **62**(8): 653-4.

Venegas, J. G., T. Winkler, et al. (2005). "Self-organized patchiness in asthma as a prelude to catastrophic shifts." Nature **434**(7034): 777-82.
Verbanck, S., D. Schuermans, et al. (2004). "Noninvasive assessment of airway alterations in smokers: the small airways revisited." Am J Respir Crit Care Med **170**(4): 414-9.
Verbanck, S., D. Schuermans, et al. (1999). "Evidence of acinar airway involvement in asthma." Am J Respir Crit Care Med **159**(5 Pt 1): 1545-50.
Verbanck, S., D. Schuermans, et al. (2006). "Small Airway Function Improvement after Smoking Cessation in Smokers without Airway Obstruction." Am J Respir Crit Care Med **174**(8): 853-7.
Verbanck, S., D. Schuermans, et al. (2003). "Nonreversible conductive airway ventilation heterogeneity in mild asthma." J Appl Physiol **94**(4): 1380-6.
Verbanck, S., D. Schuermans, et al. (2008). "The effect of conductive ventilation heterogeneity on diffusing capacity measurement." J Appl Physiol **104**(4): 1094-100.
Verbanck, S., D. Schuermans, et al. (1998). "Conductive and acinar lung-zone contributions to ventilation inhomogeneity in COPD." Am J Respir Crit Care Med **157**(5 Pt 1): 1573-7.
Verbanck, S., D. Schuermans, et al. (1997). "Ventilation distribution during histamine provocation." J Appl Physiol **83**(6): 1907-16.
Verdugo, P., P. Y. Tam, et al. (1983). "Conformational structure of respiratory mucus studied by laser correlation spectroscopy." Biorheology **20**(2): 223-30.
Verkman, A. S. (2004). "Drug discovery in academia." Am J Physiol Cell Physiol **286**(3): C465-74.
von Nieding, G., H. Lollgen, et al. (1977). "Simultaneous washout of helium and sulfur hexafluoride in healthy subjects and patients with chronic bronchitis, bronchial asthma, and emphysema." Am Rev Respir Dis **116**(4): 649-60.
Vonberg, R. P. and P. Gastmeier (2005). "Isolation of infectious cystic fibrosis patients: results of a systematic review." Infect Control Hosp Epidemiol **26**(4): 401-9.
Wall, M. A. (1985). "Moment analysis of multibreath nitrogen washout in young children." J Appl Physiol **59**(1): 274-9.
Wanger, J., J. L. Clausen, et al. (2005). "Standardisation of the measurement of lung volumes." Eur Respir J **26**(3): 511-22.
Weber, A. J., G. Soong, et al. (2001). "Activation of NF-kappaB in airway epithelial cells is dependent on CFTR trafficking and Cl- channel function." Am J Physiol Lung Cell Mol Physiol **281**(1): L71-8.
Welsh, M. J. and A. E. Smith (1993). "Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis." Cell **73**(7): 1251-4.
Wood, D. M. and A. R. Smyth (2006). "Antibiotic strategies for eradicating Pseudomonas aeruginosa in people with cystic fibrosis." Cochrane Database Syst Rev(1): CD004197.
Woodhouse, N., J. M. Wild, et al. (2007). "Hyperpolarised helium-3 MRI pre and post physiotherapy in cystic fibrosis: preliminary experiences." European Respiratory Journal **30**(Supplement 51): 450s.
Worlitzsch, D., R. Tarran, et al. (2002). "Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients." J Clin Invest **109**(3): 317-25.

Yaakov, Y., E. Kerem, et al. (2007). "Reproducibility of nasal potential difference measurements in cystic fibrosis." Chest **132**(4): 1219-26.
Yeh, M. P., R. M. Gardner, et al. (1982). "Computerized determination of pneumotachometer characteristics using a calibrated syringe." J Appl Physiol **53**(1): 280-5.
Yeh, P. and M. Perricaudet (1997). "Advances in adenoviral vectors: from genetic engineering to their biology." Faseb J **11**(8): 615-23.
Zabner, J., S. H. Cheng, et al. (1997). "Comparison of DNA-lipid complexes and DNA alone for gene transfer to cystic fibrosis airway epithelia in vivo." J Clin Invest **100**(6): 1529-37.
Zahm, J. M., S. Girod de Bentzmann, et al. (1995). "Dose-dependent in vitro effect of recombinant human DNase on rheological and transport properties of cystic fibrosis respiratory mucus." Eur Respir J **8**(3): 381-6.
Zahm, J. M., D. Pierrot, et al. (1989). "The role of mucus sol phase in clearance by simulated cough." Biorheology **26**(4): 747-52.

Appendix A – Standard operating procedure for use of Innocor to perform multiple breath washout

Contents

This manual is for the use of Innocor to lung clearance by inert gas washout. The entire process from setting up the machine to exporting data is covered. The principles of data analysis are also covered in a separate section. This is not intended to wholly replace Innocor's own manual which you may still need to refer to.

Contacts:
Alex Horsley
Molecular Medicine Centre
Western General Hospital
Edinburgh EH4 2XU
alex.horsley@ed.ac.uk

The sections on infection control and measuring LCI in children was written by:
Dr Kenneth Macleod
Department of Child Life & Health
Royal Hospital for Sick Children
Edinburgh EH9 1UW
kenny.macleod@ed.ac.uk

Part 1: Background

Lung Clearance Index

Inert gas washout is a technique for assessing the efficiency of gas mixing in the lungs. It involves measuring the elimination of a non-absorbed gas as it is exhaled during tidal breathing. The gas can either be resident nitrogen washed out by breathing 100% oxygen, or an exogenous tracer gas which has first been breathed in to equilibrium. As gas mixing becomes more uneven (or more inhomogenous) the washout is prolonged. Inhomogeneity of ventilation is a sensitive marker of airways dysfunction in early CF particularly. The reasons for this are complex, but it is likely that disease of the small airways produces regional differences in ventilation before it is sufficient to affect overall airways resistance (and hence FEV_1).

Lung clearance index (LCI) is a simple marker of deranged ventilation that can be calculated from the washout curves. Past studies using a variety of methods have demonstrated that LCI is reproducible and more sensitive than FEV_1 at identifying early lung disease in children. In addition, LCI has been shown to be further raised in children infected with *Pseudomonas aeruginosa* to be an early predictor of deteriorating lung function in children.

An additional advantage in children is that there are no techniques to learn (e.g. as with forced expiratory manoeuvres) and only tidal breathing is required. Successful tests should therefore be possible in the majority of children down to the age of five.

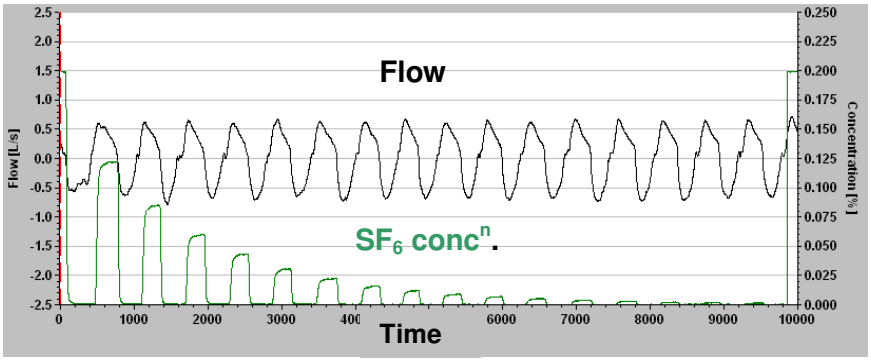


Figure 1: Example of a washout graph

In order to measure inert gas washout, a sensitive and rapid gas analyser is required. We have adapted a piece of equipment originally designed for cardiac output measurements to follow minute concentrations of sulphur hexafluoride (SF_6). This is an inert marker gas that is non-absorbed and has been used for some years in similar tests.

The LCI is the best reported measure of ventilation heterogeneity. This is calculated by dividing the cumulative expired volume required to wash the marker gas to $1/40^{th}$ of its starting concentration by the functional residual capacity (FRC, the volume of air in the lungs at the start of the washout). In other words, LCI represents the number of lung turnovers required to wash the gas out. Hence an increase in heterogeneity of ventilation distribution causes an increase in LCI.

Innocor

Innocor was developed to measure cardiac output using the technique of inert gas rebreathing. This involves the patient breathing in a mixture of a blood soluble gas (N_2O) and an insoluble gas (SF_6) for a short period. The disappearance rate of the blood soluble gas (relative to the insoluble gas) is proportional to the pulmonary blood flow. This is then used to calculate cardiac output (and other derived parameters). This procedure requires rebreathing of gas mixture from a bag.

The intended use is for the patient to carry out an incremental exercise test with Innocor continuously measuring oxygen uptake and CO_2 production (known as the breath by breath function). Periodic measurements of cardiac output are then performed at pre-programmed intervals/workloads.

Innocor measures the concentration of the gases SF_6, N_2O, CO_2 and O_2 using a novel technique known as photoacoustic mass spectroscopy. The gas molecules absorb energy from pulsed infrared light. This change in energy generates a sound wave which is measured by an extremely sensitive microphone. The microphone signal is then filtered to separate the three modulation frequencies that correspond to the different gas concentrations. The amplitude of each component of the sound wave is directly proportional to the corresponding gas concentration. This method is very sensitive to low concentrations of SF_6 (0-0.2%), with excellent signal:noise ratio and minimal drift. The downside of this is that the rise time of the gas analyser is slower than that of a standard mass spectrometer, which may limit use of this apparatus in subjects less than 5 yrs old.

The use of Innocor to measure lung clearance by inert gas washout is a non-standard adaptation of the device, and there are a number of features of Innocor that are either redundant or have to be worked around; these are covered in the relevant sections below. Innocor does not generate a measure of lung clearance index, the data have to be exported to another computer for analysis on custom built software.

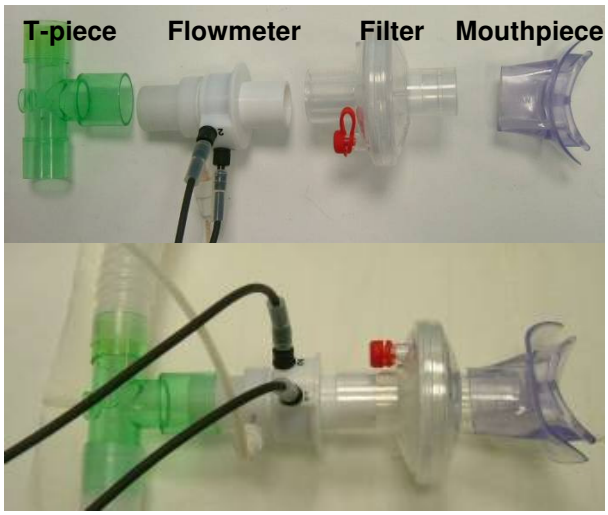
Additional background information on Innocor, and the process of validating the device for LCI measurement, can be found in Horsley et al., Thorax, 2008.

Part 2: Hardware Adaptations

The patient interface that comes with Innocor (known as the rebreathing valve unit, or RVU) cannot be easily disassembled. It is designed for exercise testing and hence has both low resistance to flow and a large dead space. For the tidal breathing involved in inert gas washout, resistance to flow is much less of an issue, but minimal dead space is important. For inert gas washout, a smaller filter and pneuomtachograph (flow sensing device) are needed, and we do not use the ones supplied with the machine. The recommended arrangement and component details are as below.

Description	Manufacturer	Part Number
Adult flowmeter	Hans Rudolph, Missouri, USA	4700A
Paediatric flowmeter		4719
Adult filter	DAR,Tyco Healthcare, UK	Barrierbac S
Paediatric Filter	Air Safety, UK	9070/01
Adult mouthpiece	Ferraris,UK	PKM0902051000000
	Hans Rudolph	Small bite mouthpiece
T-piece for flowpast	Intersurgical, UK	Code: 1981 T-Piece 22M-22F-22M

Figure 2:
Adult patient-interface for inert gas washout, disassembled (top) and ready to use (bottom).



Sample port

The end of the flowmeter furthest from the patient needs to be drilled so that a 20G needle can be placed through the hole with the tip in the middle of the stream of air (figure 3). This then needs to be secured and sealed to ensure that there is no leak around the needle as it passes through the housing. The site of the hole should be adjacent to the pressure transducer lines and sufficiently far back to not interfere with the fitting of the flow-past circuit, but otherwise to be as far from the flowmeter membrane as possible. The gas sample line on the RVU has a Luer fitting, so this can be unscrewed and attached to the needle.

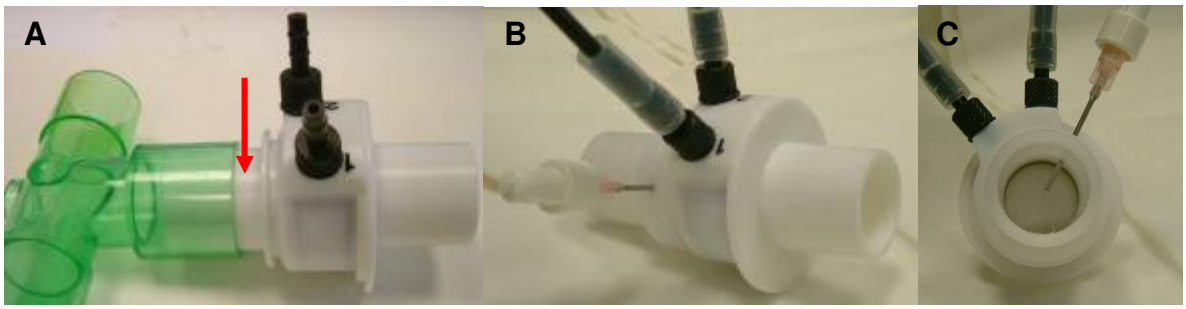


Figure 3: **A**: Site for sample port indicated by arrow on distal part of flowmeter housing, between main chamber and T-piece attachment. **B**: Flowmeter with needle hole drilled and needle in place. **C**: End-on view showing needle tip in centre of stream of air.

Attaching the pneumotachograph

Because the RVU cannot easily be dismantled, the most efficient method of connecting the flowmeter to the Innocor pressure transducer is to use a new connection entirely. Innovision can supply, on request, a connector that plugs into the RVU connection on Innocor, but only contains patent ports for the two pressure transducer lines (figure 4). These ports can then be connected to the appropriate ports on the flowmeter using equal lengths of plastic tubing (Guttasyn, Hamburg, Germany). The gas sample connects separately to the Innocor device, and has a Luer fitting which facilitates its connection to the alternative gas sample needle described above.

Figure 4: Modified pneumotachograph connector attached to RVU port on side of Innocor.

Patient Interface

An acceptable and comfortable method of positioning the mouthpiece for the patient needs to be devised. In Edinburgh we have adapted the swing arm from an anglepoise lamp, with the mouthpiece, filter and flowmeter clamped at the end of the arm and adjustable in 3 dimensions (figure 5).

When positioning the patient interface, ensure that the gas sample and flowmeter pressure transducer lines are not in a downward (6 o'clock) position. There is a theoretical risk that

moisture could condense around the gas sample needle or flowmeter membrane and run down into the lines. Ideally the flowmeter should be rotated so that the lines exit in the 12 o'clock position, but any position between 9 and 3 o'clock would be satisfactory (as in figure 5).

Figure 5: Patient interface for MBW measurements. The flow-meter is clamped to the end of a swing arm which is fixed to the side of a table.

Flow-past circuit

During the wash-in phase of the test, the patient breathes in from the flow-past circuit. This consists of two pieces of long tubing joined to a T-piece connector as shown in Figure 6. One limb of the tubing is connected to a cylinder containing 0.2% SF_6 in air, and the other limb is the exhaust. If the test room is particularly small or poorly ventilated, then it would be sensible to place the exhaust limb outside of the room.

The patient breathes from, and back into, the tubing with expired gas vented out of the tubing by the constant flow from the cylinder. The downstream limb of the circuit should be around 1m long. The upstream limb should be around 0.5m in length, with a reservoir bag placed in-line with the tubing (Figure 7). This allows a lower gas flow in the circuit, and prolongs the life of the cylinder.

Figure 6: T-piece (green) with tubing attached through which SF_6/air mix flows

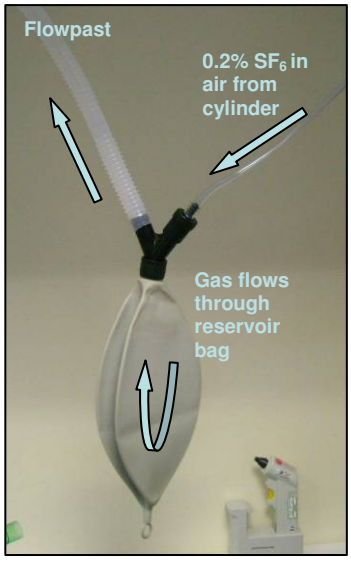
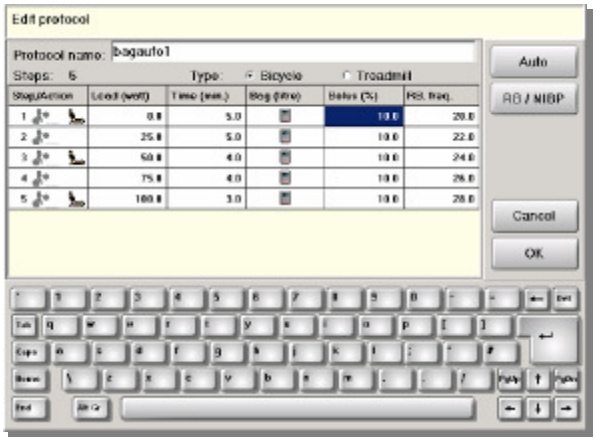


Figure 7: Reservoir bag attached to SF6 source

Washout Test

Innocor has a number of preset protocols for exercise testing, which involve incremental increases in workload and intermittent rebreathing manoeuvres to determine cardiac output. For inert gas washout, a protocol is needed which allows a prolonged period of breath by breath measurements at zero workload and without interruption for rebreathing. To set this:

- Select **Measurement,** and then **Test** from the right hand menu of the main report screen.
- A window will appear for you to enter details about patient weight, haemoglobin etc. You do not need to worry about this and can leave it blank, these data are used in cardiac output calculations. Press **OK**.
- To enter a new protocol select **New**. The following screen appears:



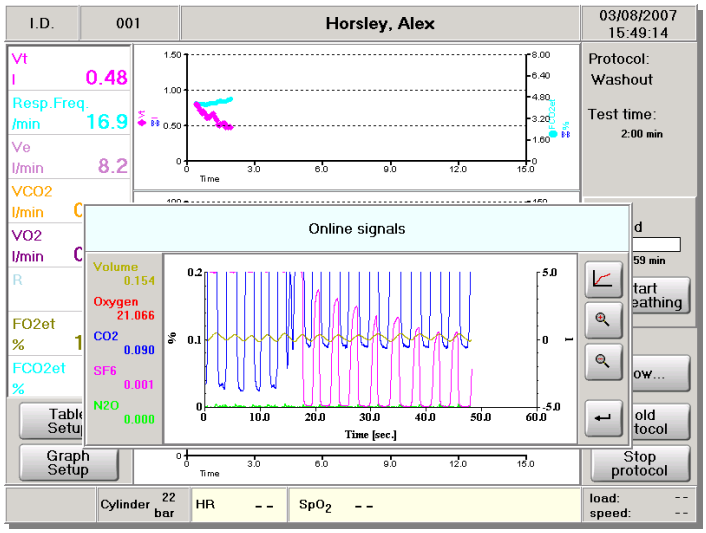
- Enter **Washout** as the protocol name.
- The protocol appears on the top line of the yellow box. Ensure this is highlighted.
- Press **RB/NIBP** repeatedly until the symbols disappear from the left hand column (the number 1 will remain).
- Set the load to 0.0 watt
- Set the time to 60 mins.
- The columns for **Bag, Bolus** and **RB freq.** should have been cleared when the symbols were cleared from the left hand column.
- Select **OK**.
- You will be returned to the previous menu. Select **BBB graphs Setup**.
- You can now select what data to display during the breath by breath manoeuvre. Bear in mind that even though you can select three graphs, one of these will be obscured by the online data graph anyway.

The suggested settings are:

- *Graph 1:* Time (*x* axis) vs Vt. This displays the tidal volume of each breath against time. Set Y axis 0-1.5L (adult) to display physiological range clearly.
- *Graph 2*: Time (*x* axis) vs V'CO_2 & V'O_2.
- *Graph 3:* blank. Cover with online data readout during test.

- **OK** to return to protocol menu.
- Press the ⇳ symbol near the bottom right corner of the menu until the exercise device for your protocol is selected as **Manual control**. Otherwise Innocor will be looking for an exercise machine to synchronise with at the start of each test.
- To set up the data display on the left hand side of a test, start a dummy run test. Select **Table Setup** from the bottom left corner.
  You do not need all the defunct cardiac output measurment data displayed. Suggested data to be displayed are:
  - Vt (tidal volume)
  - Resp Freq.
  - Ve (minute ventilation)
  - V'CO_2 (CO_2 production)
  - VO_2 (Oxygen consumption)
  - R (Respiratory quotient)
  - If using the oxygen saturation monitor, then you should also select SpO2 and HR.

The final display should look like this:



Flowmeter Linearisation

If changing a flowmeter, replacing it after cleaning, or changing the type of filter used, you must perform the following procedure. This is so that Innocor can interpret the pressure changes in the flowmeter in a linear fashion over the full range of flows. It is important that this is performed using the appropriate filter. Adding a filter to the circuit changes the flow entering the flowmeter and can lead to errors in measurement. This is

avoided by performing the linearisation with the filter in place. Both filters selected have been tested and shown not to affect the flowmeter over the physiological range, providing that the output is first calibrated as described.

- Connect the calibration syringe to the flowmeter using the appropriate sized filter (i.e. a paediatric filter when preparing the paediatric flowmeter, and an adult filter for the adult flowmeter).
- Exit to windows (see page 17).
- Select the directory **C:/Innocor/Programme** and click on **Flowmetercalibration.exe.**
- Select **Calibrate.**
- Select **Prepare Syringe size** and ensure that correct syringe size is set.
- Select **Add stroke**. A table appears with a flow/volume graph in the left.
- Following the instructions in the window at top left, complete a number of syringe empty and fill manoeuvres, covering a wide range of flow rates.
- If you make an error, that stroke can be de-selected from the right hand table by highlighting that data and pressing **Use/skip**.
- Press **Accept**.
- Unless the %error throughout is <3%, repeat the process.
- To finish press **Accept** and then **Cancel.**
- Select the option to save data, and select the file **bbblin1.cal** as the filename. You will be asked if you wish to overwrite the saved file, select **Yes**.
- **Exit** takes you back to Windows.

