

**EXPLORATION OF THE RELATIONSHIP BETWEEN
HYPOXIA AND MEASURES OF CLINICAL STATUS AND
INFLAMMATION IN CHILDREN WITH CYSTIC FIBROSIS**

By

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Declaration

I, Donald Simon Urquhart have performed all the clinical examinations and undertaken all measurements for exercise tests in the subjects recruited for this thesis along with one of the cardiac physiologists. Collection of blood and exhaled breath condensate were entirely undertaken by me.

Skeletal muscle testing (quadriceps and handgrip strength testing) were entirely undertaken by me. Respiratory muscle testing was performed jointly with Emma Scrase, Respiratory Physiologist, Great Ormond Street Hospital.

Spirometry was carried out by an experienced Respiratory Physiologist (Emma Scrase, John Rae, Cara Oliver).

Oximeters were sent to subjects and received by me. Analysis of sleep data was carried out jointly by Aidan Laverty (Respiratory Physiologist) and I.

Echocardiography studies were performed by experienced cardiac sonographers (Gill Riley, Katie Maslin, Anna Barlow) in the Department of Cardiology, Great Ormond Street Hospital.

Bone mineral density measurements were undertaken by Jane Williams.

I conducted all the CFQ questionnaires, although scoring of these was carried out by Dr Bryony Field (Psychology Assistant, Great Ormond Street Hospital).

C-Reactive Protein levels were measured in the Department of Chemical Pathology, and full blood count and erythrocyte sedimentation rate in the Department of Haematology, Great Ormond Street Hospital. DNA extraction and Angiotensin Converting Enzyme genotyping were carried out in conjunction with Dr Christina Hubbart (Research Assistant, Department of Cardiovascular Genetics, Rayne Institute, and University College London). All other laboratory analyses (ELISA, Luminex) were carried out by myself. I received some help from Dr Aris Tagalakakis (Department of Gene Therapy, Institute of Child Health, London) with day-to-day maintenance of cells, and cell counting experiments.

Following consultations with Dr Indra Narang, Consultant Respiratory Paediatrician, Great Ormond Street Hospital, and Ms Sanja Stanojevic, PhD student in Epidemiology, Portex Unit, Institute of Child Health, London, I alone performed all the statistical analyses in this thesis.

This work has not been accepted in any previous application for a degree.

DS Urquhart
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Abstract

Hypoxia in cystic fibrosis (CF) may occur during sleep, and also during exercise, chest exacerbations and air travel. No standardised definition of nocturnal hypoxia in CF exists. Theoretical evidence suggests hypoxia may have a deleterious impact on clinical status in CF, due to effects on upregulation of pro-inflammatory cytokines, changes in *Pseudomonas aeruginosa* growth patterns, and causation of pulmonary hypertension. It was hypothesised that hypoxia, and resultant inflammation would adversely affect clinical phenotype in CF.

Forty-one children with CF were studied, each undergoing home oximetry before attending for a day of clinical testing (exercise testing, lung function, respiratory and skeletal muscle testing, echocardiography, and quality of life assessment). *In vitro* work was undertaken to assess the effects of hypoxia on cell growth and interleukin-8 (IL-8) secretion in wild-type and CF airway epithelial cells. The effects of hypoxia were compared to a known pro-inflammatory stimulus - lipopolysaccharide (LPS) from *Pseudomonas aeruginosa*.

ROC statistics were used to derive the most sensitive and specific definition of sleep hypoxia in the detection of elevated levels of inflammation (WBC, CRP, neutrophil counts and IL-8 levels). This definition (SpO₂ <93% for >10% sleep) was used to dichotomise the study population. Hypoxic CF subjects (n=9) had, when compared to normoxic controls (n=32): lower exercise capacity, lower BMI, lower FEV₁ and FVC, elevated RV/TLC ratio, and higher Chrispin-Norman scores. Hypoxic subjects also had reduced quality of life, bone density, and increased RV thickness on echocardiogram. Hypoxic cell culture was suggested to be pro-inflammatory, with increased IL-8 production, and synergistically increased IL-8 secretion when cells were co-incubated with LPS under hypoxic conditions.

Hypoxia is associated with reduced clinical well-being and increased inflammation in childhood CF. The paradigm exists of whether hypoxia is merely an endpoint of severe CF lung disease; or whether hypoxia may be a causative factor (as suggested by the *in vitro* work), as well as an effect of CF lung inflammation. A trial of restoration of normoxia in children with CF, with careful re-evaluation of clinically-relevant outcomes is suggested from this preliminary work.

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List of abbreviations:

ACE – Angiotensin converting enzyme
AHA – American Heart Association
ANOVA – Analysis of variance
ASL – Airway surface liquid
ATP – Adenosine triphosphate
ATS – American Thoracic Society
BAL – Bronchoalveolar lavage
BMAD – Bone mineral apparent density
BMC – Bone mineral content
BMD – Bone mineral density
BMI – Body mass index
BTS – British Thoracic Society
CDC – Center for Disease Control
CF – Cystic Fibrosis
CFBE41₀⁻ (or CFBE) – Cystic fibrosis bronchial epithelial cells
CFQ-UK – Cystic Fibrosis Questionnaire (UK version)
CFTR – Cystic Fibrosis transmembrane conductance regulator
 χ^2 – Chi-squared
CHoP – Children’s Hospital of Philadelphia
CI – Confidence intervals
CNS – Crispin Norman chest radiograph) Score
COPD – Chronic obstructive pulmonary disease
CPET – Cardiopulmonary exercise testing
CRP - C-reactive protein
 Δ F508 – Phenylalanine substitution at the 508 position on chromosome 7
DNA – Deoxyribonucleic acid
DXA – Dual energy absorptiometry
EBC – Exhaled breath condensate
ECG – Electrocardiogram
EDTA – Ethylenediaminetetraacetic acid
EEG – Electroencephalogram
EIAH – Exercise-induced arterial hypoxia
ELISA – Enzyme-linked immunosorbent assay
EPAP – Expiratory positive airways pressure
ER – Endoplasmic reticulum
ERS – European Respiratory Society
ESR – Erythrocyte sedimentation rate
F – Female
FEF₅₀ – Forced expiratory flow at 50% lung volume
FEF₇₅ – Forced expiratory flow at 75% lung volume
FEV₁ – Forced expiratory volume in 1 second
FiO₂ – Concentration of inspired oxygen
FRC – Functional residual capacity
FVC – Forced vital capacity
GM-CSF – Granulocyte and macrophage colony stimulating factor
GOSH – Great Ormond Street Hospital
1HAE₀⁻ (or HAE) - Human airway epithelial cells

HAPE – High altitude pulmonary oedema
 Heliox₂₁TM – Air-helium gas mixture
 HRP – Horseradish peroxidase
 hsCRP – High-sensitivity CRP
 HVR – Hypoxic ventilatory responses
 I/D polymorphism – Insertion-deletion polymorphism within the Angiotensin Converting Enzyme (ACE gene)
 IL-1 β – Interleukin 1 beta
 IL-4 – Interleukin 4, etc.
 IFN- γ – Interferon gamma
 IPAP – Inspiratory positive airways pressure
 IQR – Interquartile range
 IVS_d – Interventricular septal wall diameter (measured in diastole)
 LPS – Lipopolysaccharide
 LTOT – Long-term oxygen therapy
 LV – Left ventricle
 LVD_d – Left ventricle dimensions (measured in diastole)
 LVD_s – Left ventricle dimensions (measured in systole)
 LVPW_d – Left ventricular posterior wall diameter (measured in diastole)
 M – Male
 MBL – Mannose-binding lectin
 MD – Doctor of Medicine
 MEP – Mouth expiratory pressure
 MIP – Mouth inspiratory pressure
 MIVC – Maximal isometric voluntary contraction (of quadriceps muscle)
 mRNA – Mitochondrial ribonucleic acid
 MRSA – Methicillin-resistant *Staphylococcus aureus*
 NF κ B – Nuclear factor kappa B
 Non-REM – Non-rapid eye movement sleep
 NSAIDs – Non-steroidal anti-inflammatory drugs
 OSA – Obstructive sleep apnoea
 PA – *Pseudomonas aeruginosa*
 PAMP – Pattern-associated molecular protein
 P_AO₂ – Alveolar partial pressure of oxygen
 PaO₂ – Arterial partial pressure of oxygen
 PAP – Pulmonary artery pressure
 PCR – Polymerase chain reaction
 PEF – Peak expiratory flow
 PenStrep – Mixture of procaine penicillin and streptomycin
 P_{mus} – Muscle pressure
 PRR – Pattern recognition receptor
 PSG – Polysomnography
 PVR – Pulmonary vascular resistance
 QOL – Quality of life
 r² – Correlation coefficient
 RA – Right atrium
 REC – Research Ethics Committee
 REM sleep – Rapid eye movement sleep

RER – Respiratory exchange ratio
ROC – Receiver-operator curve
RPE – R-Phycoerythrin
RV – Residual volume
RV – Right ventricle
RVW_d – Right ventricular wall diameter (measured in diastole)
SA – *Staphylococcus aureus*
SaO₂ – Arterial oxygen saturation
SpO₂ – Oxygen saturation measured by pulse oximetry
SAT₁₀ – Oxygen saturation at which 10% night spent below
sd – Standard deviation
SDS (or z score) – Standard deviation score
SnIP – Sniff nasal inspiratory pressure
sPAP – Systolic pulmonary artery pressure
SPSS – Statistical package for the social sciences
TIVAD – Total implantable venous access device
TLC – Total lung capacity
TLR2 – Toll-like receptor 2
TLR4 – Toll-like receptor 4
TMB – Tetramethylbenzidine
TNF- α – Tumour necrosis factor alpha
TR – Tricuspid regurgitation
UK – United Kingdom
V_E – Minute ventilation
V_Emax – Maximal minute ventilation
V_T – Tidal volume
V_E/VCO₂ – Ventilatory equivalent for carbon dioxide
V_E/VO₂ – Ventilatory equivalent for oxygen
VCO₂ – Elimination of carbon dioxide
VO₂ – Oxygen uptake
VO₂max – Maximal oxygen uptake
VO₂peak – Peak oxygen uptake
V/Q – Ventilation-perfusion
WBC – White blood cell count

Definitions used in this study

- Nocturnal hypoxia in CF – SpO₂ <93% for >10% study
- Normal ranges for lung function and plethysmography data – z scores constructed from:

Rosenthal M, Bain SH, Cramer D, Helms P, Denison D, Bush A, Warner JO. Lung function in white children aged 4 to 19 years: – Spirometry. *Thorax* 1993; **48**: 794-802.

- Normal ranges for respiratory muscle pressure data – z scores constructed from:

Stefanutti D, Fitting JW. Sniff inspiratory muscle pressure. Reference values in Caucasian children. *Am J Respir Crit Care Med* 1999; **159**: 107-111.

- Normal ranges for skeletal muscle pressure data – z scores constructed from:

QUADRICEPS –

Backman E, Odenrick P, Henriksson KG, Ledin T. Isometric muscle force and anthropometric values in normal children aged between 3.5 and 15 years. *Scand J Rehab Med* 1989; **21**: 105-114.

HANDGRIP STRENGTH –

Mathiowetz V, Wiener DM, Federman SM. Grip and pinch strength: Norms for 6- 19 year olds. *Am J Occup Ther* 1986; **40**: 705-11.

- Pulmonary hypertension

Defined as sPAP>30mmHg where sPAP is measured from Bernoulli equation (section 2.2.2.7) and added to right atrial pressure.

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CHAPTER 1: Introduction

1.1 Research Question

The aim of this thesis was to define a hypoxic phenotype amongst children with cystic fibrosis. The principal hypothesis of the study is that, hypoxia via up-regulation of inflammation, may exert a deleterious effect on clinical status in children with CF. The potential mechanisms by which hypoxia may stimulate inflammation are presented throughout Chapter 1, along with discussion of the potential impact of inflammation driven by hypoxia on the CF lung.

Using direct measures of inflammation, the work sets out to establish an optimal definition of hypoxia to apply to the group (Chapter 3); and then to dichotomise the group on the basis of hypoxia to examine the association with clinical (Chapters 4 and 5) variables.

Chapter 6 tests the hypothesis that hypoxia may directly upregulate inflammation in CF. The effects of hypoxia on inflammation in both CF and non-CF cell lines are explored, and compared and contrasted to a known stimulus of inflammation, namely lipopolysaccharide (LPS) from *Pseudomonas aeruginosa*.

The study hypothesises that hypoxia may directly and also indirectly propagate lung inflammation, which, in turn may potentiate a downward pathophysiological spiral of worsening disease in CF. The hypothesis is that those children defined as having significant hypoxia will be associated with adverse measures of clinical status.

1.2 Cystic Fibrosis

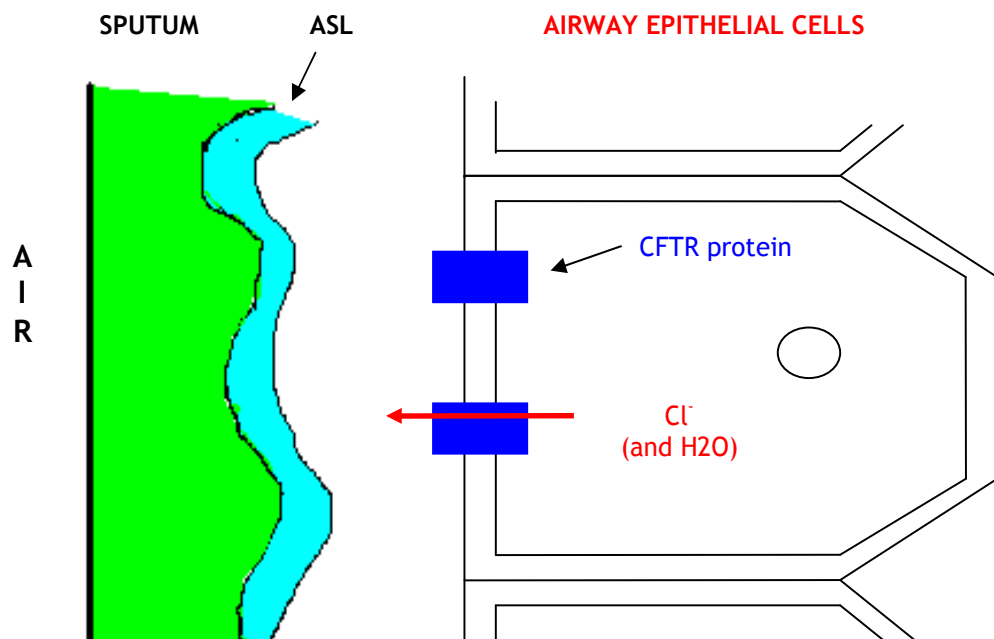
Cystic Fibrosis (CF) is the UK's commonest life-threatening inherited disease, affecting over 7500 children and young adults. Average life expectancy is quoted as 35 years (www.cftrust.org.uk) but predicted median age of survival for a baby born in the 21st century exceeds 50 years (Dodge *et al.* 2007). CF is a multisystem disorder that affects the upper and lower airways, pancreas, bowel and reproductive tracts (Davies *et al.* 2007), as well as having later endocrine (Lanng 2001) and bone effects (Aris *et al.* 2005, UK CF Trust 2007).

1.2.1 CF genetics

CF is an autosomal recessive condition, caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene on chromosome 7. This gene codes for CFTR protein, a chloride channel at the epithelial cell surface which allows co-transport of sodium and chloride along with water across the cell membrane (Figure 1.1).

Figure 1.1

Normal CFTR function



ASL = Airway surface liquid

CFTR protein = Cystic Fibrosis transmembrane conductance regulator protein

1.3 Hypoxia

1.3.1 Hypoxia and cellular respiration

Aerobic cellular respiration is dependent upon the efficient supply of oxygen substrate to the mitochondria. The efficiency of oxygen delivery is dependent upon the interaction between cardiac output, ventilatory responses and metabolic factors. Cellular respiration leads to energy production (in the form of ATP). This is at its most efficient under aerobic conditions, whereby 38 molecules of ATP are generated for every molecule of glucose. Adequate cardiorespiratory response is relied upon to supply oxygen for aerobic ATP regeneration (Wasserman *et al.* 2004). Under anaerobic conditions, only 2 ATP molecules per molecule of glucose are produced.

Air, with 21% inspired oxygen concentration is moved by convection through the airways to reach the alveoli, whence oxygen diffuses out of the alveoli and into the pulmonary capillaries. It binds to haemoglobin to form oxyhaemoglobin and is then transported to the tissues, where it diffuses out of the microcirculation, across the interstitium and cell membranes and into the cell, where it finally enters the mitochondria (Nathan and Singer 1999). The impact of hypoxaemia therefore is that less oxygen is available at cellular level for aerobic metabolism leading to a shift to anaerobic energy production, which utilises glycolysis – the catabolism of glucose.

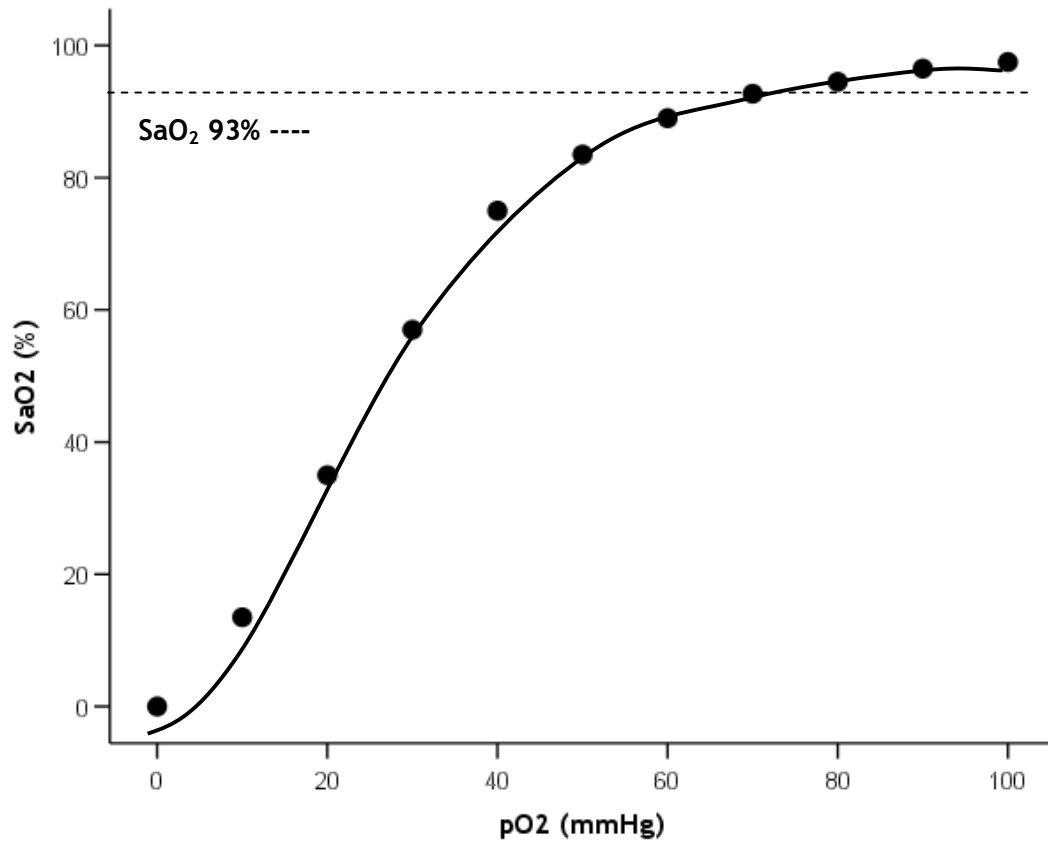
1.3.2 Defining hypoxia

The state of hypoxia can be described on the basis of arterial oxygen tensions below 8kPa (Mallory *et al.* 2005), or on the basis of oxyhaemoglobin desaturation such that arterial oxygen saturation (SaO_2) are below 90% (Mallory *et al.* 2005). Others have suggested higher cut off values for SaO_2 and hypoxia, for example SaO_2 of 93% is the point on the oxygen dissociation curve at which small changes in pO_2 result in exponential decreases in SaO_2 . Extrapolation from the following graph (Figure 1.2) suggests that a SaO_2 value of 93% equates to a pO_2 of around 70mmHg (9kPa).

Figure 1.2

The oxygen-haemoglobin dissociation curve

[Source: Ganong WF, Review of Medical Physiology (14th Edition), 1989]



Pulse oximetry provides the main assessment tool for oxygenation in children. A source of light originates from the oximeter probe at two wavelengths (665nm –red and 880nm -infrared). The light is partly absorbed by haemoglobin (Hb), by amounts which differ depending on whether it is saturated or desaturated with oxygen. By calculating light absorption at the two wavelengths the processor can compute the proportion of Hb which is oxygenated. Although freely available, and a non-invasive method of assessing oxygenation, correlation between arterial oxygen saturations measured on pulse oximetry (SpO₂) and arterial PaO₂ may be poor (Soubani 2001) and limits of accuracy for SpO₂ are +/-2% (Soubani 2001).

1.3.3 Pathophysiology of alveolar hypoxia

Hypoxia is a state of low oxygen levels, and alveolar hypoxia is a reflection of alveolar hypoventilation (West 2005). Alveolar PO_2 (P_{AO_2}) is determined by two factors – i) the rate of removal of O_2 by pulmonary capillary blood flow, and ii) breath-by-breath replenishment by alveolar ventilation (West 2005). The rate of removal of oxygen depends on oxygen uptake (VO_2) of the tissues, so is relatively constant at rest but significantly increases when VO_2 is increased, for example during exercise.

The lung is the key link in the chain of oxygen transport from air to the tissues. Having been taken up by the pulmonary capillaries and distributed by systemic arterial blood, oxygen diffuses to mitochondria at tissue level to facilitate aerobic cellular metabolism. Therefore, any reduction in arterial pO_2 (PaO_2) must result in a decrease in pO_2 at tissue level. For this reason, changes in SpO_2 are likely to reflect changes in tissue pO_2 .

1.3.4 Pathophysiology of hypoxaemia

Hypoxaemia refers to the state of an abnormally low PaO_2 due to impaired gas exchange.

There are four potential causes of hypoxaemia (West 2005), namely:

- Alveolar hypoventilation
- Shunt
- Diffusion
- V/Q mismatching

1.3.4.1 Alveolar hypoventilation and hypoxaemia

P_{AO_2} depends upon rate of removal of oxygen by the bloodstream (a rate governed by metabolic demand), and the rate of replenishment of oxygen by alveolar ventilation. If alveolar ventilation reduces, an ensuing fall in P_{AO_2} as well as a rise in alveolar pCO_2 (P_ACO_2) will occur. The alveolar gas equation (below) is used to calculate the corresponding rise in P_ACO_2 that accompanies any fall in P_{AO_2} :

$$P_{AO_2} = P_{iO_2} - \frac{P_ACO_2}{R} + F$$

where P_{iO_2} refers to inspired oxygen concentration, R is the respiratory exchange ratio - the ratio of CO_2 elimination (VCO_2) to oxygen uptake (VO_2), and F is a small correction factor.

Thus, alveolar hypoventilation will always result in hypoxaemia unless inspired oxygen concentrations are enriched.

Alveolar hypoventilation can be central in origin, relating to respiratory control and drive to breathe. Such causes can be primary, for example congenital central hypoventilation syndrome. Secondary causes of central hypoventilation include structural changes affecting brainstem function (i.e. Arnold-Chiari malformations), obesity and as a result of drug side-effects (e.g. respiratory depression due to opiates). Alveolar hypoventilation may also occur as a result of ventilatory muscle weakness or paralysis; or due to increases in airway resistance, for example in severe obstructive sleep apnoea (OSA) (Marcus 2001).

1.3.4.2 Hypoxaemia and shunting

Shunting refers to blood that enters the arterial system without going through ventilated areas of the lung (West 2005). In the healthy lung, this includes bronchial arterial blood that is collected by the pulmonary veins after it has perfused its bronchi and is thus partly depleted of O₂. The effect of adding poorly oxygenated blood depresses PaO₂. Some patients have abnormal vascular connections within the lung (pulmonary arteriovenous malformations), whilst in those with cardiac disease a direct addition of venous blood to arterial blood may occur across a right to left shunt through an intracardiac defect at ventricular or atrial level.

1.3.4.3 Hypoxaemia and impaired diffusion

As blood traverses the pulmonary capillary, PaO₂ approaches that of the P_AO₂. The alveolar-arterial pO₂ differences in the healthy lung at rest are small. During exercise, however, pulmonary blood flow is markedly increased, so that the time spent by a red cell within the pulmonary capillary is reduced to 1/3 of the resting time, meaning that the time available for oxygenation is less (West 2005). If the blood-gas barrier is thickened by disease so that oxygen diffusion is impeded, then the rate of rise of PO₂ in the red blood cells is slower, and PaO₂ may not reach that of P_AO₂ before the time available for oxygenation has run out.

1.3.4.4 Hypoxaemia and ventilation/perfusion (V/Q) mismatching

A normal V/Q ratio can be altered by changing ventilation or by changing perfusion. For example if alveolar ventilation is abolished then the V/Q ratio would be zero, whereas a complete interruption in perfusion would lead the ratio to tend towards infinity. Regional differences in gas exchange exist such that the V/Q ratio is higher at the top of the lung where blood flow is minimal, and much lower at the bottom of the lung (West 2005).

This may be important during exercise in those with a large dead space, where shallow rapid respirations may accentuate these regional V/Q mismatches and promote hypoxaemia. During infections and pneumonia, inflamed areas of lung parenchyma may have increased blood flow, yet impaired ventilation and resultant hypoxaemia may ensue.

1.3.5 Hypoxia in Cystic Fibrosis

Episodic hypoxaemia may occur at times of physiologic stress in CF, such as sleep (Bradley *et al.* 1999, Darracott *et al.* 2004), exercise (Narang *et al.* 2003), during infective exacerbations of CF, and with air travel (Buchdahl *et al.* 2001).

During sleep, minute ventilation decreases and may cause a degree of hypoventilation. Furthermore, V/Q mismatch may be accentuated by differences in regional gas exchange during sleep. Finally, obstructive hypoventilation can contribute to hypoxaemia for example, as a result of nasal polyps. This will be discussed in more detail in section 1.3.5.2.

During exercise, as a result of enlarged physiological dead space; the pattern of shallow, rapid respirations required to maintain alveolar ventilation may cause V/Q mismatch (Godfrey and Mearns 1971). Gas exchange in the upper parts of the lung is less efficient than lower regions, and this coupled with the increased blood flow on exercise that may alter diffusion kinetics, may result in hypoxaemia. This will be discussed in more detail in section 1.3.5.3.

In a CF chest exacerbation, hypoxaemia is likely to result primarily from V/Q mismatching. For example areas of lung inflammation may impair ventilation, yet blood flow to this inflamed tissue is increased accentuating mismatch. This will be discussed in more detail in section 1.3.5.4.

During commercial air travel, both barometric pressure and partial pressure of oxygen fall with altitude. This means that the passenger is breathing air with an inspired oxygen concentration (F_{iO_2}) of 15%. This alteration in inspired oxygen concentration thus affects the P_{AO_2} and results in hypoxaemia. This will be discussed in more detail in section 1.3.5.5.

1.3.5.1 Resting daytime hypoxia in CF

Studies have demonstrated significantly lower mean resting SpO_2 in children with CF when compared to controls (Betancourt *et al.* 1991). Resting hypoxia in CF has been described as awake SpO_2 less than 95% (Coffey *et al.* 1991).

1.3.5.2 Sleep hypoxia in CF

Overnight SpO_2 have been shown to be lower in stable adult (Bradley *et al.* 1999) and childhood (Darracott *et al.* 2004) CF subjects when compared to controls. Additionally, an increased incidence of desaturation events during sleep has been noted, even in those with only mildly reduced lung function (Uyan *et al.* 2007). During sleep in those with already reduced vital capacity, tidal volume falls due to reduced respiratory drive, precipitating hypoxia (Bradley *et al.* 1999) as a result of the mechanisms of alveolar hypoventilation (section 1.3.4.1) as well as accentuating regional differences in gas exchange and V/Q mismatching (section 1.3.4.4). Upper airway obstructive pathology such as nasal polyps or OSA may additionally contribute.

1.3.5.2.1 Normal sleep

Sleep is a requirement of all animals although its function remains unknown. Those who are deprived of sleep suffer physically, emotionally and intellectually, yet nobody knows how sleep restores the brain.

1.3.5.2.1.1 Sleep Patterns

There are two different kinds of sleep: Rapid eye movement or REM sleep and non-REM or slow-wave sleep. The proportion spent in REM sleep is proportionally greater in children than in adults, such that a neonate may spend 2/3 sleep time in REM compared with 20-25% in adults (Marcus 2001).

1.3.5.2.1.1.1 Non-REM (Slow-wave) sleep

Non-REM sleep is divided into four stages. These stages can be formal assessment of brain activity using electroencephalography (EEG):

Stage 1 – The stage of sleep which a person first enters on falling asleep. This stage is characterized by low-amplitude, fast frequency EEG activity.

Stage 2 – Sleep spindles appear in stage 2. These are bursts of electrical activity occurring at a frequency of 10-14 per second. They are similar in appearance to the alpha waves seen in awake individuals at rest with eyes closed.

Stage 3 – The EEG pattern becomes slower in frequency and increased in amplitude.

Stage 4 – The EEG is maximally slowed in stage 4, and deep sleep is characterized by a pattern of rhythmic slow waves.