Flowmeter re-zero

A feature of the Innocor flowmeter is that it automatically re-zeroes the flowmeter at regular intervals to correct for any drift. Whilst doing this the computer generates a non-physiological flow signal of 100L/sec for 1 second. This helps to maintain the accuracy of the flowmeter but if it occurs during expiration in the washout it will effectively wipe out that 1 second of data, as well as requiring manual adjustment of the washout analysis. In order to avoid this, we try to set the interval between re-zeroes such that the re-zero occurs at the end of wash-in (and therefore will not be repeated before washout is complete). This takes a degree of judgement and guesswork, since normal volunteers wash-in in far less time than a subject with moderately severe CF. We have found that, for adults, 5 minutes represents a reasonable compromise between an excessive wash-in time for subjects who are well, and too frequent re-zeroes affecting washout analysis. For children, 4 minutes is usually sufficient.

In order to set the time interval between flowmeter re-zero:

- Exit to Windows (see page 17)
- Unless you have a keyboard connected through the Innocor USB port, select **On-Screen Keyboard.exe** from the Startup menu.
- Select directory **C:/Innocor/setup** and select the Text file **Hardware.ini**
- Scroll down until you come to the line reading:

```
[BBB]
Installed=1
FlowzeroAfterRB=0
FlowzeroInterval=300
```

- The number after FlowzeroInterval is the time in seconds between re-zero manoeuvres. Using the On-Screen Keyboard (or a keyboard connected through the USB port) set this to the desired time interval between re-zeroes (e.g. 300 for an interval of 5 mins).
- Close the text box, and confirm that you want the changes saved when prompted.

Part 4 - Daily Checks

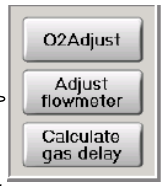
You do not need to unscrew the small cylinder at the back of the Innocor machine after use, and when prompted about this simply confirm that you have done it. This procedure is necessary with the standard RVU to prevent gas leak. Since the modified connector we use has all the gas cylinder ports occluded it is not necessary for us to do this.
Ensure that all connections are tight and leak free. Attention should also be paid to the flowmeter pressure lines and gas sample line. These should be tightly fitted to the flowmeter and gas sample needle, and should not be kinked, twisted or occluded at any point throughout their length.

Warm up

The machine should be left for 20minutes after switching on to ensure that the photoacoustic gas analyser is fully warmed up.

Calibration of pneumotachograph

This should be performed daily.
If a **new** flowmeter is being used, this should first be linearised, see page 9.



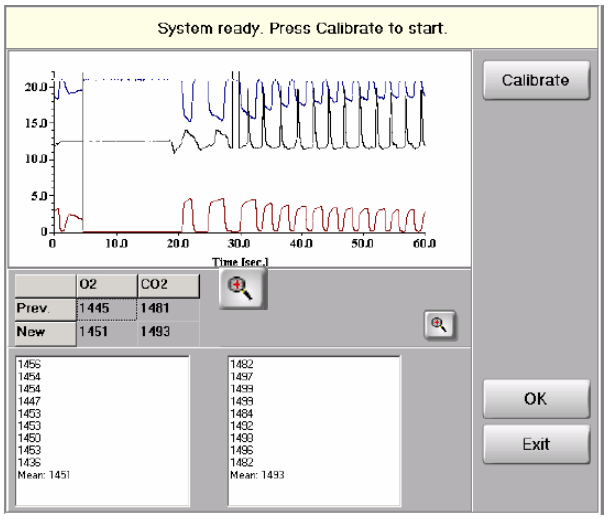
- Select **Setup** from the menu on the opening screen.
- Select **Calibration.**
- From the pop-up menu select **Adjust Flowmeter.**
- Connect a 1 or 3L calibration syringe to the flowmeter **using the appropriate filter** and set the syringe size on the screen accordingly.
- Empty the syringe.
- Press **Calibrate.**
- When instructed, fill the syringe slowly, at a low flow rate, without bumping the end.
- Continue to follow the instructions on the screen. A total 5 fill and empty procedures are performed (2 slow, 2 medium and 1 fast). After each stroke the measured volume is displayed in the table.

- At the end of the calibration, the new gain is displayed. This should be between 0.9 and 1.1. If acceptable, press **OK** and **Exit.**
- The measured syringe volume should also differ by less than +/- 3%. In other words, once the correct gain is applied the measured volume should be between 2.91 and 3.09 litres. It is preferable for the accuracy to be greater than this, and we aim to achieve an error of +/-2% (i.e. measured volume between 2.94 and 3.06 L once correct gain applied).
- If the gain, or the accuracy, are outwith this range, press **Calibrate** and repeat the process. If the gain remains unacceptable then:
    - Ensure the correct flowmeter and correct syringe are being used.
    - Ensure that the correct filter is being used. Adding a filter changes the flow and hence pressure/flow characteristics of the flowmeter, so all calibrations and linearisation procedures must be performed with the appropriate filter connected.
    - Ensure all connections between flowmeter and tubing and tubing and RVU cable are secure and leak free.
    - Ensure the syringe is securely connected to the flowmeter.
- If the problem remains then the flowmeter linearity may need to be adjusted (see page 8).
- Always record the gain in the log book, so that any trends in errors can be identified.

Flow-gas delay calibration

This should be performed daily and recorded in the log book.
It is vital that this is known before lung clearance data are calculated as accurate alignment of the signals is vital. The delay is calculated by generating rapid changes in flow on inspiration, which also causes a rapid change in CO_2 concentration. This manoeuvre should be performed by the operator, **not the patient.**

- From the opening menu select **Setup** and then select **Calibration.**
- From the pop-up menu select **Calculate gas delay.**
- Wait for the device to warm up.
- When warmed up the operator breathes through the flowmeter. The delay is specific to the equipment set-up so a filter and mouthpiece must be used exactly as if you were a patient undergoing a test, and the sample line must be connected.



- When ready press calibrate and perform 11 slow expirations followed by 11 very fast inspirations. The inspirations have to be as fast as possible to get an accurate determination of gas delay.
- The delay calculated can be shown by pressing the icon. The software filters out breaths that fail.
- Overall gas delay should not vary by more than 20-40msecs on different days. If the new delay is excessive, check all connections and repeat the calibration.
- It is the CO_2 delay that matches the SF_6 delay, this should be recorded in the log book.
- At the end of the test press **OK** and then **Exit.**

Ambient conditions

The Innocor machine generates raw data signals which, after analysis, need to be adjusted to standardised BTPS conditions (convert volumes to those they would occupy at Body Temperature, standard barometric Pressure, and water Saturated). To do this, the ambient conditions need to be known. These will also affect the flowmeter output and possibly the flow-gas delay too, though these are calibrated daily so no other adjustment is needed.

In Edinburgh, we maintain the constancy of ambient conditions by using air conditioning in the test room. This maintains the room at a reasonably constant 23^{o}C (which we check daily) and relative humidity. Fluctuations in barometric pressure are generally small and we do not measure these but assume a constant 760mmHg.

If using the apparatus in a room without air conditioning, it is important to measure temperature and relative humidity and record these daily. In addition, if there are substantial changes in ambient conditions over the course of a day, the machine should be recalibrated before use (in other words do not calibrate during a cool morning and use on a hot afternoon). The Innocor usage log allows for these factors to be recorded.

Part 5 - Performing a Test

Equipment required

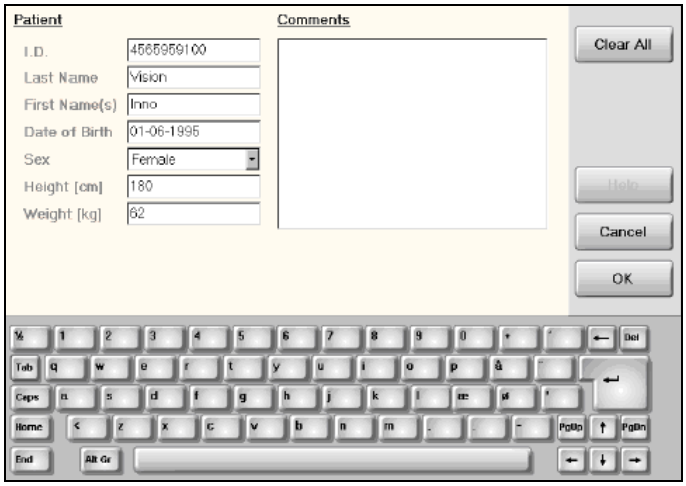
- Innocor device, modified and set up as described in previous sections.
- Supply of 0.2% SF_6 in air
- Sterile filter
- Clean mouthpiece and noseclips
- Calibration syringe
- Fan
- Visual feedback system – computer screen visible to patient
- Television
- Innocor log to record settings

1. Before starting

- Switch machine on. Ignore onscreen instructions about Innocor gas cylinder, press **OK** to continue.
- Ensure that daily checks and calibrations have been completed (page 10).
- Ensure that knob on top of 0.2% SF_6 cylinder is switched to on.
- Check that all equipment is correctly arranged and that gas sample and pressure transducer lines from Innocor are tightly connected to gas sample port and flowmeter respectively.
- Check that the pneumotach for breath volume feedback is switched on, zeroed, and correctly connected to the laptop. Ensure that the visual feedback software loads correctly, and that the screen can be seen by the subject.

2. Entering patient data & patient codes

- From the **Measurement** screen select **New Patient**.
- The following screen appears:



- Use the keyboard to enter the relevant data and then select **OK**.

- ID is the patient code, and will be used in the filename of the data generated for that patient (along with the date and time).
- See separate sheet for recommended patient codes for the different CFGT studies.

3. Instructions to Patient

Before starting

- Ensure patient is comfortably seated and suitably distracted.
- Attach noseclip (again ensure this is comfortable) and ensure that a good mouthpiece seal can be achieved.
- Demonstrate the procedure for detaching the T-piece before starting the first wash-in and instruct patient not to alter their breathing pattern whilst you are doing this during the test.

Instructions

- Instruct patient to breathe normally.
- They should breathe in and out regularly, without long pauses between inspiration and expiration.
- Warn them that you may need to give instruction to breathe more or less deeply if their tidal volume is inadequate or excessive.
- Show them the visual feedback display and indicate that you will place a target on the display once they have settled into a stable pattern. The target may require slightly deeper or shallower breaths than they have been completing spontaneously.
- If they need to cough, they can do this through the device.
- It is important that a good seal around the mouthpiece is maintained at all times.

Important points

It is important that regular breathing is maintained throughout the test. To achieve this, the patient needs to be comfortable, both with the position of the apparatus and with the fit of the mouthpiece. They also need to be adequately distracted, preferably by watching a television. Attention should be paid to the breathing pattern to ensure that it is both regular and stable with a reasonable tidal volume (0.5-1.0L for an adult).

It is also important that long expiratory pauses are avoided. The sample port has a flow rate of 120ml/min and during a long pause can sample all the expired gas in the end of the flowmeter, so that room air is then sampled. The fall in SF_6 concentration can look like the start of an inspiration and complicates data analysis.

Figure 8: Patient testing. Subject is comfortably seated, with noseclip applied, and achieving an effective mouthpiece seal.

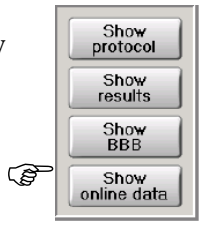
4. Starting the test

- From the opening screen of Innocor select **Measurement.**
- Select **New Pt.** to enter data for a new patient (as above) or select the patient from the list displayed. The patient details can also be retrieved from the database using the **Search** facility.
- After entering or choosing a patient press **Select.**
- Press **Test** to prepare a new measurement.
- Select "**Washout test**" from the list of possible protocols (see page 7 for details on how to set this).
- For a new patient, you will be required to confirm the height & weight data before starting. You will then be required to confirm that the load is set to zero: this is for exercise testing and the instruction can be ignored but you must select **OK** before recording can start.

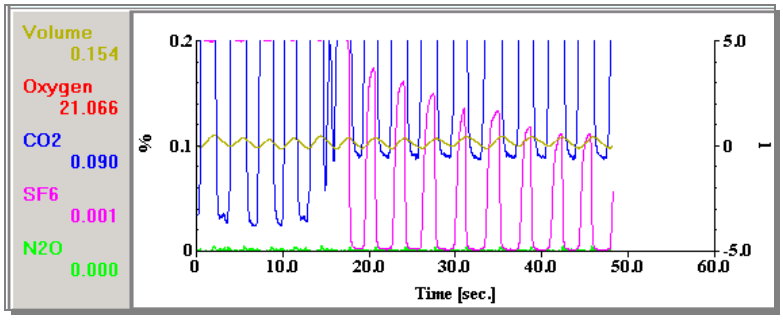
5. Wash-in Phase

- Ensure that the T-piece connects the patient to the flow past circuit and turn on the gas. The gas in the flow-past should be set at a sufficient rate to ensure that no expired air is re-inspired. For an adult this should be at least 10 L/min, but will need to be increased if the patient breathes faster or deeper than average.
- Instruct the patient to breathe normally through the system, with a target **inspiratory** volume of between 0.5 and 1.0L. They should then expire to a relaxed FRC. It is important that long pauses between inspiration and expiration are avoided.
- As the patient breathes in, the bar on the visual feedback display should rise. For the first 1-2minutes, allow the subject to achieve a comfortable tidal volume without targeting the inspiration.

- Once a steady breath volume is achieved, place target arrow on the screen at approximately the middle of the breath volume range. If this is outwith the desired breath volume range of 0.5-1L, then place the arrow within the target range and alert the patient to this.
- In order to display the real-time SF_6 concentration, select **Show results** from the right hand menu and then select **Show online data** from the pop-up menu.



- This pulls up a graph that shows real-time flow and gas concentrations. To enlarge the scale and so view the SF_6 concentration, press 
  If you want to close this graph press 



- If the SF_6 concentration suddenly falls on an inspiration it is likely that the gas flow in the flow-past is insufficient. Check for leaks and increase the flow rate to cope with the patient's minute ventilation and peak inspiratory flow.
  Note that when observing the real-time data that the gas signals are approximately 1.5 seconds (or half a breath) behind the flow signals.

6. Wash Out Phase

- Continue the wash-in phase until the inspiratory and expiratory SF_6 concentrations are equal (i.e. differ by less than 2% relative, or are within 0.004% absolute of the starting SF_6 concentration). After this, continue for a further 30 seconds before starting the washout.
- Turn on fan so that a stream of air is directed over the end of the flowmeter.
- Once the SF_6 has been fully washed in, rapidly disconnect the T-piece during expiration. It is usually possible to identify expiration by watching the patient's chest movement and/or the breath volume feedback. If this is difficult, instruct the patient to continue breathing normally, but to raise a hand during expiration.
- When disconnected plug the open arm of the T-piece so that SF_6 does not escape into the test room, and then quickly switch off the gas supply.
- The patient now breathes room air until the peak expiratory SF_6 concentration has fallen to less than 0.005%. Continue for a few breaths beyond this point.
- At the end of the test close the online data window and stop the test by pressing **Stop protocol** from the right hand menu.
  - If you have paused the test by pressing **Hold protocol,** do not press **Stop protocol** but restart before terminating.

Part 6: Special considerations in subjects under 16 yrs

Standard Procedure for Older Children (5 yrs and over)

The equipment used and procedure for adult patients is suitable for paediatric patients in this age-group. There are, however, some differences in approach that ensure the best quality data. Previous experience in communicating with children and explaining procedures is useful.

Prior explanation of procedure:

- It is important that the procedure is fully explained to the child in language that they will understand. Explain the purpose of the test as a whole, the gas, the mouthpiece and the tubing. Children, especially older ones, are able to understand difficult concepts if clear language is used. Allow questions and encourage them that they can come off if they feel unwell or find it difficult to breathe.
- The must have confidence in the person conducting the test or they may be unwilling to continue.
- A calm, relaxed approach means that the child will feel happy taking part and they will trust the researcher.

Equipment

- *General testing environment*

The room should be friendly and inviting without being too full of distractions.

Make sure the chair the child sits in is comfortable for them. A cushioned chair is not necessarily the most comfortable option as it is harder to stay still in. A swivel chair or one on wheels is very hard to sit still in. One that encourages children to sit straight and not to move around is best. The recommended chair is "q-learn chair senior" distributed by Morleys (01869320320, product code CS60281).

- *Flowmeter*

Generally a different flowmeter is used to try to minimize deadspace. The recommended pneumotach for 10-16 yrs is manufactured by Hans Rudolph (model 4719). An adult sized pneumotach is probably suitable for older children (≥5 yrs).

- *Mouthpiece*

Most children down to 5 yrs can fit the whole mouthpiece in as it is designed. This may require some encouragement. If this is impossible, then keeping their lips on the inner rim of the mouthpiece is adequate. This method is more difficult as they require a more deliberate seal with their lips and the edges of the mouthpiece often hurt the corners of the mouth after a few minutes. Some practice before starting is a good idea to familiarise them with the procedure. Explain that the mouthpiece is like a diver's mouthpiece and all their breath has to go through the machine, not out the edges. Most children are interested by, and understand, this explanation.

- *Breath volume feedback software*

Some children will be able to use this to regulate tidal volume if the concept is explained. Other children may find this confusing if they are not able to change breath volume easily. The breaths may become more erratic. Also, other children may "play" with it and make huge or very fast breaths just to make the display move. If this happens it is probably advisable simply to turn the display away.

The issues that can make multiple breath washout difficult in children are:

- Not understanding the procedure – make sure they know to keep a good seal round the mouthpiece with their lips. They may need to be reminded.
- Playing with the equipment. Make sure hands are kept away from the pneumotach, especially the open port.
- Becoming easily distracted with other things going on in the room - keep conversation and movement to a minimum.
- The feeling that they don't know what's going on – make sure there is a member of staff visible to the child all the time. If this is not possible tell the child where you are going, even if it is just behind them.
- Talking or moving during the test – it is important that they don't talk during the test as this obviously upsets the natural breathing pattern and introduces leaks. Make sure any verbal instructions are phrased to discourage a reply. Children can forget they are not to talk. Generally keep verbal communication to a minimum. It is frustrating to be talked to and not be able to reply.
- When explaining the test make sure the change-over from washin to washout is explained before the test starts. It is better not to say anything before removing the T-piece during the test as this often interrupts the breathing pattern or the child may think the test is over and detach themselves from the mouthpiece.
- Suitable distraction is vital. Children are generally easily distracted and often forget instructions they have been given. Therefore a cartoon that keeps their attention will ensure they don't start talking or moving around too much. Try to avoid videos that have singing or too much excitement as the child may join in. Make sure the screen is easily visible (i.e. straight ahead of them) and the lights are dimmed if possible. Make sure the sound is loud enough so that the child is unlikely to be distracted by other noise or movement.

Innocor in young children (<5 yrs)

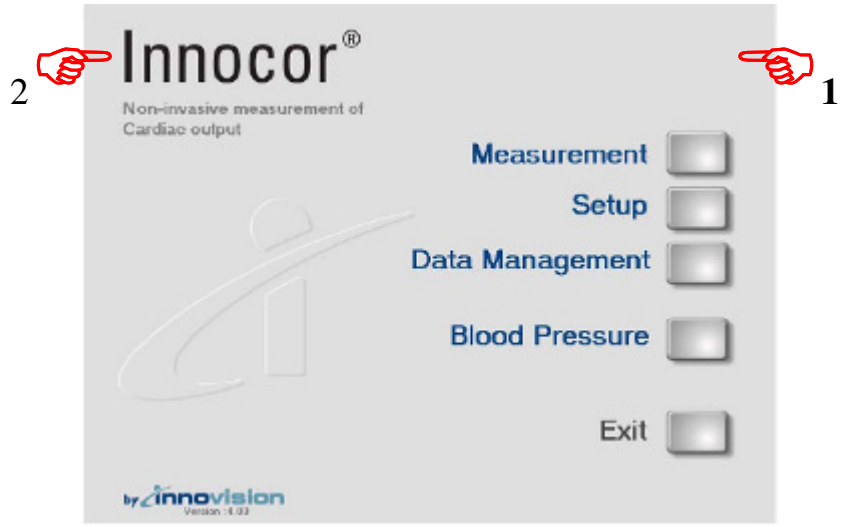
The concern about using Innocor in this age group is the speed of the gas analyser. To be able to integrate a gas concentration signal with a flow signal and calculate volume of gas expired the signal rise has to reflect the true rise in gas concentration with each breath. This is conventionally documented as the time taken for the gas concentration to rise from 10% to 90% of the concentration after a step-change. On analysis of artificial step-changes in gas concentration, a mass spectrometer has a documented10-90% rise time of 80milliseconds. This is when measuring both the rise in expired gas concentration and fall in inspired concentration. Innocor has a documented rise time of 120ms but was found to be 150ms in practice. This is considerably slower and may introduce inaccuracies in the integrated gas volumes at faster respiratory rates (e.g. in younger children). For this reason, ATS/ERS guidelines on performing multiple breath washout tests in those less than 5 yrs old have recommended a gas analyser response time of <100ms. For adults and children >5 yrs, the resting respiratory rates are slower, and any error caused by the slower rise time is minimal.

However, we cannot recommend using the current set-up in children under 5 yrs old. We are however actively looking at methods to enhance the gas analyser response in Innocor. More details on this research can be obtained from the authors.

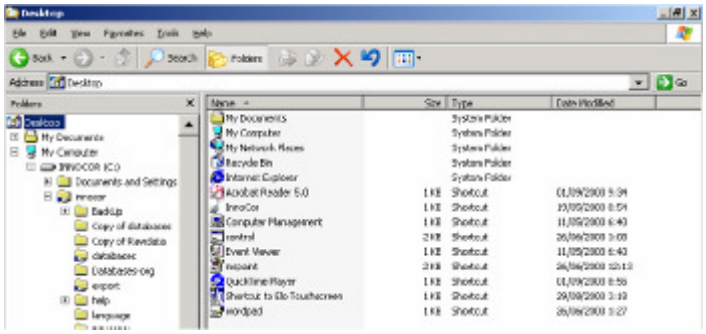
Part 7 - Data Export

USB stick

- This is the recommended method of data export.
- First exit to Windows by touching in rapid sequence the right upper followed by the left upper corner of the screen.