1.3.5.2.1.1.2 REM Sleep

During REM sleep, the EEG pattern becomes irregular with rapid, low-voltage activity as well as rapid, roving movements of the eyes. REM sleep is not however interrupted, and the threshold for arousal is much higher than in non-REM sleep. Skeletal muscle tone falls during REM sleep and contributes to changes in respiration during sleep. This occurs due to increased activity in the reticular inhibiting system in the medulla.

A typical night of sleep would involve passing through stage 1 and then stage 2 sleep, followed by a longer period in stages 3 and 4 sleep. A REM period will follow this and the cycle will be repeated at regular intervals through the night such that a total of 4-6 periods of REM will ensue (Ganong 1989). It should be noted that babies will sleep for longer periods than adults, as well as having proportionally longer periods of REM sleep (Marcus 2001). Additionally the periods spent in stage 3 and 4 sleep are longer in children than in young adults, and decrease with age (Ganong 1989).

1.3.5.2.1.2 Changes in respiration during sleep

During sleep, there is a decrease in minute ventilation, and in the childhood population such reductions may occur due to decreases in either tidal volume (Tabachnik *et al.* 1981), respiratory rate (Hoppenbrouwers *et al.* 1978), or both. Additionally there is an increase in upper airway resistance (Lopes *et al.* 1983), and a reduction in functional residual capacity during sleep (Hudgel and Devadatta 1984).

The ventilatory drive decreases, particularly during rapid eye movement (REM) sleep (Douglas *et al.* 1982), and in REM sleep breathing is erratic, with variable respiratory rate and tidal volume. Additionally, in REM sleep, the skeletal muscle hypotonia described above may affect the intercostal and upper airway musculature further impairing breathing. This is especially important in children, as they sleep more than adults, and have relatively more REM sleep (Marcus 2001).

1.3.5.2.1.3 Sleep-disordered breathing

Sleep-disordered breathing may be reflected by the presence of gas exchange abnormalities during sleep and/or the presence of arousals – the presence of broken sleep. Arousal is a defence mechanism against sleep-disordered breathing, as one breathes better whilst awake than when asleep. In general, children have a higher arousal threshold than adults (Marcus 2001). Numerous studies have shown that moderate hypoxemia is a poor stimulus to arousal in children with only 25-50% of subjects arousing (Marcus 2001). This is in contrast to hypercapnia and increased upper airway resistance which are both potent stimuli to arousal in all ages (Marcus *et al.* 1998).

However, hypoxia during sleep is known to be predictive of impairment in mathematic ability (Urschitz *et al.* 2005), whilst evidence exists that correction of sleep-disordered breathing can lead to improvement in cognitive functioning (Friedman *et al.* 2003) and school performance (Gozal 1998).

1.3.5.2.1.4 Normative values for oxygenation during sleep

Normative data for baseline arterial oxygen saturation (SpO₂) levels at night is available for infants (Hunt *et al.* 1999, Horemuzova *et al.* 2000), and older children (Gries *et al.* 1996, Urschitz *et al.* 2003). Urschitz and colleagues undertook home oximetry on 100 school-age children, and reported that median SpO₂ were 97%, whilst desaturations below 92% were unusual. Similar average SpO₂ values have been found in other childhood studies (Poets *et al.* 1993, Uliel *et al.* 2004). Urschitz's group further worked out measures of SAT₁₀ – SpO₂ below which children spent 10% of the artefact-free recording time. Median SAT₁₀ was 97% (IQR 97-98). In a hospital-based study of 350 patients including 180 aged 1-10 years and 46 aged 10-20 years (Gries *et al.* 1996), mean SpO₂ was 96.8% in the 1-10 year olds and 96.5% in the 10-20 year olds. Mean SAT₁₀ was 95.1% (sd 1.5) for the 1-10 year olds and 94.5% (sd 1.8) for those aged 10-20 years.

1.3.5.2.2 Defining sleep hypoxia in CF

Sleep-disordered breathing in CF was first described more than 25 years ago (Tepper *et al.* 1983). Desaturation (Tepper *et al.* 1983, Braggion *et al.* 1992), cough (Stokes *et al.* 1980), sleep fragmentation (Milross *et al.* 2002), and arousals (Spier *et al.* 1984), are all documented during sleep in CF patients. It is proposed that sleep disturbance in CF occurs due to coughing (Milross *et al.* 2004) as well as hypoxia, and that sleep fragmentation arising from these events affects daytime function and quality of life. Sleep quality (using the Pittsburgh Sleep Quality Index) has been assessed as poor in 38% CF patients. Patients with worse sleep quality had increased sleep fragmentation, and associations between poor sleep quality and reduced FEV₁ are also reported (Milross *et al.* 2002 B).

No single definition of sleep hypoxia in CF exists, and published work has used a number of methods to quantify nocturnal SpO₂ in CF. These include percentage of time spent with SpO₂ below 90% (Frangolias *et al.* 2001), minimum sleep SpO₂ (Tepper *et al.* 1983), mean sleep SpO₂ (Coffey *et al.* 1991, Milross *et al.* 2001), and lowest hourly mean SpO₂ (Versteegh *et al.* 1990). A definitive measure is yet to be determined, and e-mail survey of UK paediatric CF centres (2000 children) conducted by the University of Liverpool (Dr. K Southern – *Personal Communication*), concluded that less than a quarter of UK CF centres have a definition of nocturnal hypoxia in CF. A review of some of the definitions of sleep hypoxia that have been reported in CF is presented below (Table 1.1).

Table 1.1
Summary of nocturnal hypoxia definitions used in the literature for subjects with cystic fibrosis

	HYPOXIA DEFINITION	POPULATION TYPE	REFERENCE	COMMENTS
Specified time with low saturations:	Nocturnal hypoxia defined as SpO ₂ <90% for >5% night	Adult population (Canada)	Frangolias <i>et al.</i> 2001	
	Nocturnal hypoxia defined as >25% sleep time with SpO ₂ below 93%	Paediatric population Alder Hey, Liverpool, UK	Southern K, <i>Personal Communication</i> 2004	
	> 10 minutes of continuous sleep recording with SpO ₂ <92%	Paediatric population, Children's Hospital of Philadelphia, USA	Narang I, <i>Personal Communication</i> 2007	
Minimum sleep SpO₂	-	Patients aged 10-16 years (USA)	Tepper <i>et al.</i> 1983	Although minimum sleep SpO ₂ were reported in these studies, no definition of the nadir of desaturation required to constitute sleep hypoxia is given. The data collected were used to correlate with other clinical parameters.
	-	Adult population (Australia)	Bradley <i>et al.</i> 1999	
Measures of mean sleep SpO₂	-	Patients aged 14-39 years (Republic of Ireland)	Coffey <i>et al.</i> 1991	Although mean sleep SpO ₂ was reported, no definition of the mean sleep SpO ₂ below which hypoxia exists is given. Data collected were used to correlate with other clinical parameters.
Lowest hourly mean sleep SpO₂	Lowest hourly mean sleep SpO ₂ below 90%	Patients aged 10-22 years (Netherlands)	Versteegh <i>et al.</i> 1990	

1.3.5.2.3 Predicting sleep hypoxia in CF

1.3.5.2.3.1 Daytime SpO₂

It is routine practice in paediatric CF clinics to record resting SpO₂. Awake SpO₂ ≤ 93% is reported to indicate a high risk of nocturnal hypoxaemia in CF (Versteegh *et al.* 1990). Although highly specific, resting SpO₂ < 93% is poorly sensitive as an indicator of sleep hypoxia. A recent study reported 36% CF subjects with resting SpO₂ > 93% became hypoxic at night (Frangolias *et al.* 2001). Similarly, it is reported that, in children with significant nocturnal hypoxaemia (mean sleep SpO₂ < 90%), 19% had resting SpO₂ > 94% and therefore would have been missed without a sleep study (Milross *et al.* 2001).

1.3.5.2.3.2 FEV₁

Two studies suggest that a FEV₁ below 65% predicted may be a useful predictor of nocturnal hypoxia in CF. Firstly, Versteegh and colleagues studied 24 adolescents and young adults with CF (Versteegh *et al.* 1990). Extrapolation of their data suggests that whilst 88% sensitive, such a cut off is only 50% specific, and has a positive predictive value of 47%. Second, Frangolias and co-authors studied 70 older individuals with CF (Frangolias *et al.* 2001). Using the same cut-off (FEV₁ < 65% predicted), this was 93% sensitive and 44% specific, with a positive predictive value of 51%. Of note however, in the latter study was that even for individuals with an FEV₁ of 30% predicted, the time spent with SpO₂ < 90% varied from 0 to 100% of sleep time. These studies suggest that it may be useful in clinical practice, to perform a sleep study in all CF patients with a FEV₁ below 65% predicted.

1.3.5.2.3.3 Exercise SpO₂

It is previously reported that hypoxia in CF occurs more frequently during sleep than during exercise (Coffey *et al.* 1991, Bradley *et al.* 1999), suggesting that a sleep study may be indicated for all CF patients with exercise hypoxia. The mechanisms of hypoxia during exercise are likely to differ from those during sleep (as discussed in section 1.3.5.), and this is borne out by the reported relationships between %drop in SpO₂ during exercise and mean sleep SpO₂ (r²=0.13) or time spent with SpO₂ < 90% (r²=0.17) (Frangolias *et al.* 2001).

1.3.5.2.4 Prevalence of sleep hypoxia in CF

The lack of definition of nocturnal hypoxia hampers the description of the prevalence of CF hypoxia in clinical practice, and perhaps has led to both under-recognition and under-treatment of this potentially important clinical entity. Currently, only 1-2% UK CF patients receive long-term oxygen therapy (LTOT) at night (Balfour-Lynn *et al.* 2005, Southern 2004), and no guidelines exist on when to start LTOT in CF.

1.3.5.3 Exercise hypoxia in CF

Regional differences in oxygen uptake and ventilation exist within the normal lung with greater oxygen utilization in lower rather than upper zones (West 2005) due to increased perfusion in these areas. Subjects with CF may have an enlarged physiological dead space (V_D) (Godfrey and Mearns 1971), and ability to maintain alveolar ventilation during exercise is determined by increasing breathing frequency in the absence of an ability to increase tidal volumes (V_T). The implication of hyperventilation in subjects with enlarged V_D is that dynamic hyperinflation occurs in association with rapid, shallow respirations and the resultant V/Q mismatching that results from accentuation of regional gas exchange differences gives rise to hypoxaemia. An additional contribution to hypoxaemia is made from changes in diffusion characteristics related to increases in pulmonary blood flow and decreases in red cell transit time through the pulmonary capillaries on exercise (West 2005).

Exercise-testing protocols form part of CF annual assessment in many centres, and include measurement of SpO_2 on exercise (Pike *et al.* 2001, Narang *et al.* 2003). Various exercise testing protocols exist dependent upon the facilities and levels of expertise available in each CF centre. The gold-standard cardiopulmonary exercise test with breath-by-breath ventilatory gas analysis measures oxygen uptake (VO_2) and carbon dioxide elimination (VCO_2) as workload incrementally increases. This allows peak oxygen uptake (peak VO_2) to be measured, whilst SpO_2 can be monitored throughout the test. Such a test is of prognostic usefulness, as peak VO_2 has been shown to be an independent predictor of mortality in CF (Nixon *et al.* 1992, Pianosi *et al.* 2005).

More usual however, is that a non-incremental field test will be performed in an out-patient clinic setting. This may be a six-minute walk test i.e. distance walked in 6 minutes (Lammers *et al.* 2008), or a three-minute step test - stepping up and down on a single step for a 3 minute period (Balfour-Lynn *et al.* 1998). During each of these tests, changes in heart rate and SpO₂ are recorded, along with patient-derived measures of breathlessness using a visual analogue scale. The concern is that such tests are sub-maximal, except for those with severe CF lung disease, and as such, clinically significant potential desaturations may be missed.

Exercise hypoxia in CF is defined as a fall in SpO₂ of $\geq 4\%$ from baseline (Narang *et al.* 2003). This definition has also been used in healthy children (Nourry *et al.* 2004). Clearly, if this represents a fall in SpO₂ from 93% to 89%, one may deem this likely to represent a significant physiological drop in PaO₂, however the definition holds less well in an elite athlete whose SpO₂ fall from 100% to 96% at the end of exercise. Such falls in SpO₂ are known in elite athletes, and postulated to be due to intrapulmonary shunting, diffusion limitation, and ventilation-perfusion mismatching (Prefaut *et al.* 2000). Further work is required to establish clinically significant parameters.

1.3.5.4 Hypoxia during CF chest exacerbations

Children with CF face challenges to their pulmonary reserve at times of CF chest exacerbations. At such times, due to areas of consolidation and/or mucus plugging, ventilation-perfusion mismatching may be exaggerated, and hypoxaemia may ensue. Admission to hospital provides an opportunity for SpO₂ monitoring. In adult CF patients, it is reported that minimum SpO₂ are lower and time spent with SpO₂ <90% is greater in patients with chest exacerbations than those with stable CF (Dobbin 2005), and that by treating the chest exacerbation SpO₂ are significantly improved. This is supported by earlier work, which showed improvement in mean SpO₂ on discharge as compared with admission, as well as a correlation between mean SpO₂ and FEV₁ (Pond and Conway 1995).

1.3.5.5 In-flight hypoxia in children with CF

Flying heightens the risk of hypoxaemia in susceptible individuals, as both barometric pressure and partial pressure of oxygen fall with altitude. This means that a commercial flight passenger is breathing air with an inspired oxygen concentration (FiO_2) of 15% instead of the usual sea-level FiO_2 of 21%. Children with CF have additional pulmonary risks imposed on them when flying, namely the acquisition of respiratory virus infections due to recirculating cabin air, as well as in-flight dehydration that may dry up respiratory secretions (Webb 2001).

The main methods used to predict hypoxia in ‘fitness to fly’ assessments have been to perform a pre-flight hypoxic challenge challenge (monitoring of SpO_2 when in a FiO_2 of 15%), or to predict in-flight hypoxaemia on the basis of baseline PaO_2 or spirometric measures such as FEV_1 . Evidence (Buchdahl *et al.* 2001, Oades *et al.* 1994) is conflicting as to which method most usefully predicts in-flight desaturation in children and British Thoracic Society guidelines suggest that children with CF should undergo a pre-flight assessment which may include “hypoxic challenge testing in addition to spirometric tests” (BTS 2002). In-flight hypoxia in children with CF is defined as a fall in SpO_2 to <90%, necessitating the need for supplemental oxygen on long distance flights (BTS 2002).

1.3.5.6 Summary of methods of assessment of hypoxia in CF

No consensus on the definition of hypoxia in CF exists, nor which mechanism of hypoxia causation may be more important. Chapter 3 will consider a variety of definitions for sleep and exercise hypoxia, and, by using receiver-operator curve statistics, assess the relationships between measures of inflammation and hypoxia definitions. These data will be used to select an optimal definition of hypoxia in children with CF.