- When given the option to exit to Windows select **OK**.
- The following screen should appear:



- Insert the USB stick into the free port at the back of the Innocor machine and wait until this is recognised by the computer and displayed as an option in the left hand column.
- To locate the data, use the mouse to select the file **C:/Innocor/RawDataBBB**, this is the file into which all the breath by breath data are saved.
  It is advisable not to try using the touch screen for this, as it is hard to accurately select and copy the files of interest.
- Files are saved according to the formula:
  BBB[Patient ID]-[Date]-[Time].ino.
  To find the most recent files, first click on  and select the **Details** format. Then click on **Date Modified** to arrange data in date-generated order. You may need to click twice to get the most recent data displayed at the top of the column.

- Select the files for that day by clicking on or highlighting them, and click and drag them to the USB drive in the left hand menu. The files should then be copied to the USB stick.
- Once the files are saved, you can remove the USB stick without having to deactivate it first.
- It is recommended that data are copied from Innocor's hard drive at least daily (when it is in use) as this prevents data loss if anything were to happen to the machine.
- Use a dedicated memory stick, and only download to a single computer with up-to-date virus checking software.

Return to Innocor

- Select **Shortcut** from the list of files in the **C:/Innocor/** directory.
- Select **Restart Innocor**.

Important

✪ Do not alter any other settings within Innocor unless explicitly instructed.
✪ If wanting to shut the computer down, you must first return to Innocor and select **Exit** from the opening menu. Do not simply press ☒ in the top right hand corner. This will default to a screen showing the Innovision label, which you are stuck with until you restart the computer.

LAN Cable

This is a surprisingly slow method of data transfer, and for most purposes the memory stick will be sufficient.

- Plug the LAN cable into the port at the back of the Innocor machine and into the second computer.

In order for the two computers to link, the first part of their IP addresses need to be the same. The simplest way to do this is to set the IP address of your computer to be similar to that of the Innocor machine.

To find the Innocor IP address:

- In the main **shortcut** window, right click and select **New→Shortcut.**
- Enter **cmd** in the window that appears. Windows will find and direct you to the command window. Open this and type **ipconfig./all**
- This should reveal the IP address of the computer, likely to start 10.0.0.

Setting your IP address:

- On your computer, select **Control Panel→Network & Internet Connections→Network Connections→LAN connection**.
- Select **TCP/IP Internet protocol** and then select **Properties**. The default setting is an automatically configured IP address. Select **User Configured** and enter the address 10.0.0.xx , where "xx" are two digits that are different from those at the end of the Innocor IP address.

- To check that the two computers can link up, enter the command window of your computer (**Start menu→Run→**Type **cmd**). Type **ping** followed by Innocor's IP address. You should get a message back that Innocor has been successfully pinged.
- To access Innocor's files, type "\\" followed by Innocor's IP address in the **Address** window of your computer's control panel. This should pull up all the files on Innocor, though may take some time to load. You can also access Innocor from **Control Panel→Network Connections→My Network Places→Entire Network→Microsoft Windows Network→Inno_wrkgrp.**

Part 8 - Infection Control

To ensure safety of patients during studies we need to adhere to infection control guidelines that ensure minimal risk of contamination of re-usable parts that can infect other patients.
All CF patients are colonised with infectious organisms which can be passed to other susceptible patients via aerosol spread. In particular MRSA and *B.cepacia* colonised patients require special attention, but all patients should be treated as possible carriers of these organisms. Guidelines apply to all patients at all visits.

After discussion with infection control team at the Royal Hospital for Sick Children (RHSC) in Edinburgh, it was agreed that it is reasonable to have more than one patient in one day if local policy can be adhered to regarding separation of patients and adequate cleaning of equipment between patients. Two patients will not be tested or be in the test room at the same time.
Special attention has to be paid to cleaning and disinfecting parts in direct contact with exhaled breath or mucous membranes. It may be necessary to obtain similar approval from your own site.

Standard procedures recommended for cleaning and disinfecting equipment are:
(may be adapted according to local hospital policy)

- **Hard surface wipes** – Soap-based cleaning cloths (not alcohol-based).
- **Disinfection tablets** – Disifin or locally agreed alternative (e.g. Tristal). Adherance to protocol is required.
- **Disposal** – clinical waste.
- **Hand washing** – soap/ alcohol gel.

Non-disposable equipment in contact with patients during run-in studies:

- Multiple breath washout apparatus
- Other equipment: furniture etc.

Multiple Breath Washout Equipment	Procedure
Innocor	Not in contact with patient. Can be wiped with hard surface wipes (not touchscreen).
Flow-meter	Protected from patient by bacterial/viral filter. Requires periodical cleaning by dismantling and disinfection – every 10-15 patients.
Pressure lines	Protected from patient by in-line filter. Should be wiped thoroughly after each patient (hard surface wipes).
Flow-meter stand (if applicable)	Should be wiped (hard surface wipes) after each patient
Bias flow elephant tubing and T-piece	Protected from patient contamination by filter but attention should be made to any evidence of condensation inside tubing. Given that patient's breath is exhaled through exhaust end, tubing should be changed periodically – every 10-15 patients. T-piece should be disinfected
Rebreathe bag and gas tubing	?? possible special recommendations during normal clinical practice (awaiting further discussion with infection control nurses)
Gas tank and flow tap	Not in contact with patient.
Mouthpiece	Disinfected after every patient. Strict separation of clean and used.
Noseclip	Disinfected after every patient. Strict separation of clean and used.
In-line filter	Single use. Disposed of after testing. New filter for each patient and visit

Furniture etc.	Chairs, trolleys, other hard surfaces should be wiped after every patient. This should include all surfaces in contact with patient e.g. door handles etc.

Summary of recommendations

- Use a new disposable filter for each patient.
- Wash hands between patients. Either use gloves to handle filters, mouthpieces and noseclips or wash hands after doing so.
- After each patient use, wipe the machine, swing arm and seat with detergent wipes.
- Mouthpieces and noseclips should be sterilised as below.
- Although there is no evidence that any potentially harmful organisms will be missed by these procedures, it is advisable to change the flowpast circuit periodically (i.e. dispose of and replace the tubing, the T-pieces can be sterilised as per mouthpieces). This should be done every ten patients, and the date of this recorded in the logbook (under **Notes**)

Sterilisation of Mouthpieces, Masks, Noseclips & T-pieces

These items can be reused and should be sterilised as below.

- Dissolve 1 tablets of Disifin (RMP UK Ltd, London) in 0.5L of hot water.
- Clean mouthpieces or masks in warm soapy water to remove any deposits.
- Soak in Disfin solution for a minimum of 30minutes. Can be left overnight.
- Rinse in cold running water for a minimum of 60minutes.
- Allow to dry.
- Disfin solution should be discarded after 24hrs.

Cleaning of Flowmeter

Because we are using a filter, this is not necessary after every use but should be performed if there is reason to expect that the flowmeter has become contaminated. It is also good practice to routinely inspect the flowmeter and clean it if there appears to be debris on the mesh.

Cleaning is according to the manufacturer's instructions. We recommend the following steps:

Carefully disassemble the flowmeter.
Wash in Disfin solution for 30 minutes.
Rinse in cold running water for 60 minutes.
Allow to dry, preferably overnight.
Visual inspection to confirm apparatus is clean and dry and reassembly.
You will need to repeat the flowmeter linearisation after cleaning (see page 8)

Part 9 - Innocor Usage Log

The Innocor log is intended to be a daily record of the machine use and outputs. By recording flow gas delay and flowmeter gain daily, any large or sudden variations in these variables can be detected early. Similarly a log of cylinder pressure allows the operator to identify when replacements are required. Ambient conditions and flow-gas delay are necessary for the correct interpretation of the raw data, and even if stored on the patient data sheet the presence of a copy of these data on the daily log is a useful back-up.

Date		Operator		Usage	
Flowmeter Cal. Gain		**Gas Delay**		**Innocor Cylinder pressure at start**	**SF_6 cylinder pressure at start**
Fill		O_2	msecs		
Empty		CO_2	msecs	bar Remember to unscrew cylinder at end.	bar Remember to close cylinder at end.
Notes				Temp °C	RH%

Date		Operator		Usage	
Flowmeter Cal. Gain		**Gas Delay**		**Innocor Cylinder pressure at start**	**SF_6 cylinder pressure at start**
Fill		O_2	msecs		
Empty		CO_2	msecs	bar Remember to unscrew cylinder at end.	bar Remember to close cylinder at end.
Notes				Temp °C	RH%

Date		Operator		Usage	
Flowmeter Cal. Gain		**Gas Delay**		**Innocor Cylinder pressure at start**	**SF_6 cylinder pressure at start**
Fill		O_2	msecs		
Empty		CO_2	msecs	bar Remember to unscrew cylinder at end.	bar Remember to close cylinder at end.
Notes				Temp °C	RH%

Date		Operator		Usage	
Flowmeter Cal. Gain		**Gas Delay**		**Innocor Cylinder pressure at start**	**SF_6 cylinder pressure at start**
Fill		O_2	msecs		
Empty		CO_2	msecs	bar Remember to unscrew cylinder at end.	bar Remember to close cylinder at end.
Notes				Temp °C	RH%

Appendix B – Standard operating procedure for data analysis of multiple breath washouts using Innocor

Version 1.2, devised in Edinburgh on 2/2/07 by:
Alex Horsley, Kenny Macleod, Clare Saunders

Principles

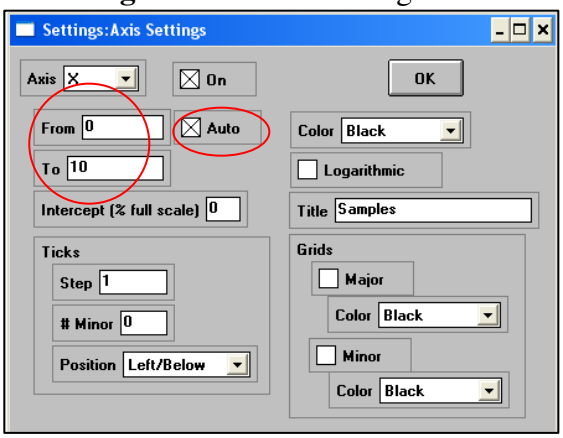
In the majority of cases analysis should be a semi-automated process. The software automatically identifies when a change from inspiration to expiration has taken place, and moves to the next phase of respiration. It does this by identifying when the flow changes from negative (inspiration) to positive (expiration), though the software is not infallible as will be discussed below. Irregular respiration, coughing, swallowing, respiratory pauses, and a host of other features, cause irregular changes in flow that can fool the automatic analysis. In most cases, little input is required from the operator, but a small number of washouts can be quite complex to analyse.

There are a number of scenarios that can lead to discrepancies between the data analysis by different operators. Following lengthy discussions, the guidelines below aim to cover the major potential discrepancies, and how to approach them. It is not possible to be exhaustive however. When encountering a scenario that is not adequately covered by the guidelines, the important principle to apply is what makes most physiological sense. For example, there may be confusion over whether the breath in question represent a true breath, is a continuation of an interrupted expiration or just a blip in flow that is not truly part of respiration (eg a swallow). If the tracing is expanded it is usually possible to work out what is going on, and adjust the analysis accordingly. If there is still doubt as to the correct approach, then consensus should be sought from the other operators.

Close inspection of a washout trace

The "inspect" function of the analysis software is not a very useful function. If you are unsure what is happening, the most effective method of examining the trace more closely is to adjust the *x* axis. To do this:

- Double click on the main tracing window
- A new window opens, select the **Axis Settings** button on the far right.
- In the **Axis Settings** window, you can alter the start and end of the *x* axis by changing the numbers in the **From** and **To** boxes in the top left corner (circled in red). You will typically need numbers in the thousands. To get an idea of the range, inspect the *x* axis of the main window and select the upper and lower limits of the region you wish to expand.



- Unclick the **Auto** box to set your range (circled in red).
- Select **OK** to return to the analysis.
- To reset the *x* axis, open the **Axis Settings** window as before, and this time reselect **Auto**.

Basic software settings

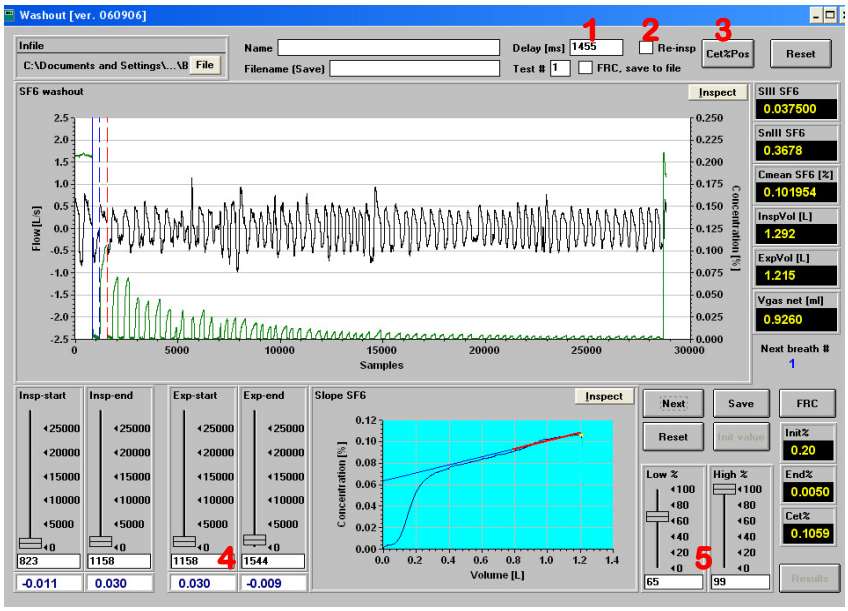
1. The **Delay** should be the CO_2 delay, which is calculated as part of the daily calibration procedure, plus 50ms. The reason for the additional time delay is to realign the signal, and thereby artificially improve the analyser response time. The rationale behind this is given elsewhere.

2. The **re-insp** box should be left unchecked. This is used if the volume of re-inspired SF_6 is to be calculated and taken into account. Again, the reasons why this is not used in the analysis of data from Innocor are covered elsewhere.

3. The **Cet%Pos** box represents the means of identification of the end tidal SF_6 concentration (Cet). Because of the signal realignment, selecting the final gas concentration value before the flow change often selects an SF_6 concentration that is from the falling part of the gas signal during early inspiration. To overcome this, click on **Cet%Pos** and select 10 for **#samples from the end (-)** and select 10 for **Window of avg cet**. This means that the Cet is the average of 10 gas samples (100ms), taken 10 samples back from the change in flow. This is also shown as a yellow bar in the **Slope SF_6** window, so you can check that this is accurate.

4. Changes can be made to the automatically identified start and end of a breath using the toggles and windows at the bottom left. We are not overly concerned with the inspired gas, though as a general principle it is important to try to be accurate with inspiration too. However, it is very important that expiration is correctly identified.
Start and End points can be moved using the toggles, which move in increments of 100 samples. Alternatively, click on the window below the toggle, and move the value using the cursor keys, or shift and cursor key which moves in increments of 10 samples.
As the start and end points are moved the corresponding flow value is shown in the lowest box in blue. Thus you can identify the start of a breath by moving the **Exp-Start** bar along using the cursor and watch for a change in flow from negative to positive.

5. These guidelines do not cover phase III slope analysis (also shown as the red line on the **Slope SF_6** window). Leave the markers at 65 and 95%.



Data Analysis: standardisation of procedure

1. The starting SF_6 concentration (Cinit)

- This should be the *alveolar* gas concentration at the start of the washout. In other words, the end tidal gas concentration (the same as is measured during washout) - not the inspiratory gas concentration which may be marginally greater, especially if wash-in is imperfect.
- **Because of the additional time delay offset, it is essential that you correct the Cinit.** This is done by clicking on the box beneath the **Insp-start** bar. Then reduce the number in the box by 5 by pressing the down arrow on your keyboard 5 times. This moves the **Insp-start** bar 5 sample (50ms) to the left.

- You should ensure, by visual inspection of the **Insp-start** bar, that the software has picked the correct start point of the first breath. This is crucial, because **Cinit** is taken from the gas concentration at this point, and is used both to calculate FRC and to define the end target Cet. If the start point is incorrect, then inspect and adjust by altering the position of the bar, as described above in point 4.
- **Cinit%** is given in a box on the right hand side of the screen. **End%**, in the box below, is defined as 1/40th of Cinit, and is the target Cet cut off for the end of the washout.

Cinit% will vary marginally from washout to washout. This is because the end-tidal (alveolar) gas concentration at the start will not be precisely the same every time.

2. Efficiency of wash-in

We are aiming for a steady state in SF_6 concentration in wash-in. In reality, there will be some variability, though this should be minimal. The wash-in inspiratory and expiratory gas concentrations can be inspected by clincking **Next** through the final few wash-in breaths. The inspiratory and expiratory gas concentrations can be read off from the **Slope SF_6** window (the graph is an in inversion of a washout curve, with concentration falling, rather than rising, to an alveolar plateau).

We should aim for a difference between inspiration and expiration of less than 2% (+/- 0.004% SF_6). If the difference is more than this, record in the washout notes that wash-in may be inadequate, but continue with the analysis.

3. End of washout

The end of the washout occurs when the end tidal SF_6 concentration (Cet) falls to 1/40th of the starting concentration (Cinit). In normal subjects, Cet falls with each breath and this end point is usually unequivocal. In CF patients, particularly the more severe subjects, abnormal gas mixing means that there is a steeply sloping alveolar plateau. Hence the Cet can vary with alterations in expiratory volume. The question then arises: at which point do you terminate the analysis?

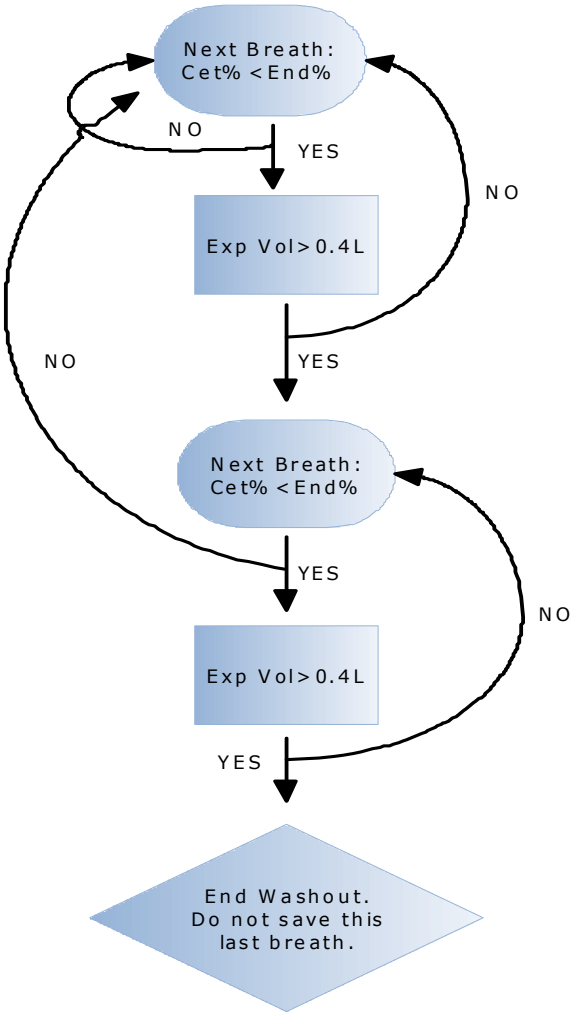
To achieve consistency, we have agreed that the washout end when there are two consecutive breaths with Cet at or below the target cut off. This means that single breath variations in Cet should not affect the analysis. In addition, very small breaths can lead to inappropriately low Cet. To avoid this, we set a minimum breath volume for identification of Cet of 400ml. In adults and children less than 50kg, this minimum volume is 8ml/kg. The breath volume of 400ml is based upon observations that this is sufficient to obtain an alveolar gas sample in adults.

To determine washout end:

- Inspect Cet%. Check that it has been correctly identified on the **Slope SF_6** graph. If it is above **End%** then continue washout, regardless of the volume of the breath.
- If Cet is the same or less than **End%**, check **ExpVol[L]**. If the volume is less than 0.4L in adults (or 8ml/kg in children and adults less than 50kg), then this Cet does not count, and you should continue the analysis.
- If volume of breath is >0.4L, then continue on to next breath. If this is also of sufficient volume and **Cet** is below **End%**, end the washout here, **without saving this last breath.**

- If **Cet** is greater than **End%** (regardless of volume), save this breath and continue the analysis. The search for two consecutive breaths with **Cet** below **End%** is reset to zero (i.e. you are still looking for 2 breaths, not just another single one).
- If Cet is below End%, but the second breath volume is <0.4L, then this breath does not count, save it and continue the analysis. However, the first breath still counts, and you are still only looking for one more breath that is of sufficient volume and with Cet<End%. When you find the 2nd breath that fulfils these criteria, end the washout without saving.

End of washout analysis algorithm



Notes:
The target of 0.4L is for adults. For children (and adults<50kg) a target of 8ml/kg is used.
To count as reaching the target End%, Cet must be the same or less than End%.

This algorithm is not perfect, and there may still occasions when the end of the washout is upset by smaller than average breaths (>0.4L) or by much larger breaths. However it represents a compromise that allows analysis of both complex and straightforward washouts, without overly complicating the latter.

Interruptions to flow

1. Flow zero

Peculiar to Innocor washout data, every 5mins the flowmeter re-zeros. At this time, it generates a flow signal of -100 for 1 second.
This is a problem if it occurs during expiration, particularly in the first few breaths.

The following algorithm to deal with flow re-zeros has been devised:

1. If the re-zero occurs up to (and including) breath 10, then it must be removed from the washout data. See below.
2. If re-zero occurs in inspiration it can be ignored, but ensure that that the -100 signal is not included in the inspiratory volume calculation.
3. If re-zero occurs during expiration, then analyse up to (but not including) the first -100. Save this breath. The next breath is the second part of that same expiration, from the end of re-zero onwards.

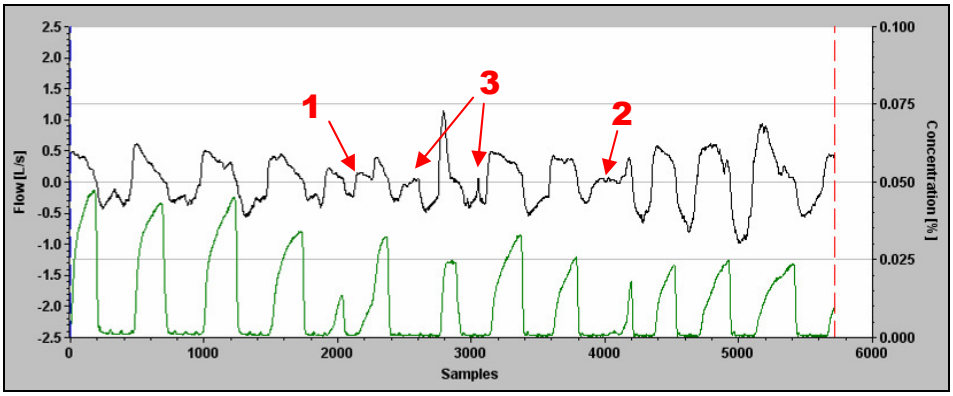
Although this loses 1 second of data, it again represents a reasonable compromise. The effect of losing these data is greater in the early part of the washout, hence the need to remove the re-zero signal if it occurs at this time.