1.4 CFTR function

CFTR protein is an adenosine triphosphate (ATP)-binding protein that acts as a unidirectional solute pump. CFTR is an epithelial ion channel expressed in the apical membranes of epithelial cells lining the airways, intestines, pancreatic ducts and renal tubules (Fuller and Benos 1992). In health, CFTR regulates liquid volumes on the surface of cells, e.g. airway surface liquid (ASL), by chloride secretion and inhibition of sodium reabsorption (Figure 1.1). In the sweat glands, however, CFTR functions in the opposite direction, by facilitating chloride reabsorption.

In CF, failure of the CFTR-gated chloride channel to function is postulated to lead to depletion of liquid on the cell surface (Davies *et al.* 2007), and ensuing thickened secretions in the airways, pancreatic ducts, biliary tree, *vas deferens* and gastrointestinal tracts of individuals with CF. Furthermore, CFTR dysfunction in the sweat gland leads to chloride efflux. This forms the basis for sweat testing, the gold-standard diagnostic test for CF.

When the chloride ion cannot be transported by CFTR at the above sites, fluid secretion is insufficient, and the protein portions of the secretions may become more viscid or precipitate and cause luminal obstruction, leading to plugging and dysfunction at the organ level. This directly contributes to a number of pathological entities including mucous plugging and bacterial entrapment within the airways (due to ASL depletion), exocrine pancreatic insufficiency, biliary stasis, infertility and distal intestinal obstruction syndrome.

Biogenesis of CFTR protein begins in the endoplasmic reticulum (ER). Conformational maturation of wild-type CFTR is inefficient, and 75% of newly-synthesised CFTR molecules are degraded by cytoplasmic proteasomes. CFTR then matures and is delivered via the Golgi apparatus to the plasma membrane where it is endocytosed into sub-apical vesicles and recycled to the plasma membrane where it can be activated (Gelman and Kopito 2002). This process may be interrupted in a number of ways, and the manifold numbers of CFTR mutations are classed within 5 groups (section 1.4.1)

1.4.1 Classes of CFTR mutation

Five classes of CFTR mutation are reported (Tsui 1992, McCauley and Elborn 2000).

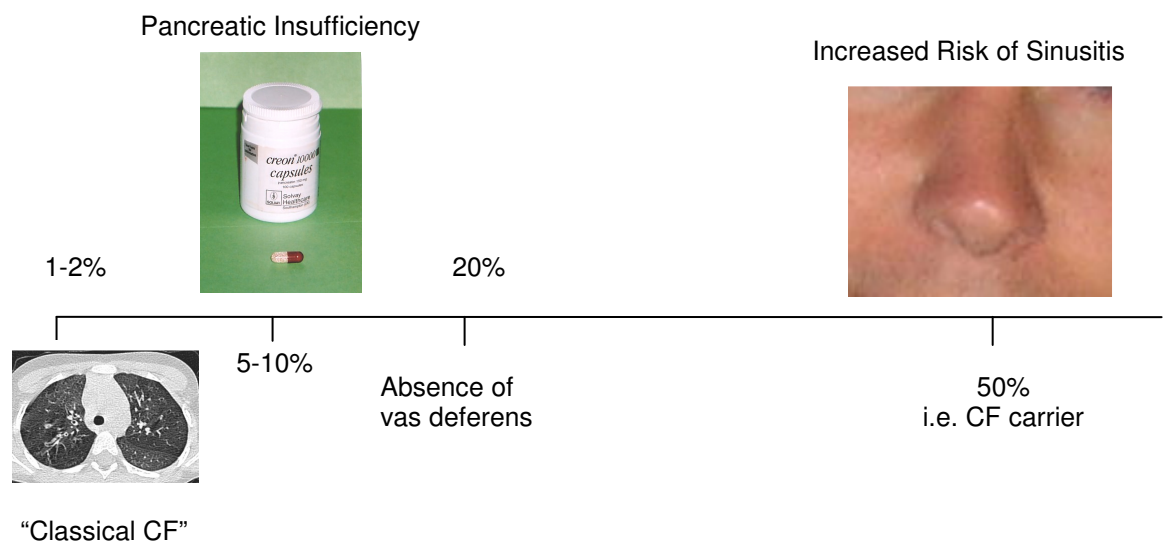
- I) Mutations associated with no production of CFTR protein
- II) Mutations altering cellular maturation of CFTR protein
- III) Mutations disturbing regulation of chloride channel
- IV) Mutations altering conduction of chloride channel
- V) Mutations associated with diminished production of CFTR protein

1.4.2 $\Delta F508$ – the commonest CFTR mutation

The majority of CF patients (>90%) have an allele coding for a mutant CFTR lacking phenylalanine at the 508 position ($\Delta F508$) (Fuller and Benos 1992). $\Delta F508$ is a class II mutation that blocks protein maturation in the endoplasmic reticulum (ER), resulting in protein breakdown (Kopito 1999). Only a small amount of mutant $\Delta F508$ CFTR makes it to the cell membrane, (Kalin *et al* 1999) and it is known that the amount of functional CFTR protein reaching the cell surface correlates with disease severity (Figure 1.3).

Figure 1.3

Correlation of clinical disease severity with amount of CFTR protein expressed at the cell surface



Improved CFTR function in $\Delta F508$ homozygotes would rely on methods to increase plasma membrane levels of CFTR (Drumm 1999). Potential methods of achieving this include elevation of $\Delta F508$ CFTR expression i.e. by improving CFTR trafficking, or by altering the intracellular environment to improve CFTR folding and processing (Drumm 1999). In the $\Delta F508$ population, work has centred on ‘molecular chaperoning’ (Accurso

2004, Morello *et al.* 2000) to guide abnormal $\Delta F508$ CFTR protein through the endoplasmic reticulum to the cell surface. A number of methods for improving CFTR function by both chemical and environmental manipulation have been described, and these are summarised in Table 1.2.

Exciting preliminary work on the role of oxygen in facilitating improvement in CFTR trafficking (Bebok *et al.* 2001, Guillembot *et al.* 2008), suggests that hypoxia may inhibit CFTR function. These reports suggest a potential novel role for oxygen therapy in increasing functional amounts of CFTR protein at the airway epithelial cell surface. The notion of hypoxia being associated with functional outcomes in CF forms the basis for undertaking this thesis.

Table 1.2
Potentiators/Inhibitors of CFTR Function

Agent/Condition	Reference
POTENTIATORS OF CFTR FUNCTION	
Hypothermia	Drumm <i>et al.</i> 1991 Denning <i>et al.</i> 1992
Glycerol	Sato <i>et al.</i> 1996
Sodium 4-Phenylbutyrate	Rubenstein <i>et al.</i> 1997 Rubenstein <i>et al.</i> 1998
Deuterated Water	Brown <i>et al.</i> 1997
Trimethylamine-N-oxide	Brown <i>et al.</i> 1997
Dimethylsulfoxide	Bebok <i>et al.</i> 1998
Genistein	Suaud <i>et al.</i> 2002
Gentamicin	Wilchanski <i>et al.</i> 2003
Curcumin <i>No effect for Curcumin</i>	Egan <i>et al.</i> 2004 Grubb <i>et al.</i> 2004, Grubb <i>et al.</i> 2006 Song <i>et al.</i> 2004
Oxygen	Bebok <i>et al.</i> 2001
INHIBITORS OF CFTR FUNCTION	
Hypoxia	Mairlbaurl <i>et al.</i> 2003 Guimbellot <i>et al.</i> 2008
Cigarette smoking	Cantin <i>et al.</i> 2006

1.4.3 CFTR function and hypoxia

In vitro evidence exists to support a beneficial role for oxygen in enhancement of CFTR function (Bebok *et al.* 2001). Marin-Darby canine kidney cell lines (which endogenously express low levels of CFTR) were cultured in air-liquid interfaces with a pO₂ gradient increasing from 2.5% to 20%. It was found that improved cellular oxygenation led to increased CFTR maturation and/or increased CFTR trafficking (Bebok *et al.* 2001). Furthermore, hypoxia is reported to reduce CFTR mRNA, protein expression and function in human cell lines (Guimbellot *et al.* 2008). Thus far, similar work in a Δ F508-homozygous CF cell-line has yet to be described.

Animal work using a murine model shows that mice subjected to hypoxia (FiO₂ of 10% for 7 days) *in vivo* had lower levels of CFTR mRNA expression in airways, gastrointestinal tissues and liver, when compared with normoxic control mice (Guimbellot *et al.* 2008).

Limited human *in vivo* work also suggests a link between hypoxia and CFTR function. First, a study of mountaineers with high-altitude pulmonary oedema (HAPE) analysed CFTR expression in those who went on to develop HAPE and a control group. At low altitude (normoxia), no difference in CFTR expression was found. However, under hypoxic conditions (4559m), a 60% decrease in CFTR expression was seen in the HAPE group (Mairbaur *et al.* 2003). Control subjects who did not develop HAPE however, had normal CFTR levels despite the same hypoxic stimulus. Secondly, CFTR mRNA expression was seen to be reduced in pulmonary tissues taken from hypoxaemic lung transplant recipients at the time of transplantation, when compared to the levels of CFTR mRNA seen in the lung epithelia of non-hypoxaemic donors (Guimbellot *et al.* 2008).

Therefore, there is *in vitro*, rodent and *in vivo* work supporting a role for hypoxia having deleterious action on CFTR function. Since it is known that the amount of functional CFTR protein reaching the cell surface correlates with clinical disease severity (Figure 1.3), a novel therapeutic role for oxygen in Cystic Fibrosis is suggested. Other reported potentiators and inhibitors of CFTR function are summarised in Table 1.2.

1.5 CF airway disease

The most widely-accepted explanation for the causation of airway disease in CF is the “low volume” hypothesis - namely that reduced ASL, as a result of CFTR dysfunction, leads to impaired mucociliary clearance and consequent failure to effectively clear inhaled bacteria (Matsui *et al.* 1998). If hypoxia does indeed inhibit CFTR function, then this may prove a contributory factor to reduction in ASL and predisposition to bacterial airways infection.

Abnormal ASL leads to bacterial entrapment and recurrent lower respiratory infections. This causes airway remodelling which, in turn, results in increased airway resistance, gas trapping, V/Q mismatching and increased work of breathing. The airway is thought to be normal at birth with no evidence of infection, and probably no inflammation is present (Davies *et al.* 2007). However, the end result of the sequence of events described above is irreversible airway damage with bronchiectasis and consequent respiratory failure.

Thus, in advanced CF, hypoxia may ensue as a result of worsening V/Q mismatch. However, a circular relationship is proposed, whereby hypoxia itself may lead to perpetuation of neutrophilic inflammation and parenchymal lung damage, contributing to a downward pathophysiological spiral. The mechanisms by which this is hypothesised to occur will be discussed below.

1.5.1 CF airway inflammation

Debate rages on the causal signalling mechanisms for inflammation in CF. Inflammation in the CF airway is typified by the presence of neutrophils along with their protein products, including elastase. Neutrophils are attracted to the airway by interleukin-8 (IL-8), a chemokine released by airway epithelial cells and macrophages as part of the innate immune response to infection. Levels of IL-8, along with other pro-inflammatory cytokines such as tumour necrosis factor -alpha (TNF α), interleukins 1-beta (IL-1 β) and 6 (IL-6) are increased in the CF airway. Levels of IL-8 (the initiating step of neutrophilic inflammation in CF) are regulated by transcription factors of the nuclear factor kappa B (NF κ B) family.

1.5.1.1 NFκB signalling and the innate immune system

The innate immune system is a host-defence system found in all multi-cellular organisms which has the ability to distinguish self from non-self (Medzhitov and Janeway Jr. 1997). The receptors of the innate immune system are known as pathogen recognition receptors (PRRs) due to their ability to recognise pathogen associated molecular patterns (PAMPs).

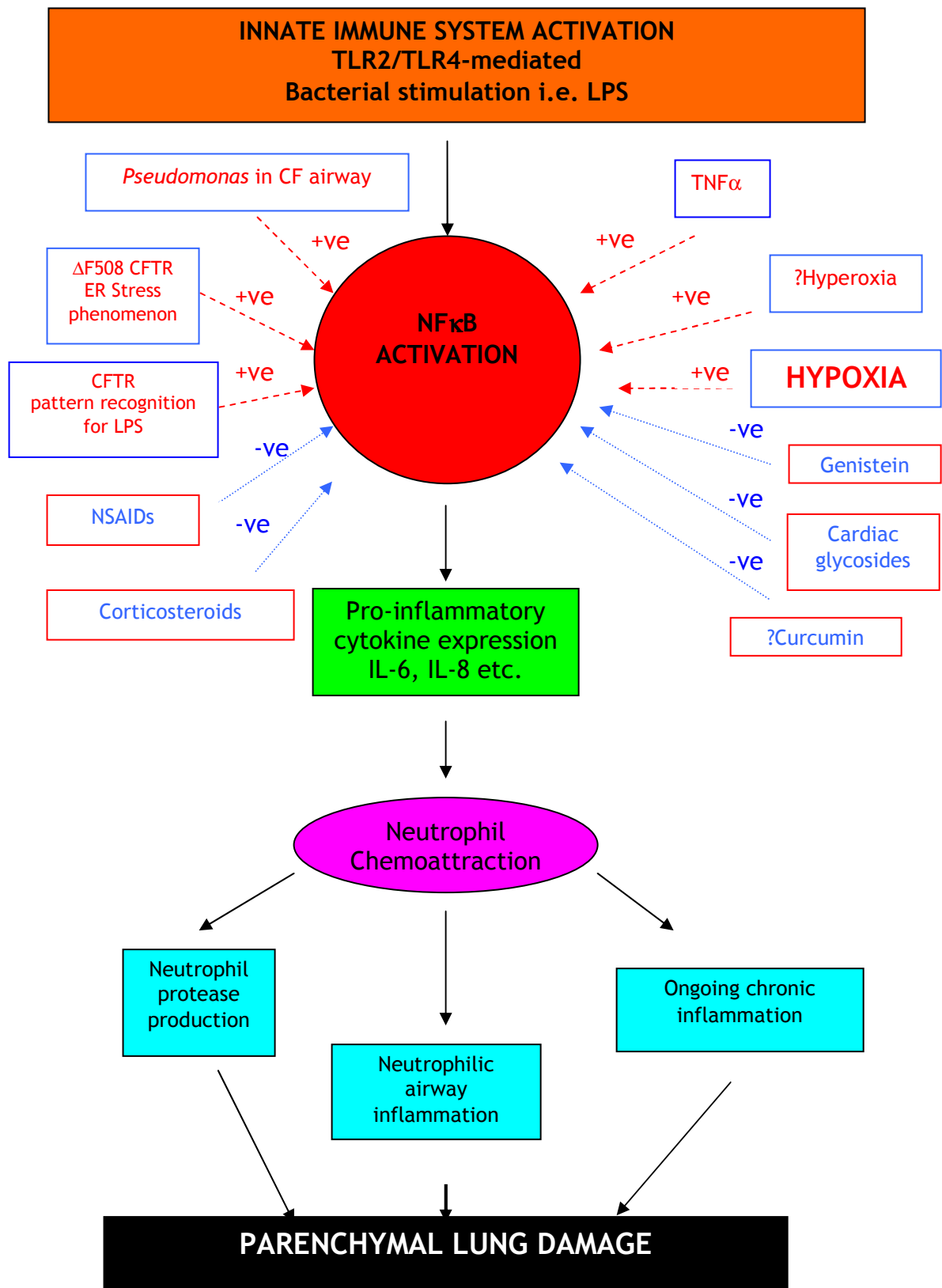
The most prominent family of PRRs are the toll-like receptor (TLR) family of proteins, and prominent PAMPs include bacterial lipopolysaccharide (LPS) – the major constituent of *Pseudomonas aeruginosa* (PA) cell wall, and lipoproteins (found in the cell walls of organisms such as *Staphylococcus aureus*). Following binding of a TLR to a pathogen-associated molecular pattern, such as binding of LPS to toll-like receptor 4 (TLR4), a cascade of protein interactions begins (Muzio *et al.* 2000). Via a series of intracellular signalling mechanisms, NFκB is activated.