Flattening the re-zero signal

This is done by first opening the washout file in a text box. Delete down to time=300000ms (5min). Save and reopen in Excel. Locate the re-zero that you wish to remove. Flatten by copying the flow signal immediately preceding it into the re-zero period. Save as a text file.
If this causes a flat flow signal during the change from expiration to inspiration, you may need to adjust the end expiration bar during analysis so that the end of expiration coincides with the peak SF_6 concentration.

2. Patient interruptions to flow.

There are often small changes in flow, or respiratory pauses, that cause confusion during analysis. The basic principle is one of physiological common sense. The following guidelines have been devised to aid this. The points are illustrated on the washout graph below with the corresponding number.

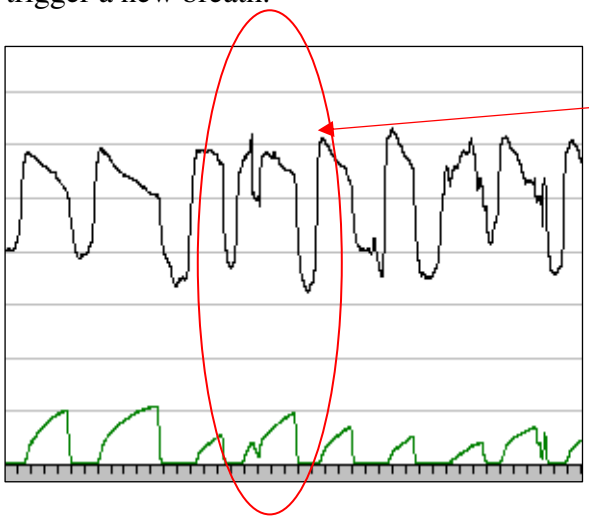


1. During expiration, ignore small inspiratory flows (<20ml).

E.g. subject may pause during expiration, and flow may even become negative (inspiratory). The magnitude of this inspiration can be assessed by following the change in **ExpVol[L]** as you move the **End-Exp** bar along. If this inspiration is of minimal volume (and certainly if <20ml), then it is insufficient to count as a proper inspiration and can be ignored. Continue to move the **End-Exp** bar along until inspiration starts properly.

Another important feature is to look at the SF_6 signal. If this continues to rise after a pause, or a tiny inspiration, then you are looking at a pause in expiration rather than a new breath, and this should be analysed as a single breath.

To complicate things further, the software does not recognise the sign of the flow signal. So it will integrate negative (inspiratory) flow as expiratory if it is included between the Exp-Start and Exp-End markers. Thus, inspiratory volumes greater than 20ml should trigger a new breath.



This counts as a single breath.
The expiration is interrupted by a brief pause, and a very small inspiration (7ml, shown as flow dipping below the central grey line). The SF_6 signal continues to rise, confirming that this is a single expiration that has been momentarily interrupted.

2. Expiratory pauses

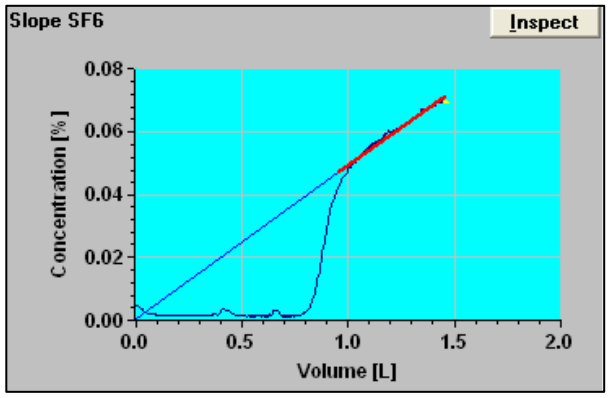
If the subject pauses at the end of expiration, end the breath once flow has returned to zero. However, if the subject pauses, then breathes out again before inspiration, include the pause and second expiratory peak, as part of the same breath.

The same rule of 20ml applies. So if there is inspiratory flow that equates to a volume>20ml during the pause, analyse the later part as a second breath. Once again, if the SF_6 continues to rise, this is a good indicator that this is all one breath.

3. Small expirations

Occasionally there are small blips in flow which occur during inspiration, or even during respiratory pauses, which the software identifies as a new breath. If these are less than 50ml in volume they can be ignored, and should not be saved. If they are greater than 50ml then they should be saved and counted as a separate breath.

4. Mis-identification of the start of expiration



Occasionally the software will identify a very small blip in flow at the start of inspiration as being the start of expiration, and will include the whole of inspiration as part of the breath. This can be spotted in the Slope-SF_6 window as the long flat part of the graph before the SF_6 signal rises on expiration (or looking like an anatomical deadspace of several hundred ml) (see figure on right). You must re-adjust the **Exp-Start** bar until it crosses zero (i.e. becomes positive) again.
A similar picture is seen with very small breaths. This is because the Slope-SF_6 graph autoscales. In this case, the volume of deadspace is only 100ml or so (read off from the x axis), and the total volume only 200ml or so. Because the deadspace represents a large portion of the total breath volume, when it is autoscaled it looks similar to the mis-identified breaths. In this case, moving the **Exp-Start** bar along, you will not see any negative flow. Save this breath as per normal.

Interruption to SF_6 signal

There have been occasional interruptions to the SF_6 signal noted. These probably occurred as a result of physical or electrical interference with the machine, but the effect is a non-physiological sine wave in the SF_6 signal. This should be treated like a flow re-zero.
In other words, integrate up to the start of the erroneous signal and again after the end of it, splitting a single breath into two. The exception to this is if the error signal occurs entirely in the first or last quarter of the breath, in which case it is reasonable not to integrate the part of the breath occurring before or after the error signal respectively. Doing so would result in a very small breath, which would affect average Vt and have little effect on overall CEV.

Intra-visit reproducibility

In accordance with published recommendations, the final FRC and LCI that we quote is the mean of at least two reproducible washouts.
In order to achieve this, we perform three washouts, so that if one is excluded we can still have two reproducible measurements.
A washout is excluded if:

1. The **FRC differs by more than 10%** from **both** the other two repeats. This is in accordance with published guidelines in children, and allows for leak or significant differences in starting point.

2. The **LCI differs by more than 20%** from **both** the other two repeats. This is an adjustment that we have included in order to allow for washouts where there is significant change in breathing pattern at the end of a washout that alters the end point to such an extent that a clearly erroneous value for LCI is obtained.

3. The **washout ends prematurely**, i.e. before target Cet is achieved.

4. The **wash-in** is inadequate (i.e. difference between inspiratory and expiratory SF_6 concentrations of more than 2% of the inspiratory [SF_6]).

5. Any other **procedural irregularities.**

In all cases the reason for exclusion must be recorded, and the number of repeats affected should be minimal.
If two reproducible washouts cannot be obtained, it may be reasonable to use the average of all three if they are technically acceptable but with differing FRC/LCI for instance.

Summary

The above represents a summary of the main problems identified during discussion, and it is likely that further issues will arise with time. Also, the volumes suggested for what qualifies as a significant inspiratory or expiratory volumes are based on a combination of common sense, experience and consensus – but are not proscriptive. Each difficulty during analysis must be considered in its own right, and difficult analyses should be discussed with other members of the group so that the analysis is both accurate and honest.

Appendix C –Published manuscripts from this thesis

Lung clearance index is a sensitive, repeatable and practical measure of airways disease in adults with cystic fibrosis

A R Horsley,[1,5] P M Gustafsson,[2] K A Macleod,[3,5] C Saunders,[4,5] A P Greening,[1,5] D J Porteous,[1,5] J C Davies,[4,5] S Cunningham,[3,5] E W F W Alton,[4,5] J A Innes[1,5]

See related editorial page 98

► Additional information is published online only at http://thorax.bmj.com/content/vol63/issue2

[1] School of Clinical and Molecular Medicine, University of Edinburgh, Edinburgh, UK; [2] Queen Silvia Children's Hospital, Gothenburg, Sweden; [3] Royal Hospital for Sick Children, Edinburgh, UK; [4] Department of Gene Therapy, National Heart and Lung Institute, Imperial College, London, UK; [5] UK Cystic Fibrosis Gene Therapy Consortium, UK

Correspondence to: Dr A R Horsley, Medical Genetics, Molecular Medicine Centre, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, UK; alex.horsley@ed.ac.uk

Received 10 May 2007
Accepted 19 July 2007
Published Online First 3 August 2007

ABSTRACT

Background: Lung clearance index (LCI) is a sensitive marker of early lung disease in children but has not been assessed in adults. Measurement is hindered by the complexity of the equipment required. The aims of this study were to assess performance of a novel gas analyser (Innocor) and to use it as a clinical tool for the measurement of LCI in cystic fibrosis (CF).

Methods: LCI was measured in 48 healthy adults, 12 healthy school-age children and 33 adults with CF by performing an inert gas washout from 0.2% sulfur hexafluoride (SF_6). SF_6 signal:noise ratio and 10–90% rise time of Innocor were compared with a mass spectrometer used in similar studies in children.

Results: Compared with the mass spectrometer, Innocor had a superior signal:noise ratio but a slower rise time (150 ms vs 60 ms) which may limit its use in very young children. Mean (SD) LCI in healthy adults was significantly different from that in patients with CF: 6.7 (0.4) vs 13.1 (3.8), $p<0.001$. Ten of the patients with CF had forced expiratory volume in 1 s ≥80% predicted but only one had a normal LCI. LCI repeats were reproducible in all three groups of subjects (mean intra-visit coefficient of variation ranged from 3.6% to 5.4%).

Conclusions: Innocor can be adapted to measure LCI and affords a simpler alternative to a mass spectrometer. LCI is raised in adults with CF with normal spirometry, and may prove to be a more sensitive marker of the effects of treatment in this group.

As part of a programme aimed at measuring the response to gene therapy in cystic fibrosis (CF), we are interested in developing more sensitive measures of changes in CF airway function and structure. In the USA the only marker of lung function currently recognised as a primary end point in CF trials is the forced expiratory volume in 1 s (FEV_1).[1] In early disease this reflects total airways resistance and is insensitive to changes in small airways, which contribute <10% of the overall resistance in healthy adult subjects.[2] Significant structural airway damage can be demonstrated on CT scanning in the presence of a normal FEV_1.[3]

In early lung disease, ventilation heterogeneity results from regional differences in small airway calibre (those beyond division 8).[4 5] This can be demonstrated both in computer models of the human lung[6] and from in vivo MR images[7] or radiolabelled tracer gas distribution.[7 8] Inert gas washout is an alternative technique which involves measuring the elimination of a non-absorbed gas as it is exhaled during tidal breathing. The gas can either be resident nitrogen washed out by breathing 100% oxygen, or an exogenous tracer gas which has first been breathed in to equilibrium. Lung clearance index (LCI), a simple marker of deranged ventilation, can be calculated from the washout curves.[9] Past studies using a variety of methods have shown that LCI is reproducible and more sensitive than FEV_1 at identifying early lung disease in children.[9–13] In addition, Aurora *et al*[10] showed that LCI is further raised in children infected with *Pseudomonas aeruginosa* and Kraemer *et al*[13] showed LCI to be an early predictor of deteriorating lung function in children.

Although an old technique,[14] measurement of LCI has always relied upon complex and bulky equipment, usually assembled from separate components by the investigators themselves, and has largely been restricted to a research setting. The current best method involves using a mass spectrometer (MS) to measure the washout of the inert tracer gas sulphur hexafluoride (SF_6). Although these are simpler than nitrogen washouts, MS are expensive to purchase and maintain.

The purpose of this study was to investigate LCI in healthy subjects and adults with CF using a modified Innocor device (Innovision, Odense, Denmark). Innocor is a compact gas analyser and flow sensor originally designed to measure cardiac output by inert gas rebreathing. The gas analyser uses photoacoustic spectroscopy to measure several gases including low concentrations of the inert tracer SF_6, making it a suitable device for ventilation distribution measurements. More information on the gas analyser is given in the online supplement (pages 2–5).

In preparation for its use as an end point in clinical trials, the aims of this study were:

1. To compare the performance (assessed by response time and signal:noise ratio) of the Innocor gas analyser with that of the current inert gas washout standard (MS).
2. To adapt the Innocor device and analysis software into a clinical system for measurement of functional residual capacity (FRC) and LCI.
3. To assess how LCI changes with age of subject in healthy volunteers.
4. To assess the intra and inter-visit reproducibility of LCI in healthy volunteers.
5. To use the adapted Innocor to measure FRC and LCI in normal adult subjects and patients with CF and to compare LCI with spirometry.

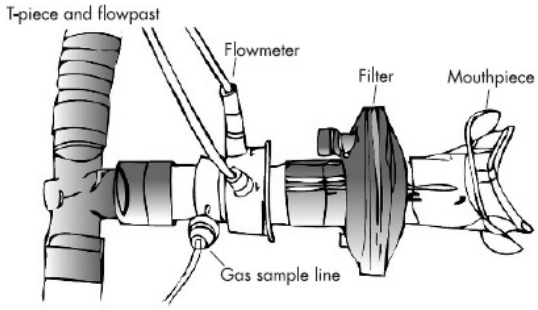


Figure 1 Patient interface used for inert gas washout with Innocor gas analyser.

METHODS

Equipment

To measure LCI, a mouthpiece fitted with a flowmeter and gas sampling port is required (fig 1). This is connected to a detachable flowpast tube which is used to supply tracer gas during the wash-in and is then removed at the start of washout. A more detailed Methods section is available in the online supplement, and the modifications to the standard Innocor patient interface are described in detail on pages 5–7.

Spirometry was measured according to American Thoracic Society/European Respiratory Society guidelines;[15] predicted values for FEV_1 are those provided by the European Community for Coal and Steel (adults $\geq$17 years)[16] and Rosenthal *et al* (children $\leq$16 years).[17]

Performance of Innocor gas analyser

The signal:noise ratio at the start and end of a washout and the rise time of the gas analyser in response to a step change in SF_6 concentration were assessed as described in the online supplement (page 8). Performance was compared with that of a MS used routinely for LCI measurements. The ability of the complete modified system to integrate flow and gas signals accurately was assessed using a gas calibration syringe which can be set to deliver different volumes (Hans Rudolph, Missouri, USA). This was filled with 0.2% SF_6 in air (BOC, Guildford, UK) to a range of different starting volumes and a washout performed by incomplete filling and emptying of the syringe around this starting point. The syringe volume derived from the calculated "expired" volume of SF_6 was then compared with the known starting volume.

Flow and SF_6 data were exported for analysis on custom-built software. FRC was derived from the total expired SF_6 volume, calculated by integration of flow and SF_6 signals. LCI is defined as the number of lung turnovers (ie, multiples of FRC) required to reduce end tidal marker gas concentration to 1/40th of the starting value (as described in the online supplement, page 11).

Subjects

Forty-nine healthy non-smokers (<10 pack-years smoking history) with no active lung disease and on no regular respiratory medications were recruited as normal adult volunteers (age range 19–58 years). Thirteen healthy child volunteers (age range 6–16 years) were recruited if they had no previous diagnosis of recurrent wheeze or asthma and were taking no current inhaled medication. There was no history of significant respiratory disease requiring hospitalisation (eg, pneumonia, pertussis, tuberculosis), no prematurity (<34 weeks gestation) and no significant co-morbidity. Thirty-three patients with CF (age range 17–49 years) were recruited from the Scottish Adult CF Service, the diagnosis being based on a combination of clinical presentation and sweat testing and confirmed by genotyping. All volunteers, patients and (where relevant) parents provided informed consent. Paediatric volunteers provided assent where appropriate. This study was approved by the Lothian research and ethics committee.

Washout test

Subjects were seated and suitably distracted by watching television. A noseclip was applied and tidal breathing established while the subject was connected to the flowpast circuit containing 0.2% SF_6 in air. This was supplied from a compressed gas cylinder with the flow rate adjusted to ensure that rebreathing did not occur. This wash-in phase continued for at least 5 min in adults or 4 min in children under 16 years and, in all cases, until inspiratory and expiratory SF_6 concentrations differed by <0.004% (absolute difference in SF_6 concentration). The flowpast circuit was then detached during expiration and the washout measured until the end tidal SF_6 had fallen to less than 1/40th of the starting concentration (ie, <0.005%). In healthy children (<16 years) an identical gas analyser and protocol were employed at a separate research site, but a smaller filter was used to reduce the precapillary dead space (36 ml vs 46 ml in adults).

Subjects completed three sets of wash-ins and washouts. A washout was discarded if the resulting calculated FRC differed by >10% from both the other two repeats.[18]

Statistical analysis

Data were analysed using Prism (GraphPad Software Inc, California, USA). The results are given as mean (SD) unless otherwise stated. Within-test repeatability for LCI was determined by calculating the coefficient of variation (CV) as 100× SD/mean. Inter-visit reproducibility was assessed using the Bland-Altman technique. Correlation with age and height were assessed by multiple linear regression analysis. Numerical values for LCI and FEV_1% predicted were compared using a Mann-Whitney U test. The 95% limits of normality for LCI were calculated as mean $\pm 1.96 \times$ residual standard deviations. A p value of <0.05 was considered statistically significant.

RESULTS

Technical validation of Innocor device

Signal:noise ratio of Innocor and MS

The Innocor device has a lower gas concentration operating range than the MS. Signal quality is therefore given at the starting and finishing concentrations of a washout, which are different for the two devices (table 1). For both devices there is a fall in signal:noise ratio as the gas concentration falls, but the Innocor signal quality remains superior throughout, despite much lower SF_6 concentrations.

Rise time and delay of gas signal

The mean (SD) SF_6 10–90% rise time was 154 (5) ms for Innocor and 64 (5) ms for the MS (p<0.001). The longer rise time of the Innocor gas analyser was allowed for by offsetting gas and flow signals during analysis by an additional 50 ms. This corresponds to the 50–80% rise time of the gas signal, and has the effect of speeding the response time by realigning the flow signal with the 80% response fraction of the gas signal.[19]

Thorax 2008;**63**:135–140. doi:10.1136/thx.2007.082628

Table 1 Signal:noise ratios of Innocor and mass spectrometer (MS) at gas concentrations encountered at start and end of washout

	SF_6 concentration (%)		Signal:noise ratio	
	Start	End	Start	End
MS	4.0	0.1	200	13
Innocor	0.2	0.005	944	53

The signal:noise ratio is calculated as the ratio of mean to standard deviation of a stable gas signal over 10 s.

The accuracy of this adjustment was then confirmed by integration of known volumes of SF_6 from a calibration syringe.

Validation of FRC measurements

Sixteen washouts were performed using a calibration syringe with the starting volume varied between 1.5 and 3 litres. There was good agreement between the measured and actual syringe volumes (see fig 1 in online supplement II). The mean (SE) error between measured and actual syringe volume was 16.3 (2.4) ml or 1.1 (0.2)%.

In vivo LCI measurement

LCI was assessed successfully in 12 healthy children, 48 adult healthy volunteers and 33 adults with CF. The demographic data of the study subjects are given in table 2. Data from two additional healthy volunteers (one adult, one child) could not be analysed because of technical difficulties (see below).

Effect of age, height and gender on LCI in non-CF subjects

Figure 2 shows the relationship between LCI and age (min 6 years, max 58 years). In those aged >16 years there was no relationship between LCI and age. When the two cohorts were combined there was a weak but statistically significant correlation with age (Pearson $r^2 = 0.16$, $p<0.002$). The small dependence of LCI on age is best summarised by a normal range (95% limits of normality) in adults of 5.9 to 7.5 and in children ($\leqslant$16 years) of 5.3 to 7.3. A weak relationship between height and LCI in the combined cohorts disappeared on multiple regression analysis. LCI was unrelated to gender of subject. By contrast, FEV_1 varied between 76% and 133% predicted in the same group of 60 healthy adults and children.

LCI in non-CF and CF adults

The group mean (SD) LCI in adult healthy controls was 6.7 (0.4) (range 6.0–7.8) with 95% limits of normality calculated as 5.9 to 7.5. In patients with CF the group mean (SD) LCI was 12.8 (3.6) (range 6.3–20.4), $p<0.001$ compared with healthy controls. The mean (SD) FEV_1 was also significantly different between the two groups (102 (12)% predicted in healthy controls vs 68 (23)% predicted in patients with CF, $p<0.001$).

Figure 3 shows the relationship between FEV_1 % predicted and LCI for healthy controls and adults with CF. In controls LCI was restricted to a narrow range but, in patients with CF, LCI increased with reducing FEV_1 % predicted ($r^2 = 0.69$, $p<0.001$).

There were 10 patients with CF with $FEV_1 \geqslant 80\%$ predicted, all but one of whom had LCI above the upper limit of normal. By contrast, LCI was marginally raised in only two healthy adults (measuring 7.7 and 7.8). The sensitivity of LCI for detecting CF was 97% compared with 70% for FEV_1.

Repeatability of washout at same visit

A washout test was excluded if the measured FRC differed by >10% from both of the other two washouts. In adult subjects this resulted in the exclusion of a total of seven tests, representing <3% of the total number of repeats from both healthy volunteers and patients with CF. Three tests were excluded from the paediatric cohort, representing 9% of the total number of washout repeats. All three washout repeats from an additional single adult healthy volunteer could not be analysed because they were unable to achieve a regular and reproducible breathing pattern. All three repeats from an additional healthy child (age 8) were also excluded because of evidence of an air leak.

After exclusion of these repeats, the mean (SD) intra-subject coefficient of variation (CV) for FRC derived from repeat washout manoeuvres on the same visit was 3.2 (1.9)% for adult healthy volunteers, 3.9 (2.1)% for healthy children and 3.5 (2.3)% for patients with CF. The mean (SD) CV for LCI was 3.6 (2.1)% for healthy adults, 5.4 (3.8)% for healthy children and 4.4 (2.8)% for patients with CF. There was no significant correlation between the LCI CV and FEV_1 % predicted.

Inter-visit reproducibility of LCI in healthy adults

Repeat measurements of LCI were performed in triplicate on 16 healthy volunteers after a mean (SE) of 36 (10) days.