NFκB is essential in driving transcription of several inflammatory cytokines (Dinarello 1996). Therefore, following NFκB activation, expression of pro-inflammatory cytokines is increased, and resultant neutrophil chemoattraction and inflammation ensues.

1.5.1.2 NFκB signalling and CF airway inflammation

There are a number of postulated mechanisms by which NFκB activation may occur in CF, namely bacterial infection, CFTR dysfunction, cytokine stimulation and possibly by hypoxia (Figure 1.4).

Figure 1.4
Simplified mechanisms for NFκB signalling and the CF airway



1.5.1.2.1 Bacterial infection leading to NFκB activation in CF

The interaction of LPS and TLR4 is of importance in the CF lung, where the LPS-producing organism, *Pseudomonas aeruginosa* (PA) is abundant. 73% children with CF have evidence of PA infection by age 3 years (Burns *et al.* 2001), and the cause of death in almost all CF patients is progressive lung inflammation associated with unremitting PA infection (Davis *et al.* 1996). The mechanism by which PA activates NFκB remains poorly understood, but differential NFκB activation in response to PA occurs in CF compared with healthy airways (Hajjar *et al.* 2002). LPS produced by PA is usually penta-acylated, but in the CF airway, PA synthesizes hexa-acylated LPS. Human TLR4 recognises this configuration change, and NFκB activation is 100 times greater with the hexa-acylated form of LPS (Hajjar *et al.* 2002). Thus, in the CF airway, differential LPS recognition may lead to massive activation of the innate immune response. The pro-inflammatory cytokine response to PA is protracted in CF cell lines with increases in IL-6 and IL-8, compared with non-CF cells (Kube *et al.* 2001). Toll-like receptor 2 (TLR2) also signals via NFκB and is likely to be important in CF, as TLR2 recognises bacterial lipoprotein associated with *Staphylococcus aureus* (Lien *et al.* 1999), another common pathogen in the CF lung.

1.5.1.2.2 CFTR protein dysfunction leading to NFκB activation in CF

Normal innate immune response to PA is thought to involve CFTR (Pier *et al.* 1996), with work suggesting that PA LPS binds to wild-type CFTR protein with CFTR acting as a PRR, facilitating translocation and activation of NFκB (Schroeder *et al.* 2002). In a ΔF508 CFTR cell-line however, there was a failure to endocytose LPS and a failure to translocate NFκB. The implication of this may be that whilst a non-CF patient quickly phagocytoses PA, the CF subject cannot; leading to persistent PA infection and inflammation. This is borne out by evidence suggesting CFTR-null mice have increased levels of inflammatory mediators (including TNFα), and increased mortality after PA infection (van Heeckeren *et al.* 1997).

One may postulate that if CFTR is lacking at the airway epithelial surface then the propensity for pattern recognition in response to PA is lost and an airway defence mechanism is lacking, but this fails to explain how patients with channel mutants of CFTR that reach the cell surface (i.e. G551D) are as susceptible to PA as patients lacking CFTR at the cell surface (e.g. ΔF508) (Chmiel and Davis 2003). *In vivo* work has shown that inflammatory response to PA assessed by cell counts and cytokine levels

in bronchoalveolar lavage (BAL) fluid was comparable between classes of CFTR mutation and independent of whether CFTR reaches cell surface (Van Heeckeren *et al.* 2004). Additionally, *in vitro* work showed increased IL-8 levels in CF cell lines exposed to PA that were independent of CFTR mutation class (Scheid *et al.* 2001).

There is however evidence suggesting that patients with $\Delta F508$ CFTR have an ongoing process of NF κ B-driven hyperinflammation, in both the presence and absence of PA. It is suggested that while NF κ B activation in response to bacterial LPS is an exogenous means of NF κ B activation, endogenous NF κ B activation occurs in patients with $\Delta F508$. The implication of this is that neutrophilic inflammation in the CF airway may be associated with CFTR dysfunction as well as with infection. This is suggested to be due to cell stress caused by the accumulation of mutant CFTR in the endoplasmic reticulum (Baeuerle and Baltimore 1995).

1.5.1.2.2.1

***In vitro* evidence for inherent inflammation due to CFTR dysfunction**

NF κ B activation and increased IL-8 expression in $\Delta F508$ CF cell lines when compared with wild-type CFTR cells is reported (Weber *et al.* 2001), along with reversal of higher baseline NF κ B activation by the introduction of normal CFTR (Stecenko *et al.* 2001). There is indirect evidence to support this from studies which have measured increased IL-8 levels and neutrophil predominance *in vitro*, in animal models, and also *in vivo*. *In vitro* work has shown increased levels of IL-6, TNF α and IL-8 secretion in CF tracheal cells when compared with non-CF cell lines (Kammouni *et al.* 1997) and also in tracheal xenografts of human CF airway in immunodeficient mice (Tirouvanziam *et al.* 2000).

There are, however, also studies showing that NF κ B activation (Scheid *et al.* 2001, Becker *et al.* 2004) and IL-8 expression (Schwiebert *et al.* 1999, Scheid *et al.* 2001, Becker *et al.* 2004) do not differ between CF and non-CF cell lines at baseline, that differences between CF and non-CF cell lines are inconsistent (Aldallal *et al.* 2002), and even evidence to show that CF cell lines secrete less IL-8 than wild type cell lines (Massengale *et al.* 1999).

1.5.1.2.2.2

***In vivo* evidence for inherent inflammation due to CFTR dysfunction**

In vivo work has found increased levels of inflammatory mediators including IL-8, and neutrophil numbers in BAL fluid of young children (Balough *et al.* 1995, Noah *et al.* 1997) and infants (Khan *et al.* 1995) with CF in the absence of clinical or microbiological evidence of infection. Additionally, IL-8 mRNA expression is increased in young CF patients compared with age-matched non-CF controls (Muhlebach *et al.* 2004).

However, it is also reported that in the absence of infection BAL profiles were comparable with control subjects and only the presence of infection was associated with raised inflammatory markers (Armstrong *et al.* 1997). Analysis of BAL fluid in children with CF and those with other chronic respiratory problems during acute infective exacerbations found increased levels of both IL-8 and neutrophils in the infected CF patients compared with the infected non-CF patients (Muhlebach *et al.* 1999). The protracted proinflammatory cytokine response in CF cell lines exposed to PA (Kube *et al.* 2001) may explain the findings of increased IL-8 expression in young children with no bacteria on BAL i.e. a prolonged response to a previous bacterial infection may be being measured.

1.5.1.2.2.3 Cytokines perpetuating NF κ B activation in CF

The proinflammatory cytokine TNF α is also known to be an activator of NF κ B (Brightbill and Modlin 2000) leading to perpetuation of inflammation, a phenomenon that is characteristic of airway inflammation in CF.

1.5.1.3 NFκB signalling and hypoxia

There is *in vitro* evidence also that hypoxia may activate NFκB (Koong *et al* 1994, Leeper-Woodford and Detmer 1999), and also increase TNFα activity (Leeper-Woodford and Detmer 1999). Although the authors suggest that it is enhanced NFκB activity which may account for TNF activation (Leeper-Woodford and Detmer 1999), it is plausible that the reverse may be true in view of the role of TNFα as an activator of NFκB (Brightbill and Modlin 2000).

The NFκB pathway is thought to have an oxygen-sensing mechanism, by which hypoxia can regulate NFκB activation and modulate inflammatory gene expression (Taylor and Cummins 2009). Thus, hypoxia, by expression of pro-inflammatory cytokines, which in turn leads to recruitment of neutrophils may initiate and propagate CF airway inflammation.

Before viewing correction of hypoxia with oxygen as a potential new anti-inflammatory therapy, one must also consider that evidence (also *in vitro*) suggests that hyperoxia also activates NFκB (Horowitz 1999), possibly via increased pulmonary TNFα expression (Shea *et al.* 1996), and that an increase in NFκB-mediated inflammatory markers in exhaled breath of chronic obstructive pulmonary disease (COPD) patients treated with oxygen (Carpagno *et al.* 2004) has been reported.

The effects of hypoxia on NFκB-mediated inflammation in CF and non-CF airway epithelial cells are presented in chapter 6, and contrasted with the effects of a known stimulus of NFκB, namely LPS from PA.

1.5.1.4 Inhibitors of the NFκB signalling pathway

NFκB is potentially an attractive target for anti-inflammatory therapy in cystic fibrosis (Wright and Christman 2003, Koehler *et al.* 2004). By diminishing production of pro-inflammatory cytokines, neutrophilic inflammation may be decreased and lung damage minimised. Corticosteroids are known to exert an inhibitory effect on NFκB signalling pathways (Kube *et al.* 2001, Escotte *et al.* 2003), and non-steroidal anti-inflammatory drugs (NSAIDs) also inhibit NFκB activation (Yin *et al.* 1998, Wahl *et al.* 1998), providing some rationale for the beneficial effects on lung function seen with both prednisolone (Auerbach *et al.* 1985) and ibuprofen (Konstan *et al.* 1995) therapy in CF.

Other suggested inhibitors of NFκB include cardiac glycoside drugs (Yang *et al.* 2004), recombinant IL-10 (Chmiel *et al.* 1999), genistein (Tabary *et al.* 1999), and curcumin (Rahman and MacNee 1998). Given that the latter two are postulated to improve CFTR trafficking - curcumin (Egan *et al.* 2004), and genistein (Suaud *et al.* 2002) - a potential mechanism for their inhibitory effects on NFκB may be suggested.

1.5.1.5 The NFκB signalling pathway in CF – A summary

NFκB signalling may be key to inflammation in the CF airway. NFκB is activated by a number of factors including cytokines, infection, and possibly CFTR dysfunction. NFκB activation will result in increased IL-6 and IL-8 expression, promoting neutrophil chemoattraction, which initiates and propagates chronic airway inflammation. A number of possible therapeutic modalities in CF may rely upon inhibition of this pathway for their beneficial action, most notably corticosteroids and NSAIDs.

Hypoxia may impact on the NFκB signalling pathway at a number of stages:

- Direct effect of hypoxia on NFκB signalling (section 1.5.1.3)
- Impairment of CFTR trafficking and resultant CFTR dysfunction (section 1.4.3)
- Propagation of *Pseudomonas aeruginosa* (section 1.6.2.1)

Hypoxia in CF is postulated to lead to activation of the inflammatory cascade that is represented in Figure 1.4, and as yet the effects of oxygen therapy on this process remain uninvestigated. Using a cell culture model, the effects of hypoxia on this inflammatory process in CF cells will be presented in Chapter 6 of this thesis.

1.6 Potential effects of hypoxia on clinical and psychological state in CF

A repeated hypoxic insult, such as that occurring on a nightly basis during sleep, and to a lesser extent the repeated periods of hypoxia that may occur on exercise or flying may be deleterious to the health of the child with CF, impacting on the pulmonary circulation and quality of life, as well as theoretical effects on exacerbating lung inflammation.

1.6.1 Hypoxia and inflammation

Hypoxia may contribute to the decline in lung function by switching on NFκB-mediated inflammation (section 1.5), as well as encouraging the growth of PA, the key pathogen associated with CF lung disease. LPS produced by PA activates NFκB and impacts on the neutrophilic inflammatory cascade of Figure 1.4. Hypoxia may exert a role on this process, as PA biofilms prove almost impenetrable for antibiotics under hypoxic conditions, leading to a more intense and prolonged innate immune response.

In non-CF models of childhood nocturnal hypoxia i.e. obstructive sleep apnoea (OSA), elevated IL-8 levels are reported when compared with healthy controls (Tam *et al.* 2006), whilst adult OSA models also report increases in IL-8 (Alzoghbi and Bahammam 2005). Other downstream cytokines of NFκB, namely IL-6 and TNF-α have also been shown to be elevated in OSA (Alberti *et al.* 2003), whilst animal models of intermittent hypercapnic hypoxia mimicking OSA resulted in corresponding increases in IL-6 (Tam *et al.* 2007).

1.6.1.1 Effect of hypoxia on *Pseudomonas aeruginosa* (PA) growth

PA is an aerobic bacterium that grows equally well in aerobic and anaerobic conditions (Worlitzsch *et al.* 2002). This is important in CF, where *in vivo* oxygen depletion has been demonstrated in PA-infected CF airways (Worlitzsch *et al.* 2002). Additionally, within PA biofilms, large regions of anoxia have been demonstrated (Borriello *et al.* 2004). PA changes phenotype under hypoxic conditions by increasing alginate production and forming biofilms. Hypoxia and the resultant biofilm state of PA leads to antibiotic resistance (Park *et al.* 1991, Borriello *et al.* 2004) and an increased (Muhlebach *et al.* 1999) and prolonged (Kube *et al.* 2001) innate immune response. These factors contribute to persistent PA infection and the chronic airway destruction that is characteristic of CF. A postulated antimicrobial benefit for oxygen is suggested, due to the potential for altering PA growth patterns i.e. if oxygen therapy penetrates

distal airways and prevents biofilm formation, increased antibiotic sensitivity and decreased PA growth and immune response may ensue. However, a steep oxygen concentration gradient exists between the airway lumen and interior of mucous in CF (Worlitzsch *et al.* 2002) meaning oxygen therapy is not only required to reach the distal airways, but must also penetrate the mucous therein.

1.6.1.2 Effect of hypoxia on *Staphylococcus aureus* growth

Under *in vitro* anaerobic conditions, *Staphylococcus aureus* (SA) also switches from a non-mucoid to a mucoid phenotype (Cramton *et al.* 2001), indicating that hypoxia may serve as a virulence factor for SA growth in the CF mucous environment.