Table 2 Demographic data, spirometric parameters and lung clearance index (LCI) of healthy volunteers and patients with cystic fibrosis (CF)

	Healthy volunteers		
	Children (age $\leqslant$ 16 years)	Adults (age $\geqslant$17 years)	CF patients
N	12	48	33
M/F	7/5	19/29	21/12
Mean (range) age (years)	11 (6–16)	33 (19–58)	30 (17–49)
Mean (SD) FEV_1 (% predicted)	95 (11)	102 (12)	66 (23)*
LCI			
Mean (SD)	6.3 (0.5)	6.7 (0.4)	13.1 (3.8)*
Range	5.6–7.1	6.0–7.8	6.3–20.4
Mean (SD) CV%	5.4 (3.8)	3.6 (2.1)	4.5 (2.7)

FEV_1, forced expiratory volume in 1 s; CV%, coefficient of variation (%) for intra-visit repeats.
*$p<0.001$ vs adult healthy volunteers (Mann-Whitney U test).

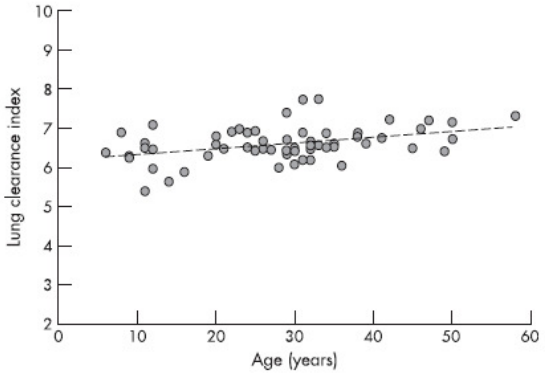


Figure 2 Effect of age on lung clearance index (LCI) in healthy volunteers. LCI remains within a narrow band of normal over an age range of 52 years. The broken line is the regression line, showing the extent of age-related increase in LCI.

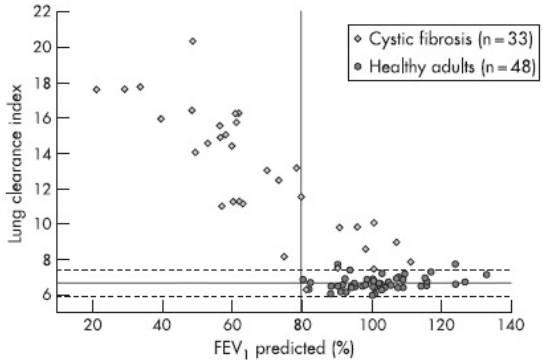


Figure 3 Lung clearance index (LCI) versus forced expiratory volume in 1 s (FEV_1) % predicted for adult healthy volunteers and patients with cystic fibrosis. The horizontal line represents the mean and the horizontal dotted lines the 95% limits of normality of the LCI, calculated from the healthy adult population. The vertical line represents the lower limit of normal for % predicted FEV_1.

A Bland-Altman plot[20] of the difference between repeat measures and the mean of the measurements for LCI is shown in fig 4. For FRC, the 95% limits of agreement between the two measurements were −0.43 to 0.45 litres and, for LCI, the 95% limits of agreement for the two measurements were −0.78 to 0.46. The inter-visit reproducibility of the FRC measurement was therefore approximately 400 ml and that of the LCI measurement was 0.6.

DISCUSSION

This study has shown that the clinical measurement of inert gas washout is practical using equipment that is cheaper, more portable and has more sensitive gas signal resolution than the current MS standard. We have also shown for the first time that, in adults with CF, a simple measure of ventilation heterogeneity is more sensitive than FEV_1 in detecting lung function abnormalities. Finally, we have shown that this measurement is reproducible both within and between visits, and that there is little change over a wide range of subject height and age.

In children with CF there is already increasing evidence that LCI is a more sensitive measure of early lung disease than FEV_1.[9–18 21] LCI has also been shown to correlate better with scores of airway damage on high-resolution CT scanning than spirometry.[22] It may therefore fill an important gap in our ability to monitor airway function and disease progression non-invasively. Since only tidal breathing is required, it is particularly suitable for airways assessment in subjects who find complex respiratory manoeuvres difficult.

The potential of multiple breath washout measurements, however, has been hampered by the lack of a convenient method of performing them.[12 21 23] The original method for assessing lung clearance was the nitrogen washout. Although this avoids the need for a wash-in first, sufficient time must be allowed between tests for the end tidal nitrogen concentration to return to normal.[18] The use of a MS to measure LCI by following changes in exogenous SF_6 is now well described in children,[12 21] and is probably the accepted gold standard technique in this population. The MS offers the additional advantage that it can measure a wider range of different gases, which is a useful option when measuring vital capacity single breath washouts.[24] However, the MS is an expensive, temperamental and bulky piece of equipment that cannot readily be taken out of the laboratory. In contrast, Innocor contains both the gas analyser and the pneumotachograph in a single unit that is both portable and robust.[25] A supply of SF_6 is required for both systems, but the concentration required for Innocor is 1/20th of that used in the MS washouts, which reduces gas wastage and the potential environmental (greenhouse) effects of SF_6.

The ideal comparison would be to compare the performance of both systems simultaneously, as has been done for other gas analysers.[26] However, washouts would have to be performed at the operating range of the Innocor gas analyser since the response is not linear above 0.2% SF_6, but the signal resolution of the MS shows excessive noise at this level. Accepting this as a limitation of the current comparison, we have shown that the gas analyser is suitable for use in a multiple breath washout apparatus. The characteristics of the two analysers are summarised in table 2 of the online supplement (page 18). Our technical validation shows that the device with our modifications is capable of measuring gas volume by dilution with high accuracy. Despite operating at a much lower SF_6 concentration, it produces washouts with a superior signal: noise ratio than a MS. Our comparison has also identified the possible limitations of the device imposed by the slower signal

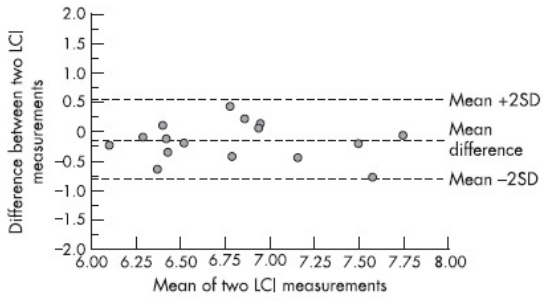


Figure 4 Bland-Altman plot of difference between lung clearance index (LCI) measured on two separate occasions (quoted as mean of triplicate repeats) and mean of the two measurements of LCI.

Thorax 2008;**63**:135–140. doi:10.1136/thx.2007.082628

rise and fall time. The system is able to integrate flow and SF_6 concentrations accurately at a physiological breathing rate of 10–30 breaths/min and should therefore be suitable for use in school-age children and adults. The response time may, however, limit the use of the method in neonates and preschool children with faster respiratory rates.[21 27] Further in vitro assessment is required before using the analyser in this age group.

To date, we have used the modified Innocor to measure LCI in more than 100 patients and volunteers. From the data presented here, over 85% of subjects are able to complete all three washout manoeuvres without difficulty and generate reproducible measurements of FRC and LCI. Even for patients with CF, the whole process (wash-in and washout) usually takes little more than 10 min, and considerably less in children. Despite the relatively uncontrolled conditions, the mean CV for repeat FRC is similar to that described in the literature, which varies from 3.5% to 6.7% for plethysmography and from 4.9% to 10.4% for helium dilution.[28] The mean CV for LCI is also better than that described in children.[11] Repeat measurements of LCI at a separate time point in a cohort of adult healthy volunteers also demonstrated good inter-visit reproducibility.

It has been shown that LCI may be influenced by large changes in tidal volume, respiratory rate or FRC.[29 30] We used tidal volume feedback to control tidal volume and respiratory rate within a range which should not affect the result. Since LCI is a ratio of cumulative expired volume and FRC, it is also independent of small changes in FRC over the physiological range. This is supported by the reproducibility of LCI and the narrow range of LCI in normal subjects found in the current study. Furthermore, because it is normalised for FRC, the normal range of LCI is largely unaffected by age, height or gender of subject. There was a weak but statistically significant rise in LCI with age. The clinical significance of this is unclear, since the magnitude of the difference (over a 52-year age range) remains very small and is less than inter-visit reproducibility. Serial dead space is known to affect LCI in infants and neonates.[31] However, the change in normal values of LCI was in the opposite direction to that which would be caused by a greater dead space/tidal volume ratio (as found in infants). It is therefore possible that this represents a true effect of age on lung elasticity and hence gas mixing. By contrast, there is a wide range of "normal" for FEV_1 % predicted, which is influenced by the choice and accuracy of the normal range selected.[32]

The mean (SD) LCI determined here is very similar to that reported in the literature in children and adolescents. In preschool children (mean age 4 years) this has been reported as 6.9 (0.4),[10] and in school-age children (mean age 11 years) as 6.5 (0.5)[11] and 6.3 (0.4)[9] in two different populations from the UK and Sweden, respectively. This supports our observation that LCI changes little with the age of the subject (>6 years). This may be especially useful during long-term follow-up studies.

These are the first data on LCI in adults with CF; previous studies have only reported measurements in subjects up to 19 years of age. Even in adult patients with normal spirometry, the LCI may be markedly elevated, indicating significant "silent" lung damage. Some of the patients with normal FEV_1 had no symptoms and were on no treatment, the diagnosis of CF having been made incidentally. Yet, despite this, there was abnormal gas mixing in almost all cases. There is a risk that the extent of lung disease in such patients will be underestimated and hence undertreated.

While FEV_1 is the currently accepted gold standard to monitor trials of new treatments for CF, the rate of decline in this parameter has steadily reduced over the last decade.[33] Power calculations show that many hundreds of patients would need to be treated over a year or more to see any beneficial effect of a novel therapeutic agent aimed at the basic defect.[34] We have therefore instituted a large programme to assess novel biomarkers which could act as surrogates for FEV_1. Ventilation heterogeneity is thought to be altered by small airways dysfunction,[4 5] and measurements of this should therefore reflect the earliest pathology in CF—as has already been shown in children.[9–11] This is also the region of the lungs which is likely to be a key target for gene therapy. The choice of which subject to recruit into trials of gene therapy represents a conflict between those with sufficiently clear airways that the gene therapy complex can be delivered into the lungs and those with sufficient abnormality in lung function so that any improvement can be measured. LCI offers the ability to measure dysfunction in the airways of interest, and also to extend the range of patients suitable for these assessments.

We have shown that there is the possibility of a robust and compact apparatus to measure LCI that can be used in multicentre studies after relatively straightforward modification. This will permit us to assess LCI routinely in patients to obtain longitudinal data and, in particular, it may serve as a more sensitive measure of lung function after changes in therapy. The value of this technology may, however, extend beyond just CF and we anticipate that it may provide valuable information about the development and treatment of airways disease in other conditions. In particular, it may be useful in conditions that initially affect the small airways such as asthma, chronic obstructive pulmonary disease and obliterative bronchiolitis.

Acknowledgements: The authors thank all the volunteers who took part in this study. They thank Eddie Bergsten for his help in developing the software used to analyse the inert gas washout data and acknowledge the assistance provided by the Welcome Trust Clinical Research Facility at the Western General Hospital, Edinburgh.

Funding: This study was performed at the Welcome Trust Clinical Research Facility at the Western General Hospital, Edinburgh and at the Department of Child Life and Health, Royal Hospital for Sick Children, Edinburgh. Funding was provided by the CF Trust as part of the UK CF Gene Therapy Consortium.

Competing interests: None.

REFERENCES

1. **Ramsey BW,** Boat TF. Outcome measures for clinical trials in cystic fibrosis. Summary of a Cystic Fibrosis Foundation consensus conference. *J Pediatr* 1994;**124**:177–92.
2. **Macklem PT,** Mead J. Resistance of central and peripheral airways measured by a retrograde catheter. *J Appl Physiol* 1967;**22**:395–401.
3. **Brody AS,** Klein JS, Molina PL, *et al*. High-resolution computed tomography in young patients with cystic fibrosis: distribution of abnormalities and correlation with pulmonary function tests. *J Pediatr* 2004;**145**:32–8.
4. **Cosio M,** Ghezzo H, Hogg JC, *et al*. The relations between structural changes in small airways and pulmonary-function tests. *N Engl J Med* 1978;**298**:1277–81.
5. **Verbanck S,** Schuermans D, Meysman M, *et al*. Noninvasive assessment of airway alterations in smokers: the small airways revisited. *Am J Respir Crit Care Med* 2004;**170**:414–9.
6. **Venegas JG,** Winkler T, Musch G, *et al*. Self-organized patchiness in asthma as a prelude to catastrophic shifts. *Nature* 2005;**434**:777–82.
7. **Samee S,** Altes T, Powers P, *et al*. Imaging the lungs in asthmatic patients by using hyperpolarized helium-3 magnetic resonance: assessment of response to methacholine and exercise challenge. *J Allergy Clin Immunol* 2003;**111**:1205–11.
8. **Venegas JG,** Schroeder T, Harris S, *et al*. The distribution of ventilation during bronchoconstriction is patchy and bimodal: a PET imaging study. *Respir Physiol Neurobiol* 2005;**148**:57–64.
9. **Gustafsson PM,** Aurora P, Lindblad A. Evaluation of ventilation maldistribution as an early indicator of lung disease in children with cystic fibrosis. *Eur Respir J* 2003;**22**:972–9.

10. **Aurora P,** Bush A, Gustafsson P, *et al*. Multiple-breath washout as a marker of lung disease in preschool children with cystic fibrosis. *Am J Respir Crit Care Med* 2005;**171**:249–56.
11. **Aurora P,** Gustafsson P, Bush A, *et al*. Multiple breath inert gas washout as a measure of ventilation distribution in children with cystic fibrosis. *Thorax* 2004;**59**:1068–73.
12. **Aurora P,** Kozlowska W, Stocks J. Gas mixing efficiency from birth to adulthood measured by multiple-breath washout. *Respir Physiol Neurobiol* 2005;**148**:125–39.
13. **Kraemer R,** Blum A, Schibler A, *et al*. Ventilation inhomogeneities in relation to standard lung function in patients with cystic fibrosis. *Am J Respir Crit Care Med* 2005;**171**:371–8.
14. **Becklake MR.** A new index of the intrapulmonary mixture of inspired air. *Thorax* 1952;**7**:111–6.
15. **Miller MR,** Hankinson J, Brusasco V, *et al*. Standardisation of spirometry. *Eur Respir J* 2005;**26**:319–38.
16. **Quanjer PH,** Tammeling GJ, Cotes JE, *et al*. Lung volumes and forced ventilatory flows. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 1993;**16**:5–40.
17. **Rosenthal M,** Bain SH, Cramer D, *et al*. Lung function in white children aged 4 to 19 years: I—Spirometry. *Thorax* 1993;**48**:794–802.
18. **Wanger J,** Clausen JL, Coates A, *et al*. Standardisation of the measurement of lung volumes. *Eur Respir J* 2005;**26**:511–22.
19. **Arieli R,** Van Liew HD. Corrections for the response time and delay of mass spectrometers. *J Appl Physiol* 1981;**51**:1417–22.
20. **Bland JM,** Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;**1**:307–10.
21. **Gustafsson PM.** Inert gas washout in preschool children. *Paediatr Respir Rev* 2005;**6**:239–45.
22. **Lindblad A,** de Jong P, Brink M, *et al*. Measurements of ventilation inhomogeneity correlate better to structural changes on high resolution CT than conventional spirometry. *Pediatr Pulmonol* 2005;310–1.
23. **Bush A,** Davies J. Early detection of lung disease in preschool children with cystic fibrosis. *Curr Opin Pulm Med* 2005;**11**:534–8.
24. **Gronkvist MJ,** Emery MJ, Gustafsson PM. Mechanisms of ventilation inhomogeneity during vital capacity breaths standing and supine. *Respir Physiol* 2002;**129**:345–55.
25. **Ghofrani HA,** Reichenberger F, Kohstall MG, *et al*. Sildenafil increased exercise capacity during hypoxia at low altitudes and at Mount Everest base camp: a randomized, double-blind, placebo-controlled crossover trial. *Ann Intern Med* 2004;**141**:169–77.
26. **Fuchs SI,** Buess C, Lum S, *et al*. Multiple breath washout with a sidestream ultrasonic flow sensor and mass spectrometry: a comparative study. *Pediatr Pulmonol* 2006;**41**:1218–25.
27. **Beydon N,** Davis SD, Lombardi E, *et al*. An official American Thoracic Society/ European Respiratory Society statement: pulmonary function testing in preschool children. *Am J Respir Crit Care Med* 2007;**175**:1304–45.
28. **Hankinson JL,** Stocks J, Peslin R. Reproducibility of lung volume measurements. *Eur Respir J* 1998;**11**:787–90.
29. **Gronkvist M,** Bergsten E, Gustafsson PM. Effects of body posture and tidal volume on inter- and intraregional ventilation distribution in healthy men. *J Appl Physiol* 2002;**92**:634–42.
30. **Bouhuys A,** Lichtneckert S, Lundgren C, *et al*. Voluntary changes in breathing pattern and N2 clearance from lungs. *J Appl Physiol* 1961;**16**:1039–42.
31. **Schmalisch G,** Proquitte H, Roehr CC, *et al*. The effect of changing ventilator settings on indices of ventilation inhomogeneity in small ventilated lungs. *BMC Pulm Med* 2006;**6**:20.
32. **Roca J,** Burgos F, Sunyer J, *et al*. References values for forced spirometry. Group of the European Community Respiratory Health Survey. *Eur Respir J* 1998;**11**:1354–62.
33. **Que C,** Cullinan P, Geddes D. Improving rate of decline of FEV_1 in young adults with cystic fibrosis. *Thorax* 2006;**61**:155–7.
34. **Davis PB,** Byard PJ, Konstan MW. Identifying treatments that halt progression of pulmonary disease in cystic fibrosis. *Pediatr Res* 1997;**41**:161–5.

Respiratory Physiology & Neurobiology 162 (2008) 197–203

Contents lists available at ScienceDirect

Respiratory Physiology & Neurobiology

journal homepage: www.elsevier.com/locate/resphysiol

Effects of cystic fibrosis lung disease on gas mixing indices derived from alveolar slope analysis

Alex R. Horsley[a,e,*], Kenneth A. Macleod[b,e], Andrew G. Robson[c], Jill Lenney[c], Nicholas J. Bell[a,e], Steve Cunningham[b,e], Andrew P. Greening[a,e], Per M. Gustafsson[d], J. Alastair Innes[a,c,e]

[a] University of Edinburgh and Department of Respiratory Medicine, Western General Hospital, Edinburgh EH4 2XU, UK
[b] Department of Child Life and Health, Royal Hospital for Sick Children, Edinburgh, UK
[c] Respiratory Function Service, Western General Hospital, Edinburgh, UK
[d] Department of Paediatric Clinical Physiology, Queen Silvia Children's Hospital, Gothenburg, Sweden
[e] UK Cystic Fibrosis Gene Therapy Consortium, UK

ARTICLE INFO

Article history:
Accepted 17 June 2008

Keywords:
Multiple breath washout
Cystic fibrosis
Innocor
Plethysmography
Diffusing capacity
Lung clearance index
S_{cond}
S_{acin}

ABSTRACT

S_{cond} and S_{acin} are derived from analysis of concentration-normalized phase III slopes (Sn_{III}) of a multiple breath inert gas washout. Studies in healthy and COPD subjects suggest these reflect ventilation heterogeneity in conducting and acinar airway zones respectively, but similar studies in cystic fibrosis (CF) are lacking. S_{cond}, S_{acin} and lung clearance index (LCI, a measure of overall gas mixing efficiency) were measured in 22 adults and 18 children with CF and 17 adult and 29 child controls. Plethysmography and gas transfer measurements were performed in adults, and spirometry in all subjects. S_{cond} was elevated in almost all CF patients, including children with mild disease and normal LCI. However, S_{cond} did not correlate with other measurements and appeared to reach a maximum; further increase in ventilation heterogeneity being restricted to S_{acin}. The nature and/or severity of CF lung disease may invalidate assumptions underlying the ability to separate phase III slope analysis of ventilation heterogeneity into proximal and peripheral components, and LCI may be a better indicator of gas mixing in this population.

1. Introduction

The last few years has seen an expansion of interest in the use of inert gas washout tests to measure non-uniformity of ventilation distribution in the lung. This has been driven both by improvements in technology and also by a growing appreciation of the need for more sensitive measures of small airway function (Venegas, 2007). These tests are now moving from the arena of experimental physiological studies to a role as outcome measures in clinical studies and trials (Davies et al., 2008).

Using the single breath washout (SBW) test, ventilation heterogeneity is determined from the slope of the alveolar plateau, also referred to as the phase III slope. Imperfect convective (bulk flow) gas mixing between regions of the lung, sequencing between these regions, and interaction between convective and diffusive gas mixing in the periphery all contribute to variations in ventilation efficiency and a sloping alveolar plateau (Prisk et al., 1996; Dutrieue et al., 2000). Although SBW tests using a single inert marker gas are useful clinical tools (Estenne et al., 2000), they are not able to inform us about the mechanisms responsible for the observed inhomogeneity.

An alternative, more complex, analysis describes how the phase III slope for an inert marker gas changes over successive breaths of a multiple breath washout, in an attempt to separate the contribution of the different gas mixing processes (Verbanck et al., 1997; Aurora et al., 2005). This analysis is explained in more detail in the online supplement. In summary however, the concentration-normalized phase III slope of the individual breaths of the washout are first plotted against the lung volume turnover (TO) (obtained by dividing the cumulative expired volume by the FRC). This profile is then divided into two separate components, each reflecting a different aspect of gas mixing. The first component can be derived from the increase in normalised phase III slope with successive breaths after the five first breaths, or 1.5 lung volume turnovers, of the washout. This occurs because successive breaths of the washout preferentially deplete the best ventilated lung regions (those with the highest specific ventilation) first, exaggerating differences in tracer gas distribution. Sequential filling and emptying among these regions results in an increasing concentration-normalized

* Corresponding author at: Department of Respiratory Medicine, Western General Hospital, Edinburgh EH4 2XU, UK. Tel.: +44 131 651 1008; fax: +44 131 651 1059.
E-mail address: alex.horsley@ed.ac.uk (A.R. Horsley).

1569-9048/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.resp.2008.06.014

phase III slope (Sn_{III}). This index is called S_{cond} since it is considered to be determined purely by convective gas mixing in the conducting airways.

The second component is not linearly progressive, but has reached an asymptote after approximately 1.5 TO. It is affected by molecular mass of the tracer gas, and is understood to originate from interaction between diffusion and convection in the zone where these processes contribute similarly to the movement of a gas molecule, i.e. in the zone of the diffusion–convection front (Paiva and Engel, 1984). This component has been termed S_{acin} because the interaction between diffusion and convection occurs in the acinar zone in healthy lungs (Verbanck et al., 1997).

Recently, we have reported on the measurement of lung clearance index (LCI) in adult patients with cystic fibrosis (CF), using a novel gas analyser that is capable of following very low concentrations of the inert marker gas sulphur hexafluoride (SF_6) (Horsley et al., 2008). LCI is a simple measure of overall ventilation heterogeneity, derived from analysis of multiple breath washouts. Early CF lung disease primarily affects the peripheral conducting airways, and spares the alveoli (Sobonya and Taussig, 1986). In adult CF patients however the picture is more complex. Possible mechanisms by which regional ventilation could be affected include different time constants between lung units of varying sizes, variations in airway calibre (due to inflammation, mucus collection or airway remodelling), reductions in the density of small airways, bullous disease and gas trapping (Hamutcu et al., 2002). In order to try to better understand how gas mixing is affected in CF, we have performed inert gas washout tests in CF patients at the same time as performing plethysmography and gas transfer. Sn_{III} analysis has been included in order to separate those effects due to variability in convective gas mixing and those due to interaction between diffusion and convection. Finally, following our initial observations, a cohort of children with CF was assessed in order to extend the range of disease severity and to investigate the relationship between age and Sn_{III} variables. We hypothesised that increase in S_{cond} would be an early event in CF, and would correlate best with overall ventilation heterogeneity, as measured by LCI, whereas S_{acin} would not increase until the lungs were more severely affected. We also hypothesised that S_{cond} would correlate with measures of central airway function, such as airways resistance and spirometry, whereas S_{acin} would correlate best with measures of peripheral airway function, such as gas transfer factor and RV/TLC.

2. Methods

2.1. Subjects

Seventeen healthy non-smokers (less than 10 pack years smoking history) with no known lung disease and on no regular respiratory medications were recruited as normal volunteers. Twenty-nine healthy child controls, with no history of wheeze, asthma or prematurity (<34 weeks), were recruited from amongst those attending follow-up of stable upper-limb fractures as well as children of hospital staff. Twenty-two CF adults were recruited from the Scottish Adult CF Service, and eighteen children with CF were recruited from the paediatric respiratory service at the Royal Hospital for Sick Children in Edinburgh. The diagnosis of CF was based upon a combination of clinical presentation and sweat testing and confirmed by genotyping. All volunteers and patients or guardians provided informed consent. Children too young to provide formal consent provided assent. This study was approved by the Lothian Research and Ethics Committee.

Paediatric healthy volunteer data are taken from subjects recruited to a previous study (Macleod K.A., Horsley A.R., Bell N. J., Greening A.P.G., Innes J.A., Cunningham S., under review), and the LCI data from some of the adult subjects has also been presented previously (Horsley et al., 2008).

2.2. Multiple breath washout

LCI was measured by multiple breath inert gas washout, using an Innocor™ gas analyser (Innovision, Odense, Denmark) adapted for the purpose, as described previously (Horsley et al., 2008). Subjects were seated and suitably distracted by watching television. A noseclip was applied and tidal breathing established. During the first half of the test (washin) subjects breathed from a flow-past circuit containing 0.2% SF_6 in air, supplied from a cylinder (BOC, Hertfordshire, UK). Exhaled volume was displayed to the adult subjects on a separate screen, but this was not used for children. Washin was deemed complete when inspiratory and expiratory SF_6 concentrations differed by less than 0.004% SF_6 (absolute). At this point, the flow-past circuit was removed during expiration, and the subject breathed room air. This washout phase continued until the end-tidal SF_6 concentration had fallen to <1/40th of the starting concentration (i.e. <0.005%).

2.3. Data analysis

Total volume of SF_6 expired is derived from integration of flow and SF_6 signals, and FRC is determined from the total amount of SF_6 washed out divided by the difference in end-tidal SF_6 concentration at the start and end of the washout. LCI is defined as the cumulative expired volume required to reduce the end-tidal SF_6 concentration to 1/40th of the initial value, divided by the FRC. Data were analysed using custom-built software prepared using Testpoint™ (Capital Equipment, Massachusetts, USA). Tests were performed in triplicate and LCI is quoted as the mean of three repeat washouts. As a quality control measure, tests where the measured FRC differed by more than 10% from both of the other two repeats were excluded (Wanger et al., 2005).

The technique of phase III slope analysis is similar to that originally described by Verbanck et al. (1997) and is described in detail in the online supplement. Unlike this study however, subjects were not restricted to an expired volume of 1 L. These volumes are impractical for young children, and adult CF patients found it hard to maintain such large breath volumes (the attempt to do so often precipitating coughing). Instead, adult subjects were encouraged to achieve an expiratory volume of 500–1000 mL using a visual feedback of the expired volume. Visual feedback was not used for the paediatric assessments. In order to allow for variability in expired volume, and in order to correct for the fact that this was often less than the 1 L volumes used in other studies, Sn_{III} was multiplied by the expired volume in litres to produce a volume-normalized Sn_{III} as described by Aurora et al. (2005). In order to make this distinction clear, tidal volume adjusted values of S_{cond} and S_{acin} have been relabelled $S_{condVTc}$ and $S_{acinVTc}$, respectively. Individual Sn_{III} data points that were inadequate, whether because of insufficient breath volume or signal noise, were removed from the analysis before calculation of S_{cond} and S_{acin}.

2.4. Lung function

All other lung physiology parameters were performed on adults using standard lung function laboratory equipment according to ATS/ERS standards (Macintyre et al., 2005; Miller et al., 2005; Wanger et al., 2005). Plethysmography was performed on a Zan 500 USB plethysmograph (Ferraris Respiratory, Hertford, UK) or a Jaeger Masterlab plethysmograph (Erich-Jaeger, Hoechberg, Germany). Lung volumes are quoted as the mean of three reproducible repeats (within 5%). Airways resistance was calculated as the spe-

Table 1
Demographics and lung function of patients and controls

	Healthy Adults	CF adults
Number	17	22
Male/female	10/7	13/9
Age (years)	31.3 (6.0) [21–47]	28.9 (10.1) [17–47]
FEV_1% predicted	106.2 (8.2) [90.5–119.2]	66.4 (18.2)* [29.4–106.8]
FEV_1 *z*-score	0.04 (0.76) [−1.26–1.77]	−3.03 (1.62)* [−6.33–0.79]
FEV_1/FVC %	80.0 (5.6) [69.6–89.7]	63.0 (13.6)* [34.8–87.1]
LCI	6.7 (0.6) [5.9–7.9]	12.8 (3.3)* [6.2–17.6]
Mean expired volume (mL)	822 (154)	836 (140)
Mean (S.D.) FRC (L) [mean percent predicted]	1.94 (1.10) [93%]	2.79 (0.77) [92%]
R_{aw} (0.5) (L/s)	0.12 (0.08) [0.03–0.29]	0.31 (0.13)* [0.11–0.57]
RV/TLC	22.6 (4.5) [16.5–33.0]	39.5 (9.5)* [20.4–56.9]
Mean (S.D.) D_LCO [mean percent predicted]	10.42 (1.97) [96.5]	9.12 (2.58) [86.3]
Mean (S.D.) D_LCO/V_A [mean percent predicted]	1.62 (0.61) [95.0]	1.8 (0.28)** [105.0]
$S_{acinVTc}$	0.112 (0.055) [0.012–0.271]	0.366 (0.208)† [0.052–0.742]
$S_{condVTc}$	0.010 (0.015) [−0.028–0.032]	0.086 (0.030)* [0.044–0.148]

Values are means (standard deviation) and [range of values], unless otherwise stated. R_{aw}(0.5) = Airways resistance; RV/TLC = Residual volume/total lung capacity; DL_{CO} = diffusing capacity for carbon monoxide (transfer factor), DL_{CO}/V_A is the alveolar volume corrected measure of diffusing capacity.
* $p < 0.0001$.
** $p = 0.03$ (*t*-test) compared to controls.
† $p = 0.0001$.

cific resistance at 0.5 L/s, R_{aw}(0.5), and is quoted as the mean of at least two reproducible repeats (within 10%). Diffusing capacity was assessed on a Collins Pulmolab (Ferraris Respiratory, Hertford, UK) using the single breath technique (Macintyre et al., 2005). Measurements of diffusing capacity are quoted as the mean of two repeats. Reference ranges were taken from Quanjer et al. (1993).

FEV_1 and FVC are quoted as the highest of three repeat manoeuvres (Miller et al., 2005). FEV_1 data are expressed as *z*-scores, as described by Stanojevic et al. (2008). For reference, percent predicted values for FEV_1 are also presented (Quanjer et al., 1993).

Lung function testing was performed within 3 h of LCI measurement, and though the order of the tests was not fixed there was at least a 30 min interval between the completion of lung function testing and start of MBW. Exhaled gas volumes were converted to body temperature, ambient pressure, and saturated water vapour (BTPS) conditions.

Paediatric volunteers completed triplicate washouts followed by spirometry only, using an Easyone spirometer (ndd Medizintechnik, Bern, Switzerland), according to ARTP Guidelines (1994). Full lung function was not performed in these patients. FEV_1 data have been presented and analysed as *z*-scores (Stanojevic et al., 2008), but percent predicted values are also presented for reference (Rosenthal et al., 1993).

Table 2
Demographics and lung function of paediatric patients and controls

	Healthy children	CF Children
Number	29	18
Male/female	18/11	12/6
Age (years)	11.1 (3.3) [5.3–16.2]	12.5 (3.5) [7.8–16.7]
FEV_1 % predicted	90.6 (11.9) [64.1–116.6]	89.9 (32.0) [47.7–164.8]
FEV_1 *z*-score	−0.81 (0.97) [−3.32–1.23]	−1.32 (2.38) [−5.04–4.75]
LCI	6.2 (0.5) [5.1–7.1]	7.3 (2.3)† [4.8–14.0]
Mean expired volume (mL)	538 (253)	487 (149)
FRC (L) [%predicted]	2.14 (1.02) [110.7]	2.01 (0.83) [120.7]
$S_{acinVTc}$	0.117 (0.062) [0.019–0.286]	0.192 (0.123)* [0.038–0.497]
$S_{condVTc}$	0.015 (0.019) [−0.031–0.058]	0.068 (0.029)** [0.022–0.124]

Values are means (standard deviation) and [range of values], unless otherwise stated.
* $p = 0.014$.
** $p < 0.0001$ compared to controls (Mann–Whitney *U* test).
† $p = 0.022$ (*t*-test).

2.5. Statistical analysis

Data were analysed using Prism (GraphPad Software Inc, CA, USA). Parametric data are quoted as mean (S.D.), unless otherwise stated, and were compared using *t*-tests. Non-parametric data, and small datasets, are quoted as median (inter-quartile range), and compared using the Mann–Whitney *U* test. Correlations were analysed using Spearman's rank correlation. A *p* value of 0.05 was considered significant. A Bonferroni correction for multiple comparisons of independent variables was applied to the correlations in Table 3, and for this analysis a *p* value of below 0.01 was considered statistically significant. The upper limit of normality for LCI, $S_{acinVTc}$ and $S_{condVTc}$ were defined as the mean +1.96 × standard deviation of the combined control populations.

3. Results

Twenty-two CF adults completed MBW, spirometry and diffusing capacity assessments. Plethysmography was not completed in one adult patient because of technical problems with the apparatus. Seventeen adult healthy volunteers completed inert gas washout, and diffusing capacity. Plethysmography was not completed in 5 controls. A single CF adult was excluded from Sn_{III} analysis because she was only able to produce two reproducible washouts, which due to variability in breathing pattern could not be analysed accurately for Sn_{III} analysis.

Eighteen children with CF and 29 healthy child volunteers completed MBW and spirometry. No other lung function assessments were performed in children.

Demographics and lung function data for all 39 adult subjects are presented in Table 1 and for all 47 paediatric subjects in Table 2. The upper limit of normality for LCI was calculated as 7.50, for $S_{acinVTc}$ was 0.230 and for $S_{condVTc}$ was 0.048.

3.1. Comparison between CF adults and healthy controls

CF adults had lower mean FEV_1 *z*-scores than healthy controls (−3.03 vs 0.04, $p < 0.0001$), and higher mean LCI (12.8 vs 6.7, $p < 0.0001$).

CF patients also had higher mean $S_{acinVTc}$ (0.366 vs 0.112, $p < 0.0001$) and higher mean $S_{condVTc}$ (0.086 vs 0.010, $p < 0.0001$) than healthy controls. Mean DL_{CO}/V_A was significantly greater in the CF patients ($p = 0.03$), but there were no significant differ-

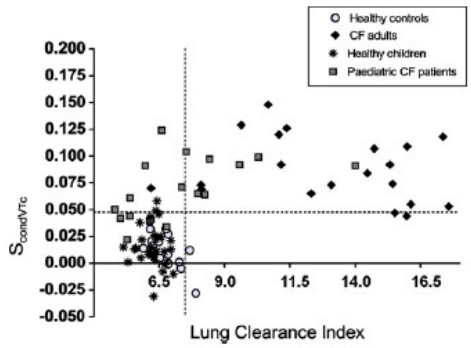


Fig. 1. Relationship between $S_{condVTc}$ and LCI for healthy subjects and cystic fibrosis patients. Healthy adults are represented as light grey circles and healthy children as asterisks. CF adults are represented as black diamonds and CF children as dark squares. The horizontal and vertical dotted lines represent the upper limit of normal $S_{condVTc}$ and LCI, respectively. A colour version of this graph is provided in the online supplement (Fig. 4).

ences in DL_{CO} or percent predicted DL_{CO}/V_A between the two groups.

3.2. Comparison between CF children and healthy controls

There were no statistically significant differences in spirometry between the patients with CF and healthy controls. As with the adult subjects, children with CF had higher mean LCI (7.3 vs 6.2 in controls, $p = 0.022$), and higher mean $S_{acinVTc}$ (0.192 vs 0.117, $p = 0.007$) and mean $S_{condVTc}$ (0.068 vs 0.015, $p < 0.0001$).

There was no significant difference between mean $S_{condVTc}$ in CF children and in CF adults ($p = 0.06$), but mean LCI and mean $S_{acinVTc}$ were both significantly lower in CF children than CF adults ($p < 0.0001$ and $p = 0.0036$, respectively).

3.3. Association between LCI and phase III slope

The relationships between LCI and $S_{condVTc}$ and $S_{acinVTc}$ are presented in Figs. 1 and 2, respectively. Elevation of $S_{condVTc}$ appears to be an early event in CF, occurring in both adults and children with normal LCI (Fig. 1). However, $S_{condVTc}$ did not increase further with increasing disease severity, and appeared to reach an asymptote with a maximum value of 0.150. $S_{acinVTc}$ on the other hand, was within the normal range in the majority of children (Fig. 2). In CF adults, $S_{acinVTc}$ showed a significant correlation with LCI (Spearman $r = 0.86$ ($p < 0.0001$)) but remained within the normal range until LCI was greater than 10.

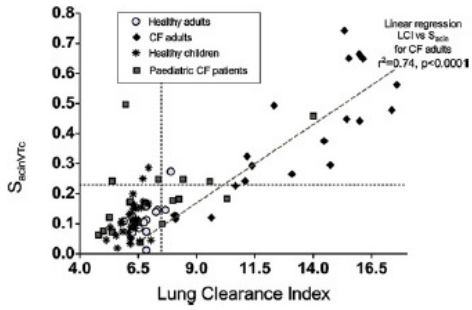


Fig. 2. Relationship between $S_{acinVTc}$ and LCI for healthy subjects and cystic fibrosis patients. Healthy adults are represented as light grey circles and healthy children as asterisks. CF adults are represented as black diamonds and CF children as dark squares. The horizontal and vertical dotted lines represent the upper limit of normal $S_{acinVTc}$ and LCI, respectively. Linear regression of $S_{acinVTc}$ versus LCI for CF adults is represented by the diagonal dotted line. A colour version of this graph is provided in the online supplement (Fig. 5).

3.4. Association between S_{cond} and S_{acin} and other markers of lung function

In CF adults, $S_{acinVTc}$ showed significant correlations with RV/TLC ($r = 0.72$, $p = 0.0003$), a measure of gas trapping, and with FEV_1 z-scores ($r = -0.73$, $p = 0.0002$). $S_{acinVTc}$ was also correlated with R_{aw} ($r = 0.56$, $p = 0.01$) but there was no association between $S_{acinVTc}$ and diffusing capacity in either CF adults or controls. In CF adults, $S_{condVTc}$ was not significantly correlated with any other measures of lung function. Table 3 shows how the main outcome measures correlate with each other for CF adults. DL_{CO}/V_A percent predicted is the quoted measure of diffusing capacity, but there were no significant correlations between any of the markers and DL_{CO}, DL_{CO} percent predicted or DL_{CO}/V_A.

3.5. Relationship between age and phase III slope analysis

There was no relationship between age and either $S_{acinVTc}$ ($p = 0.98$) or $S_{condVTc}$ ($p = 0.22$) for all healthy control subjects. These data are presented in Figs. 6 and 7 in online supplement.

3.6. Effects of modifying the phase III slope analysis

All data presented here are for $S_{condVTc}$ and $S_{acinVTc}$, the breath volume corrected derivations of S_{cond} and S_{acin} described by Aurora et al. (2005). When the uncorrected data were analysed, a similar pattern of associations and correlations was seen. This is because both $S_{condVTc}$ and $S_{acinVTc}$ correlate strongly with their uncorrected values ($r = 0.80$ and $r = 0.91$, $p < 0.0001$, respectively, for adults).

Since we did not include tight expiratory volume control, the findings could be criticised as being an artefact of small breath volumes. In order to investigate the effect of this, a number of supplementary analyses were performed to exclude those washouts with small breath volumes or excessive variability in breath volume. The results of these are described in more detail in the online supplement. In summary, the association described above between LCI and both S_{acin} and S_{cond} was robust even when only breaths of greater than 850 mL were analysed or when only selected washouts (mean VT 950 mL) were included.

3.7. Effect of altering the lung turnover range on S_{cond}

It was apparent during data analysis that in some washouts from CF patients there was a levelling off of the Sn_{III} values within the TO range 1.5–6. This is illustrated in Fig. 9 in online supplement. If the slope flattens off before the upper TO limit of 6, any calculation of linear regression within this range is erroneously diminished. The solution to this is to use a lower TO range (1.5–4) for calculation of S_{cond}. Full details of this analysis are presented in the online supplement (p. 28). Mean $S_{condVTc}$ was higher when the reduced TO range was employed (0.102 vs 0.088 L^{-1}), but this difference was not statistically significant ($p = 0.25$, paired t-test). Overall this adjustment had little effect on the graph of $S_{condVTc}$ versus LCI (see Fig. 13, online supplement), which continued to show progression of $S_{acinVTc}$ with worsening gas mixing, but limited progression of $S_{condVTc}$.

Table 3
Summary of correlations between different markers of lung function in patients with cystic fibrosis

	LCI	$S_{acinVTc}$	$S_{condVTc}$	FEV_1% z-score
$S_{acinVTc}$	**0.87; <0.0001**			
$S_{condVTc}$	−0.22; ns	−0.38; ns		
FEV_1 z scores	**−0.86; <0.0001**	**−0.73; 0.0002**	0.15; ns	
R_{aw} (0.5) (L/s)	0.42; ns	0.56; 0.011	−0.13; ns	−0.42; ns
RV/TLC	**0.73; 0.0002**	**0.72; 0.0009**	−0.21; ns	**−0.80; <0.0001**
D_LCO/V_A % predicted	0.10; ns	0.03; ns	0.48; 0.03	−0.19; ns

Correlations were assessed using Spearman's rank correlation coefficient. p values of greater than 0.05 are recorded as ns (not significant). Correlations with a p value less than 0.01 are highlighted in bold.

3.8. Effect of anatomical dead space on ventilation inhomogeneity

Anatomical dead space was calculated from the first three breaths of each washout for 21 CF adults and 12 healthy adults according to the method described by Fowler (1948). The method is described in more detail in the online supplement (Section 2.10 in online supplement). There were no significant differences between healthy subjects and CF patients in percent predicted dead space or dead space:tidal volume ratio (V_D/V_T). Moreover, there were no associations between dead space and LCI or $S_{condVTc}$. A weak association between V_D/V_T and $S_{condVTc}$ was in the opposite direction to that which would explain the increase in LCI or S_{cond} with increased disease severity. These data are presented in Table 4 and Fig. 15 in online supplement.

4. Discussion

From first principles it might be expected that CF patients with more severe lung disease would have more inhomogenous convective gas mixing. However, we were unable to demonstrate this using a method based upon phase III slope analysis. Although the convection dependent component (S_{cond}) was elevated in almost all CF subjects, including children with mild disease and normal LCI, S_{cond} did not continue to rise with increasing disease severity (as expressed by deteriorating FEV_1 or LCI) and appeared to reach an early asymptote. In contrast, the contribution to the normalised phase III slope of diffusion–convection interaction (S_{acin}) was correlated with severity of lung disease and hyperinflation (RV/TLC). Furthermore, increases in heterogeneity of gas mixing appeared to occur largely in the S_{acin} component. These findings were seen even after eliminating potential methodological differences between this and previously published studies.

Unlike previous studies (in COPD patients) we did not detect a relationship between S_{acin} and diffusing capacity (Verbanck et al., 1998, 2004). This may reflect differences in the pathology, since alveolar structure and diffusing capacity are well preserved until late in CF (Sobonya and Taussig, 1986; Espiritu et al., 2003). S_{acin} does however show a convincing correlation with RV/TLC, measured at plethysmography. This is a measure of hyperinflation which, particularly in those with normal alveolar function, one would expect to be caused by disease of the small conducting airways, and would therefore be measured by S_{cond} (Macklem et al., 1971; Cosio et al., 1978). King et al. (2005) showed that in healthy controls, it was S_{cond} that related to the volume of gas trapping at FRC, whereas S_{acin} was not.