1.6.1.3 Effector mechanisms for reduction in inflammation with oxygen

A potential anti-inflammatory role for oxygen therapy may lie in inhibition of NFκB. While NFκB activation due to hypoxia may be a direct effect, it is likely that other signalling pathways are involved to effect this action. The effect of oxygen on CFTR trafficking (section 1.4.3) is one such area of potential importance, which may impinge on NFκB-driven inflammation, and if CFTR trafficking were improved with oxygen therapy, as suggested in an *in vitro* model (Bebok *et al.* 2001), then less CFTR degradation in the ER could be expected to take place with a resultant reduction in NFκB-driven inflammation. Additionally, a reduction in PA burden and its' associated immune response (section 1.6.1.1) could also be a potential effector mechanism by which oxygen therapy may decrease inflammation in CF.

1.6.1.4 Sequelae of inflammation as a result of hypoxia in CF

Inflammation is increased in CF. Whether this is intrinsic hyperinflammation, or a heightened response to microbial stimuli remains debatable, as discussed in previous sections. It is known that existence in a pro-inflammatory state promotes body wasting. Levels of pro-inflammatory cytokines including IL-6, IL-8 and TNF-α are raised in chronic obstructive pulmonary disease (COPD) (Gan *et al.* 2004), promoting catabolism, and muscle-wasting (Debigare *et al.* 2003); and the catabolic effects of ongoing lung inflammation in CF have been shown to be associated with reduced bone mineral density (Haworth *et al.* 2004).

Therefore if hypoxia activates NFκB, and leads to upregulation of proinflammatory cytokines, it is possible that this may have a detrimental effect on muscle bulk, bone density, and nutritional status.

1.6.2 Hypoxia and the pulmonary circulation

The first case report of *cor pulmonale* in a CF patient was published in 1946 (Wiglesworth 1946). The pulmonary circulation in CF responds to alveolar hypoxia by increasing pulmonary arterial pressure (PAP) and pulmonary vascular resistance (PVR). Graded decreases in $P_{A}O_2$ produce similar increases in PVR (Bright-Thomas and Webb 2002). Chronic alveolar hypoxia results in pulmonary artery remodelling, with muscularisation of pulmonary arterioles proliferation of intimal smooth muscle in the pulmonary arteries, and progressive intimal fibrosis. Post-mortem studies in children with CF have shown that all had some degree of pulmonary artery muscle hypertrophy, and that muscle wall thickness was related to degree of right ventricular hypertrophy (Ryland and Reid 1975).

Systolic PAP (sPAP) can be estimated by measuring the peak velocity of tricuspid regurgitation blood using Doppler echocardiography, and correlates closely with invasive sPAP (Yock and Popp 1984). A significant association between sPAP and mean SpO_2 during sleep ($r = -0.56$) and exercise ($r = -0.78$) in adults with CF is reported (Fraser *et al.* 1999), although these associations have yet to be quantified in children.

Various agents have been tried in CF to reduce pulmonary vascular resistance (PVR), including calcium-channel antagonists (Davidson *et al.* 1989) and pulmonary vasodilators (Geggel *et al.* 1985), with no evidence of benefit. The only selective pulmonary vasodilator shown to decrease PAP and PVR (Davidson *et al.* 1989) and improve RV performance (Alpert *et al.* 1987) in CF is oxygen. Therefore, oxygen in CF may be beneficial to the right heart, protecting against the development and progression of pulmonary hypertension.

1.6.3 Hypoxia and quality of life

The only reported randomised controlled trial of oxygen therapy ever undertaken in CF (Zinman *et al.* 1989) reported that, over a 12-month period, school and work attendance was significantly better maintained in the oxygen-treated group, compared with those randomised to air (83% versus 20%, $p < 0.01$), which may suggest a reduced quality of life for hypoxic subjects.

1.6.4 Hypoxia and sleep quality

Sleep quality (Pittsburgh Sleep Quality Index), and sleep duration are reported to correlate with minimum sleep SpO_2 (Milross *et al.* 2002B).

1.7 Oxygen as a therapy in CF

Oxygen use in CF can be divided into long-term use, or use on a short-term basis such as during exacerbations, on exercise, or during air travel.

1.7.1 Long-term and nocturnal oxygen therapy

Little has been written on the criteria for starting oxygen in children with CF, and currently only 1-2% of UK paediatric CF patients receive long-term oxygen therapy (Balfour Lynn *et al.* 2005, Douglass *et al.* 2008). The Cochrane review of oxygen as a therapy in CF (Mallory *et al.* 2005) suggests that "...oxygen should be reserved for those individuals with objective evidence of hypoxemia whether awake or during exercise or sleep." Given the lack of a uniform definition of what constitutes important hypoxia in each of these situations, implementation of such a policy appears fraught.

Dinwiddie and colleagues (Dinwiddie *et al.* 1999) suggested the following as indications for home oxygen therapy in CF: Resting SpO₂ <90%, PaO₂ <7.3 kPa, PaCO₂ >6 kPa and adult lung function measures of FVC < 2.0L and FEV₁ < 1.5L. The US consensus guidelines on adult CF care (Yankaskas *et al.* 2004) recommend night-time oxygen if SpO₂ are below 88-90% for ≥10% of sleep time, whilst previous guidelines suggested long-term oxygen therapy in CF should be reserved for bringing about symptomatic relief (Schidlow *et al.* 1993), a view shared by the British Thoracic Society (BTS) Home Oxygen Working Party (Balfour Lynn *et al.* 2009).

In assessing short-term benefit, SpO₂ during sleep in CF improve with supplemental oxygen (Spier *et al.* 1990, Gozal 1997) although no change in sleep architecture and no reduction in number of arousals is reported (Spier *et al.* 1990). In the only long-term oxygen trial in CF (Zinman *et al.* 1989), twenty-eight CF subjects were randomised to receive either air or oxygen therapy, and followed up for up to 3 years. Although no differences in mortality or hospitalisation were found, 83% of those in oxygen maintained school/work attendance at 12 months compared with only 20% of the air group (p<0.01). Further work is needed on the role of oxygen during sleep in patients with CF, with regards to improving daytime function and potentially conferring survival benefits (Milross *et al.* 2004). Additionally non-invasive ventilation (NIV) may become more widely used in hypoxic individuals if accompanying hypercapnia co-exists.

1.7.1.1 Non-invasive ventilation (NIV) in CF

The commencement of NIV along with supplementary oxygen may be indicated in hypoxic CF individuals if accompanied by hypercapnia (Hodson *et al.* 1991, Moran *et al.* 2007, Noone 2008, Young *et al.* 2008). Although the BTS NIV standards of care document was cautious about routine NIV use in CF because of excessive secretions (BTS 2002), NIV actually augments airway clearance in CF (Bradley *et al.* 2006). Clear benefit on nocturnal hypoventilation and daytime functioning was seen from the use of nocturnal NIV over oxygen or placebo was seen in a 6-week cross-over trial in a small number (n=8) of hypoxic and hypercapnic Melbourne CF patients (Young *et al.* 2008). NIV may also be used during exercise, with documented benefits on exercise endurance as well as reduction in dyspnoea (Menadue *et al.* 2009).

1.7.2 Oxygen therapy during exercise

Exercise should be recommended for all patients with CF, and disease severity should not be an exclusion to participation in exercise (Webb *et al.* 1995). The 2004 adult CF care consensus report (Yankaskas *et al.* 2004) advocates the use of oxygen during exercise if SpO₂ falls below 88-90% during exercise, whilst McKone and colleagues studied the role of oxygen therapy during sub-maximal exercise in a group of eight patients, and found that patients were able to exercise for longer and maintained significantly higher SpO₂ in oxygen therapy (McKone *et al.* 2002). Previous authors have also reported improved SpO₂ and increased exercise tolerance in CF patients who received supplemental oxygen during exercise (Marcus *et al.* 1992). The mechanism by which oxygen is thought to act to improve exercise tolerance is that by minimising hypoxic drive to breathe, the rate of increase in respiratory rate in hypoxic subjects is lower (Snider 2002), leading a reduction in dynamic hyperinflation and improvement in physiological lung volumes (O'Donnell *et al.* 2001). Thus dead space is lower and instead of rapid, shallow respirations, more useful gas exchange can take place, reducing fatigue and improving tolerance.

Although SpO₂ improve with oxygen during exercise, studies have reported minimal (Marcus *et al.* 1992) or no (Nixon 1990) improvement in VO₂max during exercise in oxygen in patients with CF. A cross-over design study (n=14) undertaken at both low altitude (Dead Sea) and sea-level (Falk *et al.* 2006) reported modest improvements in both SpO₂ and increased VO₂max at low altitude, however, with the assumption being that the enriched FiO₂ at lower altitude may account for these differences.

In CF subjects with exercise-induced hypoxia, oxygen supplementation appears to prevent hypoxia and may have benefits on exercise capacity and duration. Although it is tempting to speculate that longer-term oxygen supplementation during exercise in CF subjects might have cumulative benefits, there are no studies upon which to base this recommendation (Mallory *et al.* 2005). In an analogous chronic lung disease – chronic obstructive pulmonary disease (COPD), however a beneficial role for oxygen therapy alongside a pulmonary exercise rehabilitation programme has been reported (Emtner 2003) and this may be an area that warrants further investigation in patients with CF.

1.7.3 Oxygen during CF chest exacerbations

It is known that nocturnal hypoxia may be exaggerated during chest exacerbations, (Pond and Conway 1995, Dobbin 2005), and that treating the exacerbation leads to improved SpO₂. In a recent review (Smyth and Elborn 2008) on the management of exacerbations in CF, oxygen is not mentioned other than in the treatment of young children with chest exacerbations as a result of respiratory syncytial virus (RSV). Although no evidence exists, a pragmatic view would be that oxygen is indicated during a chest exacerbation if periods of hypoxia are apparent on either nursing observations or oximetry recordings

1.7.4 In-flight Oxygen

In-flight hypoxia in children with CF is defined as a fall in SpO₂ to <90%, necessitating the need for supplemental oxygen on long distance flights (BTS 2002). It should also be noted that there are cost implications for in-flight oxygen ranging from nil (with Virgin airlines), to £100 each way with British Airways.

1.7.5 Potential risks and benefits of oxygen as a therapy in CF

Caveats exist to starting oxygen therapy in CF however, which fall broadly into 3 groups. First, children with CF already carry a heavy burden of care (Hunter 2003). Oxygen may be poorly tolerated due to wheeze or tightness (Dodd *et al.* 1998), has household safety implications (Laubscher 2003), and may necessitate changes in parental behaviour, namely smoking cessation. Oxygen may be perceived as palliative rather than active therapy, and one should not underestimate the accompanying psych morbidity. The only trial of long-term oxygen therapy in CF (Zinman *et al.* 1989) highlights this, as, of 146 subjects approached to take part, only 28 entered the study. Secondly, oxygen therapy may blunt respiratory drive, and although no rise in PaCO₂ was seen after one year of oxygen therapy in CF (Zinman *et al.* 1989), other

studies report small (but probably clinically insignificant) rises in transcutaneous CO₂ in CF adults receiving supplemental oxygen (Gozal 1997, Young *et al.* 2008). Finally, hyperoxia may itself cause toxicity and activate lung inflammation (Carpagno *et al.* 2004).

1.8 Summary

Review of the basic science and rodent literature suggests that hypoxia may have a deleterious impact on measures of clinical status by mechanisms including inhibition of CFTR function and also activation of NFκB-mediated inflammation, as well as promotion of growth of bacteria including *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Although human data are sparse, such data as do exist would also support this conjecture: CF patients with periods of hypoxia may have increased pulmonary artery pressures, increased lung inflammation, greater levels of *Pseudomonas aeruginosa* burden, reduced exercise ability and skeletal muscle strength, and perhaps most importantly of all worse sleep quality and quality of life.

Perhaps due to the lack of adequate definitions and subsequent lack of appropriately-powered trials, there is no consensus as to when (and if) oxygen therapy should be initiated, nor for its mode or duration of delivery. A uniform approach to defining hypoxia needs to be developed, along with guidelines for prescribing oxygen therapy in children with CF.

The need for studies to document the prevalence and potential adverse effects of hypoxia in CF patients is thus apparent. Following this, a trial of oxygen therapy (or other means of restoration of normoxia) in those with demonstrable hypoxia may be worthy of further exploration.

1.9 Study aims

The aims of this thesis were to interrogate the relationship between hypoxia and inflammation in order to select an optimal definition of hypoxia in children with CF (Chapter 3). This definition was then used to study the association of hypoxia and measured clinical and psychological parameters (Chapters 4 and 5), whilst the *in vitro* effects of varying degrees of hypoxia upon NFκB-mediated inflammation in a CF airway epithelial cell culture model were studied (Chapter 6).

1.9.1 Selection of optimal definition of hypoxia

The effects of hypoxia upon inflammation in CF have been discussed throughout Chapter 1. Hypoxia may directly stimulate the NFκB pathway (Leeper-Woodford and Detmer 1999, Taylor and Cummins 2009), and may exert indirect effects on NFκB-mediated inflammation by promoting biofilm growth of *Pseudomonas aeruginosa* - PA (Worlitzsch *et al.* 2002) and *Staphylococcus aureus* (Cramton *et al.* 2001), as well as inhibition of CFTR trafficking (Bebok *et al.* 2001, Guillembellot *et al.* 2008).

Thus, a number of existing and *de novo* definitions of nocturnal hypoxia were considered and analysed for their ability to detect abnormal inflammatory measures in a group of children with CF (Chapter 3). These included measures of inflammation that have direct relevance to the NFκB pathway namely interleukin-8, C-reactive protein (CRP), and peripheral blood neutrophil counts. NFκB is a key transcription factor for IL-8 (which promotes neutrophil chemoattraction), and also IL-6, which in turn is a precursor of CRP production (Heikkila *et al.* 2007). The sensitivity and specificity of each hypoxia measure were studied using receiver-operator (ROC) characteristics, such that a definition of hypoxia that performed best in the detection of CF inflammation could be selected.

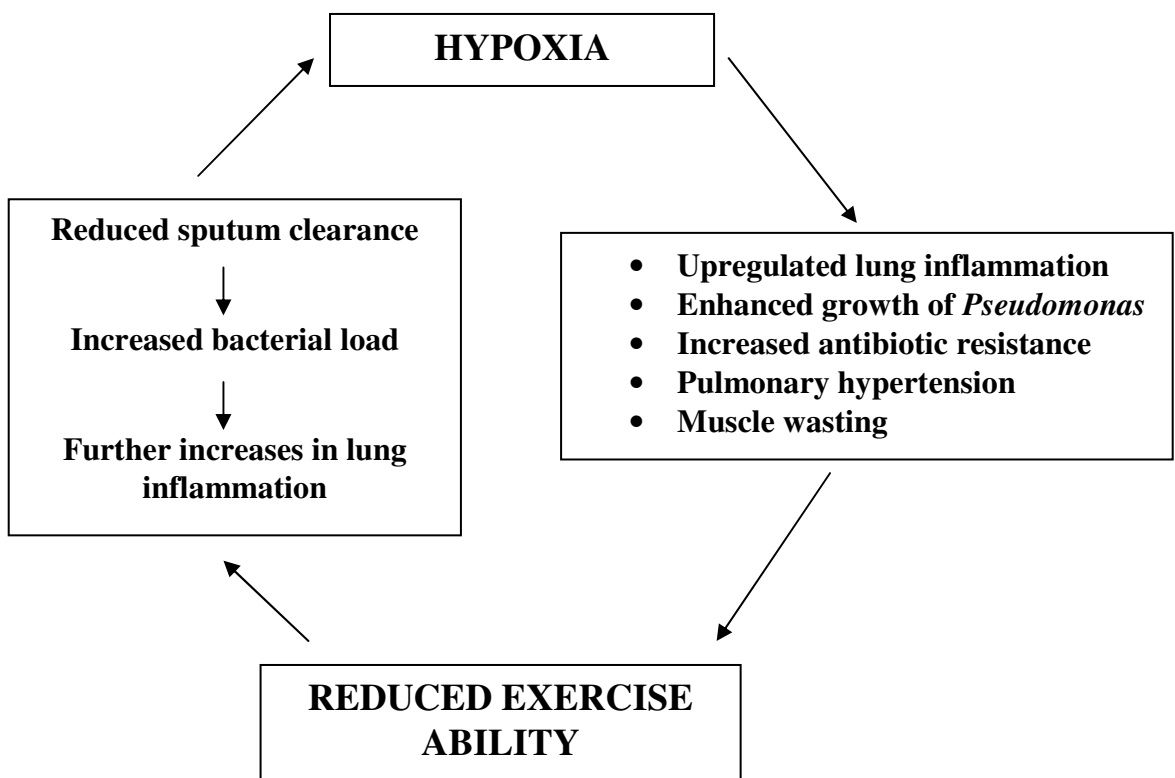
Upon selection of an optimal hypoxia definition, dichotomisation of the study population into hypoxic groups could be carried out and comparison of hypoxic subjects with their normoxic counterparts enabled the study of the association of hypoxia and clinical status (Chapters 4 and 5).

1.9.2 Outcome Measures for analysing the effects of hypoxia

The effect of hypoxia on exercise capacity was selected as the primary clinical outcome measure because of the suggested mechanism by which hypoxia may exert effects on function. As discussed above, hypoxia switches on inflammation (Leeper-Woodford and Detmer 1999) and encourages PA growth in the CF lung (Worlitzsch *et al.* 2002). These factors each may worsen lung damage and limit exercise ability. Additionally, hypoxia accelerates skeletal muscle wasting (Gan *et al.* 2004), and contributes to the aetiology of pulmonary hypertension (Fraser *et al.* 1999), which may also impact on exercise capacity.

Exercise may be key to halting decline in lung function, and ability to exercise has proven beneficial effects on airway clearance (Baldwin *et al.* 1994), aerobic exercise capacity (Orenstein *et al.* 1981), and quality of life in CF (Klijn *et al.* 2004). Deteriorating lung function combines with reduced muscle mass to limit exercise capacity, setting up a 'vicious circle' (Figure 1.5) whereby decreased exercise ability reduces sputum clearance and encourages bacterial growth within the CF lung. Inflammation may thus be initiated or perpetuated by hypoxia, leading to further reductions in both lung function and exercise ability.

Figure 1.5
Hypoxia and its purported effects on exercise



Furthermore, exercise capacity has been shown to be a measurable predictor of survival in children with CF. Exercise capacity has been shown to be an independent predictor of mortality in CF (Nixon *et al.* 1992), with peak VO_2 levels $< 32\text{mls.kg}^{-1}.\text{min}^{-1}$ associated with a significantly increased mortality over the following 8 years (Pianosi *et al.* 2005).

Exercise capacity (VO_2) at anaerobic threshold (AT) was chosen as the primary outcome measure for this study. Because cardiopulmonary exercise testing is a volitional test, peak VO_2 measures rely on a near-maximal effort, whereas VO_2 at AT (AT VO_2) occurs earlier and is an objective measure of cardiopulmonary exercise capacity that can be obtained on sub-maximal testing (Wasserman *et al.* 2004). AT VO_2 is a repeatable measure both within subjects and also between subjects.

Secondary outcome measures included the effects of hypoxia on measures of lung function (spirometry and plethysmography), lung structure (X-ray scoring), respiratory and skeletal muscle strength, echocardiographic measures, bone mineral density and quality of life.

The hypothesis tested in the laboratory work of Chapter 6 is that hypoxia exerts an *in vitro* effect on NF κ B-mediated inflammation in CF airway epithelial cells. The effects of variable degrees of hypoxia upon NF κ B-mediated inflammation (as assessed by cellular IL-8 production) in both CF and non-CF cell lines was analysed over periods up to 96 hours. Furthermore, the effects of hypoxia on inflammation in CF and non-CF cell lines was compared and contrasted to the effects of a known stimulus of the NF κ B cascade, namely bacterial lipopolysaccharide (LPS) from *Pseudomonas aeruginosa*.

CHAPTER 2: Subjects, Equipment and Methods

2.1 The Study Population

2.1.1 Subjects

Subjects were recruited to the study when attending Great Ormond Street Hospital for Children (GOSH), London for their Cystic Fibrosis (CF) annual review appointment. Eighty-eight patients aged 8-16 years of age were identified within the GOSH CF service, and 41 subjects were recruited (Figure 2.1) and completed the full testing protocol (Figure 2.2). The exclusions were for a number of reasons including current oxygen therapy, *Burkholderia cepacia* infection, size, infrequent clinic attenders who were not approached, and 11 who declined. The main reason for declining to take part was the need for an additional hospital visit and the time involved.

Recruitment took place at annual review, as each child with CF under the care of GOSH attends for annual review. Recruitment at other times could introduce a selection bias as some children had more routine follow-up visits than others.

2.1.2 Ethics Approval

An application was made to the Institute of Child Health/Great Ormond Street Hospital Research Ethics Committee. Ethics approval for the study was granted in March 2005 (REC number 05/Q0508/19). Copies of the confirmation of ethics approval are attached in Appendix 1.

Figure 2.1
CF cohort studied

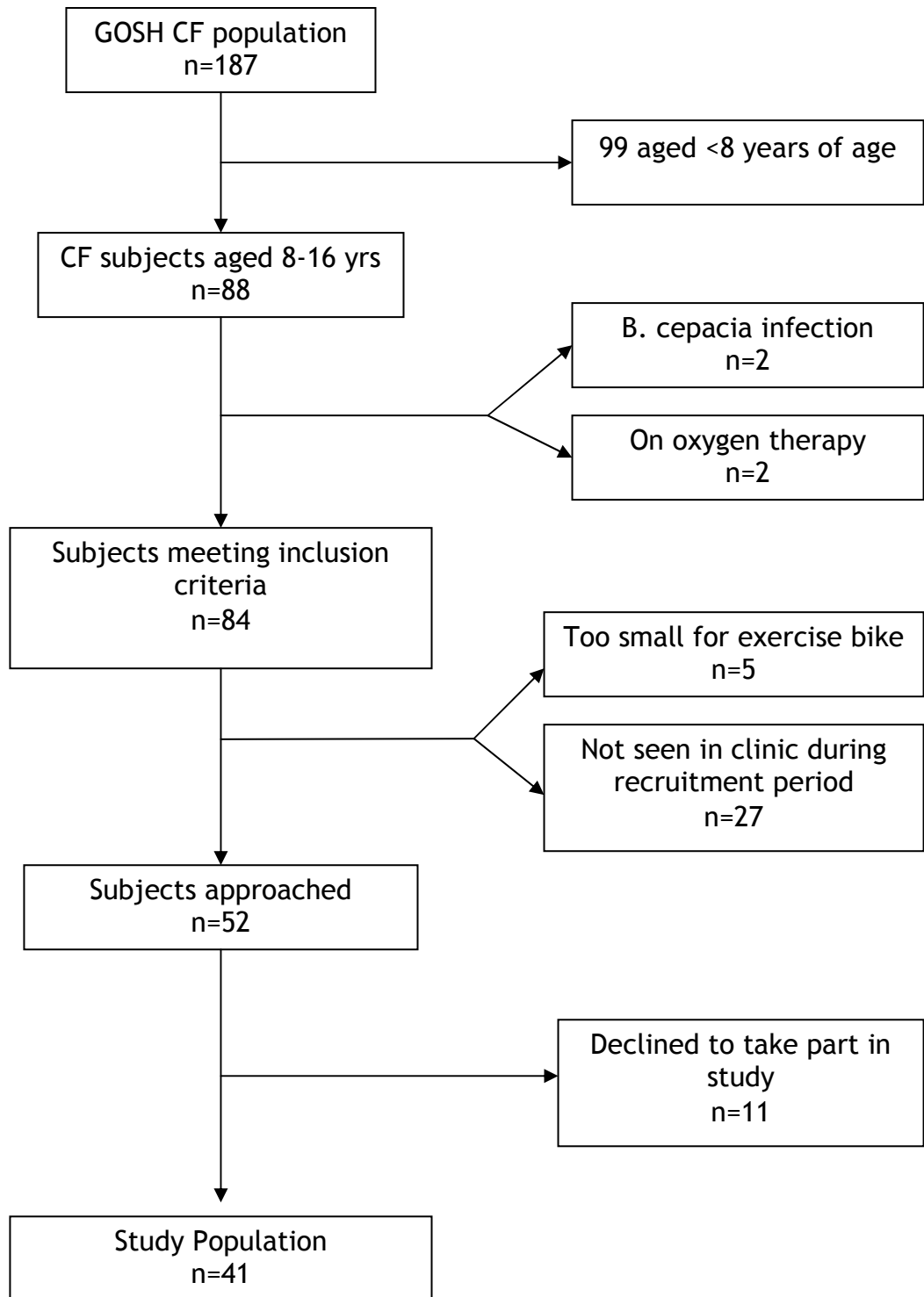
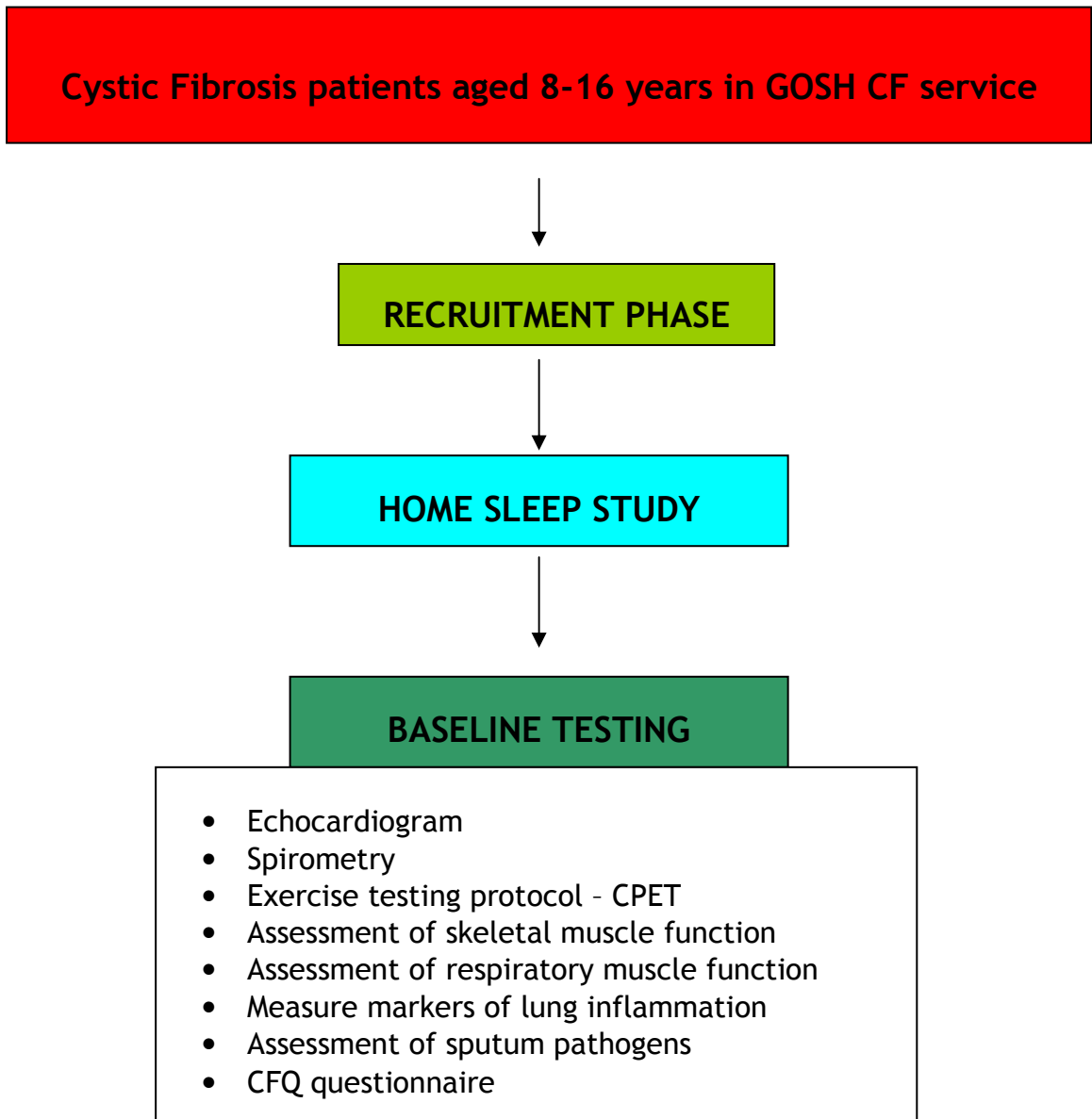


Figure 2.2
Testing Schedule



2.1.3 Power calculation

Previous work from an exercise intervention study in CF (Blau *et al.* 2001) suggested that 10% differences in oxygen uptake at anaerobic threshold (AT VO₂) were clinically significant. From the baseline measures of AT VO₂ undertaken in the Blau study, a standard deviation of 8% in measures of AT VO₂ across the study group was predicted.

Differences of 10% in AT VO₂ between hypoxic and normoxic CF patients that are thought to be of clinical importance:

$$d=10$$

The standard deviation in AT from previous evidence (Blau *et al.* 2001) is 8%.

$$\sigma = 8$$

A level of significance of 5% ($p < 0.05$) and a power of 80% were considered sufficient (i.e. the study will incorrectly lead us to believe that a difference of 10% in AT exists when it doesn't 1 time in 20, but correctly identifies the difference 80% of the time when it is there).