The earliest effects on gas mixing appear to occur in the conducting airways, and S_{cond} was elevated in almost all children with CF, including those with LCI well within the normal range. This corresponds with our understanding about the site of earliest pathology in CF (Brownlee, 2006). Impairment of S_{acin} on the other hand appears to be a relatively late event, not occurring until there is already established ventilation heterogeneity. However, S_{cond} did not continue to rise with increasing disease severity, even though aerosol bolus dispersion studies show continued deterioration in convective flow in CF with increasing overall ventilation heterogeneity (Brown et al., 1998). The failure of S_{cond} to reflect increasing convective ventilation heterogeneity in this study may partly be related to the low tidal flows employed. Faster breathing, or greater breath volumes, might reveal dynamic differences in flow-resistance between lung units that would increase measurements of convective gas mixing heterogeneity beyond those seen at tidal breathing. However, this would still fail to explain why S_{acin}, rather than S_{cond}, is so strongly correlated with gas trapping. The ceiling value of S_{cond} appeared to be around 0.150. Inspection of previously published data on Sn_{III} reveal that, in a range of different airways diseases, S_{cond} values of this level are rarely achieved—suggesting that this may indeed be a true ceiling for this parameter (Verbanck et al., 1999, 2003, 2004, 2006; Aurora et al., 2005; Downie et al., 2007). Yet in this study, adults and children with mild CF lung disease were routinely obtaining S_{cond} values of around 0.100.

Phase III slope analysis is based upon persuasive experimental data and modelling, and now supported by a growing number of clinical studies (Verbanck et al., 1997, 1998, 1999, 2003, 2006; King et al., 2005; Downie et al., 2007). However, these assumptions and modelling were derived from histological data on normal lungs (Paiva and Engel, 1984; Crawford et al., 1985). Furthermore, all the studies that have so far reported on Sn_{III} analysis have been in those with relatively normal or mild airways disease. The pathology in CF is quite different from that of asthma or COPD. Adult CF patients in particular may have marked suppurative lung disease, with radiological evidence of bronchiectasis, regional lung collapse, bullous lung disease, and small airways obstruction (Helbich et al., 1999). Even in subjects without such marked structural airways disease, bronchiectasis, mucus plugging and gas trapping are commonly described features (Helbich et al., 1999). It is not clear what effect these fundamental differences will have on the assumptions behind the phase III slope analysis, but it is of note that S_{cond} shows no correlation in CF adults with any other measures of lung function, including those such as R_{aw} that might have been anticipated to reflect similar processes (King et al., 2005). There are two possible interpretations of these findings. The first is that, compared to other airways diseases (including COPD and asthma), CF represents the severe end of a spectrum. The limitations seen on S_{cond} are thus applicable in all diseases, but are apparent much earlier in disease progression in CF. This also would explain why other authors have so far failed to describe S_{cond} values greater than 0.150. Indeed such a limitation was described by Paiva (1975) more than 30 years ago in a two-compartment lung model. He showed that Sn_{III} would increase sequentially during a washout, and that this increase would be steeper with increasing difference in ventilation distribution. However, he also showed that this increase in Sn_{III} would reach an asymptote, and that this would occur earlier in the washout with more pronounced ventilation heterogeneity. This is precisely what we have described in patients with CF. It might be possible to make inferences about convective ventilation from the

level of this asymptote. Unfortunately however real life data do not perform as neatly as lung models and the reality is that the progression of Sn_{III} tails off rather than coming to an abrupt end, making it hard to determine with precision the level of the asymptote or more particularly the breath at which it is reached.

As an adjunct to this, rather than necessarily an alternative, the derivation of S_{cond} may be particularly susceptible to the nature of airways disease in CF. The effects on ventilation of small airway obstruction due to mucus plugging may be what makes LCI such a sensitive measure of early disease in CF. It is also possible that, as disease progresses, these same pathological processes invalidate some of the assumptions behind phase III slope analysis. Complete obliteration of small airways means that they will not contribute to convective ventilation (S_{cond}). Furthermore, as larger airways become obstructed the diffusion front will be moved proximally. The resulting regional differences in gas distribution therefore occur at very low or zero gas flows and are present at the start of the washout. They would thus be measured by the S_{acin} component of phase III slope analysis. This would explain the association between gas trapping (a result of small airways obstruction), S_{acin}, and LCI. This would also explain why S_{acin} correlates with measures of conducting airway obstruction, such as R_{aw} and FEV_1.

4.1. Methodological differences between this and previous studies

In previous studies, very precise expiratory volume targeting of one litre has been employed (Verbanck et al., 1997; Crawford et al., 1985). CF patients however, especially those with poorer lung function, found it hard to maintain such large tidal volumes without difficulty or coughing, and in children these breath volumes are impractical. Expiratory volume correction has therefore been applied in order to allow comparison with earlier studies (Aurora et al., 2005). The data from the two analysis protocols show strong correlation with each other and conclusions from the uncorrected data were the same as those presented here. It would therefore appear that the conclusions are robust regardless of analysis protocol or tidal volume, and despite adjustment of the lung TO range.

Most previously published studies of phase III slope analysis have been performed on nitrogen washouts. SF_6 is denser than nitrogen, and diffusivity is less, which may accentuate the diffusive differences in the lungs and lead to an increase in S_{acin}. Gronkvist et al. (2002) showed a significant increase in S_{acin} of 30% when measured with SF_6 compared to He. The difference between SF_6 and nitrogen however is likely to be less than this and cannot explain the findings entirely. The values of S_{acin} obtained in CF are similar to those derived from nitrogen washouts in patients with CF (0.307) (Gustafsson, 2007) and COPD (0.480) (Verbanck et al., 1998). Furthermore, S_{acin} in healthy controls reported here is similar to that previously reported in control groups (Verbanck et al., 1997).

The values for S_{cond} in controls presented here were lower than those reported in nitrogen washouts in healthy adults (Verbanck et al., 2004) and are similar to values reported by Aurora et al. (2005) in healthy children up to 18 years. The phase III slope of healthy subjects was usually around zero (i.e. near horizontal) and changed very little during the course of a washout. This makes S_{cond} in healthy subjects more susceptible to the effects of outlier values of Sn_{III} and partly explains the poor repeatability of the measurement in control subjects, since small absolute changes in Sn_{III} within and between washouts translate into substantial percent changes.

4.2. Summary

This study presents novel data on phase III slope analysis in both children and in adults with CF. Although S_{cond} is sensitive to early changes in airway physiology, it does not correlate with other measures of gas mixing or airway function due to an early ceiling value that is reached even in those with apparently mild disease. In these patients, this analysis appears to offer few advantages over LCI. This study highlights the fundamental differences in airway pathology and physiology between CF other airways diseases.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.resp.2008.06.014.

References

Aurora, P., Kozlowska, W., Stocks, J., 2005. Gas mixing efficiency from birth to adulthood measured by multiple-breath washout. Respir. Physiol. Neurobiol. 148, 125–139.
Brown, J.S., Gerrity, T.R., Bennett, W.D., 1998. Effect of ventilation distribution on aerosol bolus dispersion and recovery. J. Appl. Physiol. 85, 2112–2117.
Brownlee, K.G., 2006. Small airways disease in cystic fibrosis. Eur. Respir. Momgr. 35, 21–37.
Cosio, M., Ghezzo, H., Hogg, J.C., Corbin, R., Loveland, M., Dosman, J., Macklem, P.T., 1978. The relations between structural changes in small airways and pulmonary-function tests. N. Engl. J. Med. 298, 1277–1281.
Crawford, A.B., Makowska, M., Paiva, M., Engel, L.A., 1985. Convection- and diffusion-dependent ventilation maldistribution in normal subjects. J. Appl. Physiol. 59, 838–846.
Davies, J.C., Cunningham, S., Alton, E.W., Innes, J.A., 2008. Lung clearance index in CF: a sensitive marker of lung disease severity. Thorax 63, 96–97.
Downie, S.R., Salome, C.M., Verbanck, S., Thompson, B., Berend, N., King, G.G., 2007. Ventilation heterogeneity is a major determinant of airway hyperresponsiveness in asthma, independent of airway inflammation. Thorax 62, 684–689.
Dutrieue, B., Vanholsbeeck, F., Verbanck, S., Paiva, M., 2000. A human acinar structure for simulation of realistic alveolar plateau slopes. J. Appl. Physiol. 89, 1859–1867.
Espiritu, J.D., Ruppel, G., Shrestha, Y., Kleinhenz, M.E., 2003. The diffusing capacity in adult cystic fibrosis. Respir. Med. 97, 606–611.
Estenne, M., Van Muylem, A., Knoop, C., Antoine, M., 2000. Detection of obliterative bronchiolitis after lung transplantation by indexes of ventilation distribution. Am. J. Respir Crit. Care Med. 162, 1047–1051.
Fowler, W.S., 1948. Lung function studies; the respiratory dead space. Am. J. Physiol. 154, 405–416.
Guidelines for the measurement of respiratory function, 1994. Recommendations of the British Thoracic Society and the Association of Respiratory Technicians and Physiologists. Respir. Med. 88, 165–194.
Gronkvist, M., Bergsten, E., Gustafsson, P.M., 2002. Effects of body posture and tidal volume on inter- and intraregional ventilation distribution in healthy men. J. Appl. Physiol. 92, 634–642.
Gustafsson, P.M., 2007. Peripheral airway involvement in CF and asthma compared by inert gas washout. Pediatr. Pulmonol. 42, 168–176.
Hamutcu, R., Rowland, J.M., Horn, M.V., Kaminsky, C., MacLaughlin, E.F., Starnes, V.A., Woo, M.S., 2002. Clinical findings and lung pathology in children with cystic fibrosis. Am. J. Respir. Crit. Care Med. 165, 1172–1175.
Helbich, T.H., Heinz-Peer, G., Eichler, I., Wunderbaldinger, P., Gotz, M., Wojnarowski, C., Brasch, R.C., Herold, C.J., 1999. Cystic fibrosis: CT assessment of lung involvement in children and adults. Radiology 213, 537–544.
Horsley, A.R., Gustafsson, P.M., Macleod, K.A., Saunders, C., Greening, A.P., Porteous, D.J., Davies, J.C., Cunningham, S., Alton, E.W., Innes, J.A., 2008. Lung clearance index is a sensitive, repeatable and practical measure of airways disease in adults with cystic fibrosis. Thorax 63, 135–140.
King, G.G., Downie, S.R., Verbanck, S., Thorpe, C.W., Berend, N., Salome, C.M., Thompson, B., 2005. Effects of methacholine on small airway function measured by forced oscillation technique and multiple breath nitrogen washout in normal subjects. Respir. Physiol. Neurobiol. 148, 165–177.
Macintyre, N., Crapo, R.O., Viegi, G., Johnson, D.C., van der Grinten, C.P., Brusasco, V., Burgos, F., Casaburi, R., Coates, A., Enright, P., Gustafsson, P., Hankinson, J., Jensen, R., McKay, R., Miller, M.R., Navajas, D., Pedersen, O.F., Pellegrino, R., Wanger, J., 2005. Standardisation of the single-breath determination of carbon monoxide uptake in the lung. Eur. Respir. J. 26, 720–735.
Macklem, P.T., Thurlbeck, W.M., Fraser, R.G., 1971. Chronic obstructive disease of small airways. Ann. Intern. Med. 74, 167–177.
Miller, M.R., Hankinson, J., Brusasco, V., Burgos, F., Casaburi, R., Coates, A., Crapo, R., Enright, P., van der Grinten, C.P., Gustafsson, P., Jensen, R., Johnson, D.C., MacIntyre, N., McKay, R., Navajas, D., Pedersen, O.F., Pellegrino, R., Viegi, G., Wanger, J., 2005. Standardisation of spirometry. Eur. Respir. J. 26, 319–338.
Paiva, M., 1975. Two new pulmonary functional indexes suggested by a simple mathematical model. Respiration 32, 389–403.
Paiva, M., Engel, L.A., 1984. Model analysis of gas distribution within human lung acinus. J. Appl. Physiol. 56, 418–425.
Prisk, G.K., Lauzon, A.M., Verbanck, S., Elliot, A.R., Guy, H.J., Paiva, M., West, J.B., 1996. Anomalous behavior of helium and sulfur hexafluoride during single-breath tests in sustained microgravity. J. Appl. Physiol. 80, 1126–1132.
Quanjer, P.H., Tammeling, G.J., Cotes, J.E., Pedersen, O.F., Peslin, R., Yernault, J.C., 1993. Lung volumes and forced ventilatory flows. Report Working Party Standardiza-

tion of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. Eur. Respir J. Suppl. 16, 5–40.
Rosenthal, M., Bain, S.H., Cramer, D., Helms, P., Denison, D., Bush, A., Warner, J.O., 1993. Lung function in white children aged 4 to 19 years: I—Spirometry. Thorax 48, 794–802.
Sobonya, R.E., Taussig, L.M., 1986. Quantitative aspects of lung pathology in cystic fibrosis. Am. Rev. Respir. Dis. 134, 290–295.
Stanojevic, S., Wade, A., Stocks, J., Hankinson, J., Coates, A.L., Pan, H., Rosenthal, M., Corey, M., Lebecque, P., Cole, T.J., 2008. Reference ranges for spirometry across all ages: a new approach. Am. J. Respir. Crit. Care Med. 177, 253–260.
Venegas, J., 2007. Linking ventilation heterogeneity and airway hyperresponsiveness in asthma. Thorax 62, 653–654.
Verbanck, S., Schuermans, D., Meysman, M., Paiva, M., Vincken, W., 2004. Noninvasive assessment of airway alterations in smokers: the small airways revisited. Am. J. Respir. Crit. Care Med. 170, 414–419.
Verbanck, S., Schuermans, D., Noppen, M., Van Muylem, A., Paiva, M., Vincken, W., 1999. Evidence of acinar airway involvement in asthma. Am. J. Respir. Crit. Care Med. 159, 1545–1550.
Verbanck, S., Schuermans, D., Paiva, M., Meysman, M., Vincken, W., 2006. Small airway function improvement after smoking cessation in smokers without airway obstruction. Am. J. Respir. Crit. Care Med. 174, 853–857.
Verbanck, S., Schuermans, D., Paiva, M., Vincken, W., 2003. Nonreversible conductive airway ventilation heterogeneity in mild asthma. J. Appl. Physiol. 94, 1380–1386.
Verbanck, S., Schuermans, D., Van Muylem, A., Melot, C., Noppen, M., Vincken, W., Paiva, M., 1998. Conductive and acinar lung-zone contributions to ventilation inhomogeneity in COPD. Am. J. Respir. Crit. Care Med. 157, 1573–1577.
Verbanck, S., Schuermans, D., Van Muylem, A., Paiva, M., Noppen, M., Vincken, W., 1997. Ventilation distribution during histamine provocation. J. Appl. Physiol. 83, 1907–1916.
Wanger, J., Clausen, J.L., Coates, A., Pedersen, O.F., Brusasco, V., Burgos, F., Casaburi, R., Crapo, R., Enright, P., van der Grinten, C.P., Gustafsson, P., Hankinson, J., Jensen, R., Johnson, D., Macintyre, N., McKay, R., Miller, M.R., Navajas, D., Pellegrino, R., Viegi, G., 2005. Standardisation of the measurement of lung volumes. Eur. Respir. J. 26, 511–522.

Respiratory Medicine (2009) xx, 1–7

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/rmed

REVIEW

Lung clearance index in the assessment of airways disease

Alex Horsley [a,b,*]

[a] Department of Respiratory Medicine, Edinburgh Royal Infirmary, 51 Little France Crescent, Edinburgh EH4 2XU, United Kingdom
[b] UK Cystic Fibrosis Gene Therapy Consortium, United Kingdom

Received 17 October 2008; accepted 29 January 2009

KEYWORDS
Lung clearance index;
Multiple breath washout;
Lung physiology;
Small airways;
Cystic fibrosis

Summary
In the last few years there has been a growing interest in lung clearance index (LCI), a measure of lung physiology derived from multiple breath washout tests. This resurgence of interest was initially driven by the recognition that such assessments were capable of detecting early airways disease in children, and are more sensitive and easier to perform in this population than conventional lung function tests [Aurora P, Kozlowska W, Stocks J. Gas mixing efficiency from birth to adulthood measured by multiple-breath washout. *Respir Physiol Neurobiol*, 2005;**148**(1–2):125–39]. With an appreciation of the importance of earlier identification of airways dysfunction, and prevention of irreversible structural airway changes, methods of following airways disease in these "silent years" are especially important. LCI has now been reported in studies involving all age groups, from infants to adults [Lum S, Gustafsson P, Ljungberg H, Hulskamp G, Bush A, Carr SB, et al. Early detection of cystic fibrosis lung disease: multiple-breath washout versus raised volume tests. *Thorax*, 2007;**62**(4):341–7; Horsley AR, Gustafsson PM, Macleod K, Saunders CJ, Greening AP, Porteous D, et al. Lung clearance index is a sensitive, repeatable and practical measure of airways disease in adults with cystic fibrosis. *Thorax*, 2008;**63**:135–40], and has a narrow range of normal over this wide age range, making it especially suitable for long-term follow-up studies. In cystic fibrosis (CF) particularly, there is a pressing need for sensitive and repeatable clinical endpoints for therapeutic interventions [Rosenfeld M. An overview of endpoints for cystic fibrosis clinical trials: one size does

* Department of Respiratory Medicine, Edinburgh Royal Infirmary, 51 Little France Crescent, Edinburgh EH4 2XU, United Kingdom. Tel.: +44 131 5372415; fax: +44 131 5371101.
E-mail address: alex.horsley@ed.ac.uk

0954-6111/$ - see front matter
doi:10.1016/j.rmed.2009.01.025

Please cite this article in press as: Horsley A, Lung clearance index in the assessment of airways disease, Respiratory Medicine (2009), doi:10.1016/j.rmed.2009.01.025

not fit all. *Proc Am Thorac Soc*, 2007;4(4):299–301], and LCI has been proposed as an outcome measure in future CF gene therapy studies [Davies JC, Cunningham S, Alton EW, Innes JA. Lung clearance index in CF: a sensitive marker of lung disease severity. *Thorax*, 2008;63(2):96–7].

This review will consider how LCI is derived, how it differs from conventional lung function testing, and its applications and limitations.

Contents

In the last few years there has been a growing interest in lung clearance index (LCI), a measure of lung physiology derived from multiple breath washout tests. This resurgence of interest was initially driven by the recognition that such assessments were capable of detecting early airways disease in children, and are more sensitive and easier to perform in this population than conventional lung function tests.[1] With an appreciation of the importance of earlier identification of airways dysfunction, and prevention of irreversible structural airway changes, methods of following airways disease in these ''silent years'' are especially important. LCI has now been reported in studies involving all age groups, from infants to adults,[2,3] and has a narrow range of normal over this wide age range, making it especially suitable for long-term follow-up studies. In cystic fibrosis (CF) particularly, there is a pressing need for sensitive and repeatable clinical endpoints for therapeutic interventions,[4] and LCI has been proposed as an outcome measure in future CF gene therapy studies.[5]

This review will consider how LCI is derived, how it differs from conventional lung function testing, and its applications and limitations.

Multiple breath washout tests

LCI is derived from Multiple Breath Washout (MBW) tests. The basic principles behind MBW are relatively simple, and were first described more than 50 years ago.[6] The test involves following the washout of an inert tracer gas from the lungs during relaxed tidal breathing. The tracer gas can be nitrogen that is normally resident in the lungs, washed out when the subject is switched to breathing 100% oxygen. Alternatively, it can be an exogenous tracer gas that must first be washed into the lungs to equilibrium. Each approach has its own advantages and challenges, but the principle is the same: namely that the tracer gas should be inert and neither absorbed nor excreted by the body to any significant degree. With each successive breath of the washout, there is a fall in the peak concentration of exhaled tracer (Fig. 1).

As shown by hyperpolarised helium MRI studies, airways disease tends to be patchy.[7] Airway narrowing due to factors such as mucus retention, inflammation and airway wall structural damage causes unevenness of ventilation. This unevenness, or ventilation heterogeneity, affects the overall gas mixing efficiency of the lung, and can be measured by following the washout of a tracer gas during tidal breathing. In disease, the washout will take longer to complete, requiring a greater number of breaths.

A number of different indices of deranged ventilation can be calculated from the washout tracings, but one of the most robust and sensitive, and hence one of the most widely reported, of these is the lung clearance index (LCI).

Derivation of lung clearance index

For calculation of LCI, the washout is deemed completed when the end-tidal tracer gas concentration has fallen to 1/40th of the starting concentration. The reason for using 1/40th is largely historical, as this represents the limits of the linear operating range (2–80%) of the early nitrogen analysers. However, it has stood the test of time and represents a workable compromise between ending a washout too soon (and therefore losing sensitivity) and an excessively protracted procedure.

Functional residual capacity (FRC) is calculated from multiple breath washouts from the starting end-tidal fraction of tracer gas (C_{Init}), the final fraction of tracer (C_{End}), and the total volume of tracer gas exhaled up to the end of the washout ($V_{[Tracer]}$):

$$FRC = V_{[Tracer]}/(C_{Init} - C_{End})$$

LCI is then defined as the cumulative expired volume (CEV), divided by the FRC:

Please cite this article in press as: Horsley A, Lung clearance index in the assessment of airways disease, Respiratory Medicine (2009), doi:10.1016/j.rmed.2009.01.025

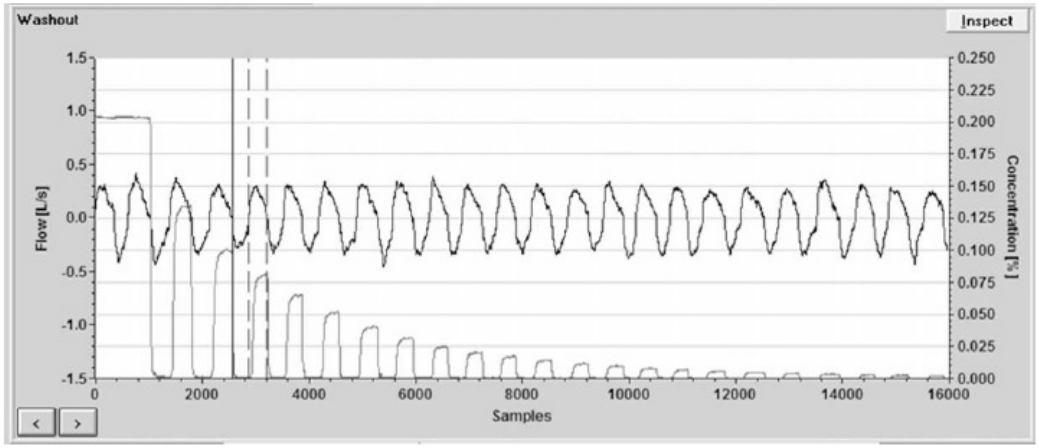


Figure 1 Typical washout tracing of a healthy adult subject. Flow is shown in the upper trace (expiration upwards), with the scale on the left hand *y*-axis. Tracer gas (in this case, 0.2% SF_6) concentration is shown in the lower trace — with each successive breath, there is a fall in peak expiratory SF_6 concentration.

$$LCI = CEV/FRC$$

In other words, LCI represents a measure of the number of times the volume of gas in the lung at the start of the washout (the FRC) must be turned over in order to wash out the tracer to the pre-defined endpoint. With increasing disease severity, LCI increases.

Clinical use of LCI

Because no complex respiratory manoeuvres are required, MBW tests are especially useful in children, and the majority of recent studies reflect this. The earliest work on lung gas mixing was performed using nitrogen washout apparatus. A number of studies, comparing small numbers of groups of subjects with different respiratory diseases, were performed in the 1970s—1980s.[8–10] The gas analyser and washout analysis technology used in these studies was relatively crude and constructed in-house. Although abnormalities in gas mixing indices were demonstrated in disease, there was little suggestion that these would be useful clinical assays. A combination of improved analyser technology and data analysis software, as well as a general increased interest in the need for robust infant lung function techniques, has been a major driving force behind the recent resurgence of interest in LCI.

In 2003, a Swedish group described the use of a mass spectrometer to perform washouts using 4% sulphur hexafluoride (SF_6) as the inert tracer gas.[11] They reported that LCI was elevated in 43 children with cystic fibrosis (CF) (aged 3—18 years) compared to 28 healthy controls. More importantly, they showed that LCI was more sensitive than spirometry, being elevated in 22 of the 33 CF patients with normal spirometry. A similar system, using the same technique and analysis software, was subsequently established at Great Ormond Street Children's Hospital in London. Using this apparatus, Aurora et al. confirmed the Swedish findings in school age children,[12] and then went on to use the technique in younger age groups.[13] In pre-school children LCI was higher in those patients infected with *Pseudomonas aeruginosa*, an important lung pathogen in CF and one that is known to be associated with a poorer prognosis.[14] More recently, the same group have measured LCI in infants as young as 10 weeks old and showed that LCI was elevated in those with CF (mean age 41 weeks) compared to age matched healthy controls.[2] Using a different analyser technology altogether, a third group have reported on LCI in adults and children with CF.[3] The normal range of LCI in the healthy volunteers in all of these studies was almost identical, despite differences in subject age, location, and technology. Cumulatively, these studies have demonstrated that multiple breath washouts can be performed in large numbers of subjects in clinical studies, and that LCI can be reliably and reproducibly measured, even in young subjects. The group mean coefficient of variation of intra-visit repeat LCI measurements, a simple measure of reproducibility, ranged from 7.8% in pre-school children with CF (mean age 4.4 years), to 3.2% in healthy adults (mean age 33 years).[3,13] Although no association between age and reproducibility has been described in children,[12] our own observations suggest that reproducibility declines with deteriorating LCI.

Longitudinal studies of LCI are particularly important in establishing how LCI tracks disease progression. A large Swiss cohort has been followed between the ages of 6 and 20 years.[15] 142 children with CF have had at least 4 serial annual evaluations of conventional lung function (spirometry, specific airway resistance and FRC at plethysmography), *P. aeruginosa* infection status, and LCI (performed using a nitrogen washout apparatus). They demonstrated that LCI was the earliest measurement to deteriorate, followed by FEF_{50}, FVC and finally FEV_1. LCI was elevated in more than half of those with FEV_1 within the normal range. Furthermore LCI continued to increase, along with pulmonary hyperinflation and trapped gas volume, beyond the age of 12 years, whereas FEV_1 z-scores stabilised. In subsequent papers derived from the same dataset they

Please cite this article in press as: Horsley A, Lung clearance index in the assessment of airways disease, Respiratory Medicine (2009), doi:10.1016/j.rmed.2009.01.025

also showed that LCI was more elevated in those with ABPA, and that the slope of longitudinal progression of LCI was greatest in those chronically infected with *P. aeruginosa*.[16] LCI was also the most sensitive discriminator between groups divided on the basis of chronic and intermittent *P. aeruginosa* colonization of the lower airway.

It is not possible to know exactly what LCI represents at a histological level, since airway histopathology in subjects with mild disease is not available. Most of the work published in LCI so far has looked at CF, and our understanding of this disease is that it affects primarily the small airways, at least initially.[17] This, and other evidence, leads us to believe that LCI is particularly sensitive to small airways dysfunction. Further support for this came from a study of CT appearances, spirometry and LCI in 44 children (age 5–19 years) with CF.[18] Sensitivity of FEV_1 to structural abnormalities on CT was poor. LCI was the most sensitive measure of structural lung abnormalities, particularly air trapping, for which it had a sensitivity of 94%. In addition, LCI was also elevated in one third of those with a normal CT score, which may represent the presence of physiological abnormalities due to disease that is below the limit of resolution of the CT scanner. Normal LCI in a patient with CF almost excluded the presence of structural abnormalities on HRCT.

LCI also has a potential role in asthma and wheeze. It is known that asthma has a number of different phenotypes in childhood, some of which may be associated with structural airway wall changes (airway remodelling).[19] There is also evidence from bronchial biopsy studies of structural airway changes in children with wheeze as young as 3 years old. This has clinical implications as, in many cases, asthma symptoms diminish in late childhood but inflammation and airway remodelling may be progressive in adulthood.[20] There is now a recognised need for robust and repeatable surrogate measures to detect and track early lung function abnormalities in the presence of progressive airway remodelling.

Two studies have measured LCI in asthmatic children with a view to investigating evidence of ventilation heterogeneity and its response to acute treatment. In the first of these, Gustafsson reported on LCI and spirometry in children with asthma and CF.[21] He showed that LCI was elevated in asthma, and fell in response to nebulised bronchodilator. The CF patients had a similar degree of impairment in FEV_1 to the asthmatics (groups mean FEV_1 72% and 77% predicted, respectively), but had significantly higher LCI, which did not respond to bronchodilators. More recently, Macleod et al. reported that LCI in a cohort of children with well-controlled asthma (mean FEV_1 z-score of −1.26) was significantly elevated compared to age matched healthy controls, although the absolute elevation in LCI was modest (mean LCI 6.69 in the asthmatics versus 6.24 in controls). Despite adequate preventative treatment and clinical stability, FEV_1 improved significantly following inhaled salbutamol. LCI did not improve, but remained significantly higher than controls, indicating evidence of non-bronchodilator responsive residual airways disease that was not detected by spirometry. LCI may offer an alternative method of assessing airway physiology in this and similar populations.

Advantages of LCI

It is the sensitivity to small airways dysfunction that makes LCI such a valuable measure of airway physiology. Spirometry, essentially a measure of airway resistance at flow limitation, has long been known to be an insensitive measure of small airways disease. The small airways (those less than 2 mm diameter) have a large combined cross-sectional surface area and therefore have low mean flow rates and combined resistance. In healthy adults, they contribute less than 10% of the total airways resistance.[22] Considerable structural damage to these airways can therefore occur before there is any impairment of FEV_1.[23] Using hyperpolarised helium MRI to image the distribution of inhaled helium, it has also been shown that FEV_1 is insensitive to disturbances in ventilation distribution.[7] LCI therefore fills an important gap in our ability to follow airways disease non-invasively – the so called ''silent zone'' between onset of pathology and detection of this with standard lung function tests.

Several studies have now shown LCI to be considerably more sensitive to disease than spirometry.[3,11–13] The particular sensitivity of LCI to CF lung disease may reflect the underlying lung pathology, which is one of uneven small airways inflammation and obstruction.[24] There is growing interest in the early identification and treatment of lung disease, and this is particularly true in CF where untreated disease leads to a progressive and irreversible decline.

Because only tidal breathing is required, LCI is ideal for use in children. No complex or forced respiratory manoeuvres are required. The test can therefore be successfully performed in the majority of those down to pre-school years,[1] though very young children may require sedation. In infants, the challenges are greater because of the technical demands on the apparatus, but the demonstration that this can be done successfully is an exciting development in this field.[2]

A particular problem with other measures of small airway function, such as mid-expiratory flows and single breath washout tests, is that they are poorly reproducible.[25] LCI however has good intra-visit reproducibility (with a coefficient of variation of repeat measurements of around 3–8%[3,12]), which is as good, or better, than most lung physiology assessments in the lab.[26] Inter-visit reproducibility is also good in healthy volunteers.[3]

The range of LCI in normal subjects is remarkably narrow across a wide age range, and consistent throughout the various studies.[1,3,11–13] Unlike spirometry, it is also unaffected by height or gender. Because it is derived using the FRC, differences due to physical size are already accounted for, leaving only the effects of gas mixing. This is especially important for longitudinal studies, particularly in children. Since spirometry changes with age, height and gender, it is normally expressed as a percent predicted. But this means accepting a wide range of FEV_1 which would be considered ''normal'' for any individual, and the equations most commonly used to determine normal range change in late teens.[27,28] Use of different prediction equations for ''normality'' can have profound effects on the measured rate of decline in ''% predicted'' values for spirometry.[29] These problems are particularly acute when assessing lung

Please cite this article in press as: Horsley A, Lung clearance index in the assessment of airways disease, Respiratory Medicine (2009), doi:10.1016/j.rmed.2009.01.025

function during the adolescent growth spurt, which itself may be affected by disease processes (e.g. CF). Normal LCI however remains unchanged, allowing any deviation to be easily identified. Although LCI was slightly higher in the infants, this may be due to differences in protocol (the test was performed supine) or due to the effects of serial deadspace.[30]

Disadvantages of LCI

The test takes much longer to perform than simple spirometry, and (for an exogenous tracer) requires both a wash-in and a washout phase. In normal children or those with mild disease, the entire process takes little more than 5 min, but is conventionally repeated in triplicate and therefore takes around 15 min to complete. In adults, a single wash-in and washout may take twice as long. A good mouthpiece seal is required, and this can be difficult to sustain for prolonged periods.

The sensitivity of LCI means that, whilst it is a simple and useful test in those with early airways disease, it is much less informative and more protracted in those with significant airflow obstruction. In addition, particularly in those with severe CF, interventions that reduce the burden of airway infection and inflammation may open up previously poorly ventilated lung regions. This may actually increase the heterogeneity between well and poorly ventilated units, paradoxically worsening LCI. In these subjects, spirometry is probably a more useful indicator of the state of their airways.

Practicalities of measuring LCI

Although well established in a research setting, the technology required for multiple breath washout assessment is considerably more complex than it at first appears. Until recently there have been no commercial apparatus available, and no universal standards for performing the tests. Studies so far have therefore relied upon apparatus and protocols developed in-house. During a nitrogen washout, the fractional nitrogen and oxygen concentrations alter during the course of both individual breaths and the washout as a whole. This alters the viscosity of the expirate, and hence the measured flow, by up to 12%.[8] In order to accommodate this, continuous adjustment of flowmeter output is required according to the measured nitrogen concentration. Although this can be done by computer, in the absence of an off-the-shelf commercial system it requires individual programming by the user.

In addition, with nitrogen washout systems the contribution of additional body nitrogen excreted during the lungs may become significant with prolonged washouts, though is not felt to be significant in normal subjects.[31] Sufficient time must be left between washouts for additional oxygen to be expired or absorbed, and the resting gas concentrations return to baseline — this is recommended to be at least 15 min.[32]

The alternative approach of using an exogenous inert marker gas requires the subject to first wash in the tracer until inspiratory and expiratory marker gas concentrations are equal. The supply of gas is then disconnected and as the subject breathes room air the marker gas is washed out from the lungs in the same way as nitrogen is during the nitrogen washout. This approach relies on the availability of an inert marker gas, and the two gases that have been used in previous studies are helium and SF_6, although only data from SF_6 washouts have been reported.[1,11–13]

The advantages of using a mass spectrometer to follow SF_6 concentrations are that it offers a stable gas signal, with a rapid analyser response time.[1] This has been crucial in the development of this technology in the assessment of very young subjects, including infants.[2] The mass spectrometer also offers the possibility of measuring more than one gas, so that simultaneous washouts of gas species with different diffusion coefficients (helium and SF_6) can be performed in order to explore the effects of diffusion on gas mixing.[33,34]

Mass spectrometers however are costly and temperamental devices, and a separate supply of tracer gas is required (unlike the nitrogen washout system which can use the hospitals' piped oxygen). An alternative photoacoustic gas analyser, known as Innocor™, has also been employed to measure LCI in washouts from 0.2% SF_6.[3] This has the advantages of being considerably more compact and robust than the mass spectrometer, but cannot measure the same range of different gases and has a slower analyser response time which may limit its use to school age children and older. Innocor also requires modification and custom-built software in order to use it in this way[3] (see Fig. 2).

A third system has also been used, based upon an ultrasonic flowmeter to measure both flow and molar mass (a measure of gas density) (ndd medizintechnik, Berne, Switzerland).[35] This is used in the mainstream position, which reduces apparatus deadspace and response time (both important for use in very young subjects with low tidal volumes and high respiratory rates). However the gas density is also affected by heat, moisture and CO_2 content of the gas sample, so complex experimentally derived algorithms are required to correct for this, and dynamic

Figure 2 Subject performing a washout. The supply of wash-in gas (0.2% SF_6 in air) is provided by the cylinder in the background. An Innocor™ gas analyser is used to measure flow and SF_6 concentration and expiratory volume is displayed to the subject on a separate screen.

Please cite this article in press as: Horsley A, Lung clearance index in the assessment of airways disease, Respiratory Medicine (2009), doi:10.1016/j.rmed.2009.01.025

change in these variables remains difficult to correct for.[36,37]

At the moment, MBW tests remain restricted to a small number of laboratories, where they are used primarily as research tools. However, the simplicity and reproducibility of the technique make it especially useful for long-term follow-up in children and a few units have successfully integrated these measurements into their annual assessments of CF lung function.[15,38] In addition, LCI is now also being worked up for use as a possible endpoint in trials of CF gene therapy.[5] There is now considerable interest in using MBW to assess airways function, and progression, in a range of other diseases.

Recognising the importance of clear guidelines in assisting development of this technology, the European Respiratory Society and American Thoracic Society are currently preparing guidelines on the performance and analysis of multiple breath washouts. It is hoped that this will standardize the procedures used in different units. Clear guidance should also permit and encourage manufacturers to develop commercial apparatus for the measurement of LCI. If this happens, it will allow units without specialist technical and computing support to access the technology, and should further facilitate progress in this emergent field.

Acknowledgements

The contribution of Dr Alastair Innes and Dr Kenny Macleod, for their ongoing input and discussions, as well as valuable feedback on this review, is gratefully acknowledged.

Competing interests

None.

Funding

The author's research has been funded by the Cystic Fibrosis Trust Gene Therapy Consortium.

References

1. Aurora P, Kozlowska W, Stocks J. Gas mixing efficiency from birth to adulthood measured by multiple-breath washout. *Respir Physiol Neurobiol* 2005;**148**(1—2):125—139.
2. Lum S, Gustafsson P, Ljungberg H, Hulskamp G, Bush A, Carr SB, et al. Early detection of cystic fibrosis lung disease: multiple-breath washout versus raised volume tests. *Thorax* 2007;**62**(4):341—347.
3. Horsley AR, Gustafsson PM, Macleod K, Saunders CJ, Greening AP, Porteous D, et al. Lung clearance index is a sensitive, repeatable and practical measure of airways disease in adults with cystic fibrosis. *Thorax* 2008;**63**:135—140.
4. Rosenfeld M. An overview of endpoints for cystic fibrosis clinical trials: one size does not fit all. *Proc Am Thorac Soc* 2007; **4**(4):299—301.
5. Davies JC, Cunningham S, Alton EW, Innes JA. Lung clearance index in CF: a sensitive marker of lung disease severity. *Thorax* 2008;**63**(2):96—97.
6. Becklake MR. A new index of the intrapulmonary mixture of inspired air. *Thorax* 1952;**7**(1):111—116.
7. Mentore K, Froh DK, de Lange EE, Brookeman JR, Paget-Brown AO, Altes TA. Hyperpolarized HHe 3 MRI of the lung in cystic fibrosis: assessment at baseline and after bronchodilator and airway clearance treatment. *Acad Radiol* 2005;**12**(11): 1423—1429.
8. Saniie J, Saidel GM, Chester EH. Real-time moment analysis of pulmonary nitrogen washout. *J Appl Physiol* 1979;**46**(6): 1184—1190.
9. Fleming GM, Chester EH, Saniie J, Saidel GM. Ventilation inhomogeneity using multibreath nitrogen washout: comparison of moment ratios and other indexes. *Am Rev Respir Dis* 1980;**121**(5):789—794.
10. Lutchen KR, Habib RH, Dorkin HL, Wall MA. Respiratory impedance and multibreath N_2 washout in healthy, asthmatic, and cystic fibrosis subjects. *J Appl Physiol* 1990;**68**(5): 2139—2149.
11. Gustafsson PM, Aurora P, Lindblad A. Evaluation of ventilation maldistribution as an early indicator of lung disease in children with cystic fibrosis. *Eur Respir J* 2003;**22**(6):972—979.
12. Aurora P, Gustafsson P, Bush A, Lindblad A, Oliver C, Wallis CE, et al. Multiple breath inert gas washout as a measure of ventilation distribution in children with cystic fibrosis. *Thorax* 2004;**59**(12):1068—1073.
13. Aurora P, Bush A, Gustafsson P, Oliver C, Wallis C, Price J, et al. Multiple-breath washout as a marker of lung disease in preschool children with cystic fibrosis. *Am J Respir Crit Care Med* 2005;**171**(3):249—256.
14. Schaedel C, de Monestrol I, Hjelte L, Johannesson M, Kornfalt R, Lindblad A, et al. Predictors of deterioration of lung function in cystic fibrosis. *Pediatr Pulmonol* 2002;**33**(6): 483—491.
15. Kraemer R, Blum A, Schibler A, Ammann RA, Gallati S. Ventilation inhomogeneities in relation to standard lung function in patients with cystic fibrosis. *Am J Respir Crit Care Med* 2005; **171**(4):371—378.
16. Kraemer R, Delosea N, Ballinari P, Gallati S, Crameri R. Effect of allergic bronchopulmonary aspergillosis on lung function in children with cystic fibrosis. *Am J Respir Crit Care Med* 2006; **174**(11):1211—1220.
17. Brownlee KG. Small airways disease in cystic fibrosis. *Eur Respir Momgraph* 2006;**35**:21—37.
18. Gustafsson PM, De Jong PA, Tiddens HA, Lindblad A. Multiple-breath inert gas washout and spirometry versus structural lung disease in cystic fibrosis. *Thorax* 2008;**63**(2):129—134.
19. Kiley J, Smith R, Noel P. Asthma phenotypes. *Curr Opin Pulm Med* 2007;**13**(1):19—23.
20. van den Toorn LM, Overbeek SE, de Jongste JC, Leman K, Hoogsteden HC, Prins JB. Airway inflammation is present during clinical remission of atopic asthma. *Am J Respir Crit Care Med* 2001;**164**(11):2107—2113.
21. Gustafsson PM. Peripheral airway involvement in CF and asthma compared by inert gas washout. *Pediatr Pulmonol* 2007;**42**(2):168—176.
22. Macklem PT, Mead J. Resistance of central and peripheral airways measured by a retrograde catheter. *J Appl Physiol* 1967;**22**(3):395—401.
23. Brody AS, Klein JS, Molina PL, Quan J, Bean JA, Wilmott RW. High-resolution computed tomography in young patients with cystic fibrosis: distribution of abnormalities and correlation with pulmonary function tests. *J Pediatr* 2004;**145**(1): 32—38.
24. Bedrossian CW, Greenberg SD, Singer DB, Hansen JJ, Rosenberg HS. The lung in cystic fibrosis. A quantitative study including prevalence of pathologic findings among different age groups. *Hum Pathol* 1976;**7**(2):195—204.
25. Timonen KL, Randell JT, Salonen RO, Pekkanen J. Short-term variations in oscillatory and spirometric lung function indices among school children. *Eur Respir J* 1997;**10**(1):82—87.

Please cite this article in press as: Horsley A, Lung clearance index in the assessment of airways disease, Respiratory Medicine (2009), doi:10.1016/j.rmed.2009.01.025

26. Hankinson JL, Stocks J, Peslin R. Reproducibility of lung volume measurements. *Eur Respir J* 1998;**11**(3):787—790.
27. Quanjer PH, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC. Lung volumes and forced ventilatory flows. Report working party standardization of lung function tests, European community for steel and coal. Official Statement of the European Respiratory Society. *Eur Respir J Suppl* 1993;**16**:5—40.
28. Rosenthal M, Bain SH, Cramer D, Helms P, Denison D, Bush A, et al. Lung function in white children aged 4 to 19 years: I — spirometry. *Thorax* 1993;**48**(8):794—802.
29. Merkus PJ, Tiddens HA, de Jongste JC. Annual lung function changes in young patients with chronic lung disease. *Eur Respir J* 2002;**19**(5):886—891.
30. Schmalisch G, Proquitte H, Roehr CC, Wauer RR. The effect of changing ventilator settings on indices of ventilation inhomogeneity in small ventilated lungs. *BMC Pulm Med* 2006;**6**:20.
31. Crawford AB, Makowska M, Paiva M, Engel LA. Convection- and diffusion-dependent ventilation maldistribution in normal subjects. *J Appl Physiol* 1985;**59**(3):838—846.
32. Wanger J, Clausen JL, Coates A, Pedersen OF, Brusasco V, Burgos F, et al. Standardisation of the measurement of lung volumes. *Eur Respir J* 2005;**26**(3):511—522.
33. Gronkvist MJ, Emery MJ, Gustafsson PM. Mechanisms of ventilation inhomogeneity during vital capacity breaths standing and supine. *Respir Physiol* 2002;**129**(3):345—355.
34. von Nieding G, Lollgen H, Smidt U, Linde H. Simultaneous washout of helium and sulfur hexafluoride in healthy subjects and patients with chronic bronchitis, bronchial asthma, and emphysema. *Am Rev Respir Dis* 1977;**116**(4):649—660.
35. Schibler A, Hall GL, Businger F, Reinmann B, Wildhaber JH, Cernelc M, et al. Measurement of lung volume and ventilation distribution with an ultrasonic flow meter in healthy infants. *Eur Respir J* 2002;**20**(4):912—918.
36. Buess C, Pietsch P, Guggenbuhl W, Koller EA. A pulsed diagonal-beam ultrasonic airflow meter. *J Appl Physiol* 1986;**61**(3): 1195—1199.
37. Latzin P, Sauteur L, Thamrin C, Schibler A, Baldwin D, Hutten GJ, et al. Optimized temperature and deadspace correction improve analysis of multiple breath washout measurements by ultrasonic flowmeter in infants. *Pediatr Pulmonol* 2007;**42**(10):888—897.
38. Gustafsson PM, de Jong PA, Tiddens HA, Lindblad A. Multiple-breath inert gas washout and spirometry vs. structural lung disease in cystic fibrosis. *Thorax*; 2007.