A table of F values (Wade 1999) based on significance levels and power required, shows that accepting a 5% significance level with 80% power, leads to a value for F of 7.85.

$$F=7.85$$

The following power calculation formula was used (Wade 1999):

$$n > 2F (\sigma/d)^2$$

$$n > 2 \times 7.85 (8/10)^2 = 10.04$$

Therefore, 11 patients would be required in each group if sample sizes were equal.

However, our projected groups sizes were likely to be unequal, and the normoxic group was estimated to be likely to be 4 times larger than the hypoxic group.

Based on GOSH CF Annual Review data from 2004, 6/72 patients aged 8 -16 years (8.3%) had evidence of daytime hypoxia (daytime SpO₂ ≤93%), and 6/72 patients (8.3%) experienced significant desaturation on exercise, 2 of whom had low daytime SpO₂ also. Alder Hey Hospital, Liverpool had carried out overnight oximetry on their cohort of children with CF (Southern – *Personal communication* 2004). Defining hypoxia as SpO₂ <93% for >25% of sleep time, 12% of their study population (n=75) were hypoxic. The overlap between sleep and daytime hypoxia is hard to quantify. It has been shown that, in a group of adolescent and adult CF patients, hypoxia was more likely to occur during sleep than exercise (Coffey *et al.* 1991), so that although some patients with sleep hypoxia desaturate on exercise, there will be others in whom the desaturation only occurs during sleep. Also, whilst resting SpO₂ are a highly specific predictor of nocturnal hypoxia, a significant proportion of patients with normal resting SpO₂ will be hypoxic during sleep (Frangolias *et al.* 2001, Versteegh *et al.* 1990), indicating the need for overnight sleep study to confidently rule in/rule out sleep hypoxia.

Extrapolating the daytime and exercise measures from GOSH, along with the Liverpool data, then we expected 8% to have daytime hypoxia and 8% to have exercise-induced hypoxia (25% of whom would also have low resting SpO₂). Additionally, 12% might be expected to have nocturnal hypoxia. Therefore the median number of hypoxic patients likely to be recruited is 20% (range 14-26), with 80% (74-86) normoxic controls.

The following formula (Wade 1999) for unequal sample sizes was the applied:

Total number required:

$$N = \frac{2n(1+k)^2}{4k} \quad \text{where } k = n_1/n_2 = 4$$

$$N = \frac{2 \times 11 \times (1+4)^2}{4 \times 4} = \frac{22 \times 25}{16} = 34.3$$

Group sizes required are: **Hypoxic group:** $N/1+k = 34.3/5 = 6.9$
 Normoxic group: $kN/1+k = 137.5/5 = 27.5$

Therefore, our study required the recruitment of 35 patients to confidently detect a 10% difference in AT with 80% power and a level of significance of 5%. This equates to 28 in the normoxic group and 7 in the hypoxic group.

2.2 Clinical Study Methods

2.2.1 Home

2.2.1.1 Home Oximetry

Sleep studies were carried out in the subjects' home by way of overnight pulse oximetry recording using the Minolta Pulsox-3i oximeter (Konica Minolta Holdings inc., Singapore) as shown in Figure 2.3 below.

Figure 2.3

Minolta Pulsox 3i device in use



Oximeters were despatched via the Royal Mail, and returned in the same fashion using a pre-paid envelope. Oximetry recordings were carried out over a minimum of 2 consecutive nights to control for night-to-night variability (Milross 2002) and data downloaded using the Download 2001 software package (Stowood Scientific Instruments, Oxford, UK). Home oximetry appears to be a reproducible measure of nocturnal hypoxaemia with good between occasion repeatability (Montgomery *et al* 1989) and limited night-to-night variability (Milross *et al.* 2002).

Oximeters are calibrated during manufacture and automatically check their internal circuits when they are turned on. They are accurate in the range of SpO₂ of 70 to 100% (+/-2%), but less accurate below 70%. The oximeter utilizes a light source originating from the finger-probe at two wavelengths (665nm –red and 880nm - infrared). The light is partly absorbed by haemoglobin (Hb), by amounts which differ depending on whether it is saturated or desaturated with oxygen. By calculating the absorption at the two wavelengths the processor can compute the proportion of oxygenated Hb.

Continuous measures of oxygen saturation (SpO₂) and pulse rate were recorded. The SpO₂ value is moving averaged over a period of 3 seconds, and displayed every second on the LCD. SpO₂ and pulse rate values are sampled and stored every 5 seconds, allowing downloading at a later date. The Minolta Pulsox 3i oximeter is accurate over a

range of SpO₂ from 50-100% with precision of +/- 2%. Measured outcome measures were mean SpO₂, lowest SpO₂, and percentages of sleep time spent with SpO₂ below 94%, 93%, 92%, and 90%. Various methods of quantifying nocturnal SpO₂ in CF are reported as detailed in Table 1.1.

2.2.2 Hospital Visit

2.2.2.1 Clinical Examination

- 1) Height was measured using a Harpenden height stadiometer (Holtain Ltd, Crymych, Dyfed, UK) to the nearest millimetre.
- 2) Body mass was measured with the subject wearing minimal clothing, using electronic scales (Seca [Model 861], Vogel and Halke, Germany) to the nearest 0.1 kilogram.
- 3) Height, weight and body mass index were converted to standard deviation scores using the British 1990 growth reference data (Freeman *et al.* 1995, Cole *et al.* 1995), and associated computer package from the Child Growth Foundation.

Body surface area was calculated using the following equation (Mosteller 1987):

$$\text{Surface area (m}^2\text{)} = \sqrt{\frac{\text{Height (cm.)} \times \text{Weight (kg)}}{3600}}$$

- 4) All subjects underwent a cardiopulmonary physical examination.

2.2.2.2 Resting Oximetry

Transcutaneous oxygen saturations (SpO₂) and heart rate were recorded at rest over a 3-minute period, using a pulse oximeter (Nonin, USA) which was placed over the right supraorbital artery. Daytime hypoxia was defined as a resting SpO₂ <95%, in accordance with the definition of Coffey and colleagues (Coffey *et al.* 1991).

Following clinical examination and oximetry, it was determined if the subject was allowed to proceed with cardiopulmonary exercise testing. A subject was to be excluded from testing if SpO₂ was < 90%; if there was evidence of an acute upper/lower respiratory tract infection; if wheeze was present on examination; or they had a lower limb injury that would limit exercise performance.

2.2.2.3 Spirometry and measures of other lung parameters

2.2.2.3.1 Spirometry methods

Incentive spirometry was performed using a laboratory spirometer (Jaeger Masterscreen 4.65) with computer package (Figure 2.4). The spirometer was calibrated before each set of measurements with a three-litre syringe. Three technically acceptable manoeuvres were performed and forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC), mid-expiratory (FEF_{75} and FEF_{50}) flows and peak expiratory flow (PEF) were recorded in accordance of the joint American Thoracic Society and European Respiratory Society (ATS/ERS) standards for spirometry (Miller *et al.* 2005).

Figure 2.4

Child performing incentive spirometry on Jaeger Masterscreen system



2.2.2.3.2 Spirometry reference data

The published Brompton paediatric reference data for spirometry (derived from a cross-sectional study of 772 caucasian children) were used. Values from our study subjects were converted to standard deviation scores (SDS) using this validated reference data (Rosenthal *et al.* 1993).

2.2.2.3.3 Plethysmography

Plethysmography was undertaken at Annual Review as part of each CF patient's clinical evaluation. Plethysmography estimates measures of both residual volume (RV) and total lung capacity (TLC), and from these measures, an estimate of gas trapping can be made from the RV to TLC ratio. This was undertaken in accordance with the methodology of Stocks and Quanjer (Stocks and Quanjer 1995).

2.2.2.3.4 Modified Chrispin Norman chest radiograph scoring

The original Chrispin-Norman scoring system involves assessment of both frontal and lateral chest radiographs (Chrispin and Norman 1974). The modified Chrispin-Norman scoring system (Benden *et al.* 2005) assesses only a frontal postero-anterior chest radiograph, which is scored according to a standardised scoring system for the typical abnormalities seen in CF. These include hyperinflation, increases in lung volume and the degree of diaphragm depression. Each individual item is given a score of: 0 - not present; 1 – present but not marked; 2 – marked; depending on the degree of change.

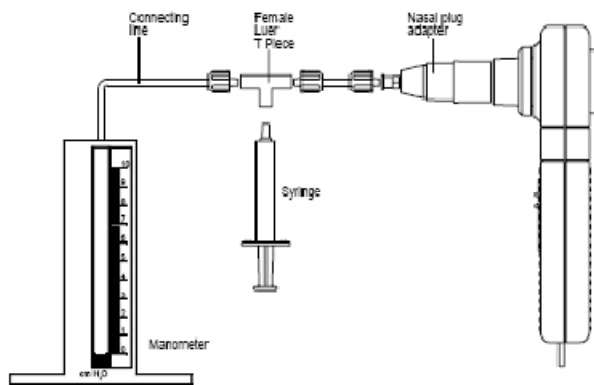
The lung fields are then divided into four quadrants; right upper, left upper, right lower, left lower. Each area is then reviewed for parenchymal lung changes which are a consequence of bronchial mucus plugging and infection. These are seen as bronchial wall thickening, ring shadows, mottled shadowing and areas of confluent consolidation – large soft shadows. A score of 0, 1 or 2 is given according to severity for each zone in relation to these changes. Scoring is performed by two observers simultaneously in order to reduce inter-individual error. The proforma used for the scoring process is included in Appendix 2.

2.2.2.4 Respiratory Muscle Pressure Measurements

Sniff nasal inspiratory pressure as well as mouth inspiratory and expiratory pressures were measured using a hand-held pressure meter (Micro RPM01, Micro Medical Ltd., Chatham, Kent, UK). Pressure is measured as that transduced across a piezoelectric crystal within the meter. Calibrations are factory set and are reported to remain stable indefinitely. However, in order to check pressure calibration, a 3-way tap connected to a syringe and attached to a manometer at one end, and the RPM01 at the other is used (Figure 2.5). The syringe is aspirated until a negative pressure of 200cm H₂O is reached on the manometer. The respiratory muscle pressure meter should be reading the same pressure value. Adjustments can be made by turning a calibration screw in the outer housing of the meter until calibrations is reached.

Figure 2.5

Calibration of the RPM01 respiratory muscle pressure meter (Micro Medical, UK)



Reproduced from operating manual of RPM01 respiratory muscle pressure meter

2.2.2.4.1 Sniff Nasal Inspiratory Pressure (SnIP)

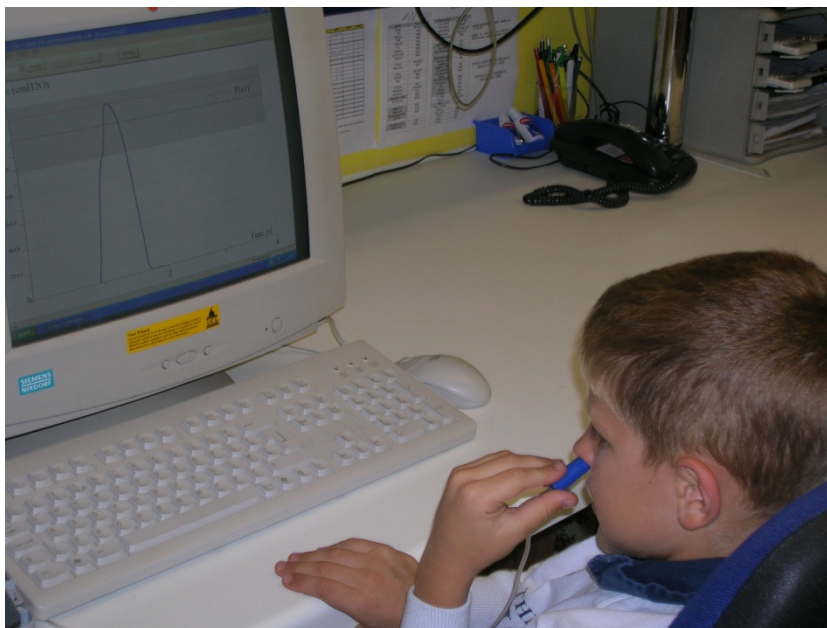
The SnIP is a short, sharp voluntary manoeuvre performed whilst seated (Figure 2.6). Pressure is measured by wedging a nasal plug (Micro Medical Ltd., Chatham, Kent, UK) in one nostril whilst the other nostril remains unoccluded. The nasal plug allows a leak-free fit inside the nostril, and is connected to a 1.0mm ID polyethylene catheter that is attached to the pressure transducer of the hand-held pressure meter. The respiratory pressure meter was connected to a respiratory pressure database and analysis software (PUMA, Micro Medical Ltd.), via a desktop personal computer. The subject sniffs through the obstructed nostril from relaxed end-expiration (Functional Residual

Capacity, FRC), to a maximum of 10 attempts. The pressure in the obstructed nostril corresponds to the transmitted pressure from alveoli to the nasopharynx, as a measure of respiratory muscle strength. Most subjects are able to attain a maximal sniff with practice (ATS statement), and there appears little gain in carrying out a greater number of sniffs (Fitting 2006).

SnIPs were recorded using the software above, and maximum SnIP of three manoeuvres which varied by less than 5% was noted. Sniff measurements were rejected if the pressure tracing did not show smooth upstroke, or the total duration of the sniff was > 500ms as suggested by previous authors measuring SnIP in children (Rafferty *et al.* 2000).

Figure 2.6

SnIP being performed in a young child



2.2.2.4.2 Mouth Inspiratory Pressure (MIP)

Mouth inspiratory pressures were measured using a standard flanged mouthpiece connected to the hand-held pressure meter. Maximum pressure that is sustained over 1 second was computed. Pressure-time plots were viewed in real-time using the PUMA analysis software package

A nose clip was worn during testing, and the subject was asked to perform a minimum of 5 and a maximum of 10 maximal inspiratory efforts from FRC, whilst seated. Each effort was undertaken 1 minute apart. Subjects were asked to maintain maximal pressures for at least 2-3 seconds, in order that maximum pressure sustained for 1 second could be recorded. A leak is built into the mouthpiece in order to allow some airflow and prevent glottic closure on inspiration. Care was taken to ensure an adequate seal around the mouthpiece to minimise airleak, and the highest pressure of 3 manoeuvres which varied by less than 5% was noted.

2.2.2.4.3 Mouth Expiratory Pressure (MEP)

MEP is a method of assessing global expiratory muscle strength by measuring the maximal static pressure that can be generated at the mouth by performing a maximal expiratory effort (Valsalva manoeuvre). Mouth expiratory pressures were measured using a standard flanged mouthpiece connected to the hand-held pressure meter, and maximum pressure that is sustained over 1 second was computed. A leak is built into the mouthpiece in order to prevent buccal muscle involvement. Pressure-time plots were viewed in real-time using the PUMA analysis software package.

The subject was asked to perform a minimum of 5 and a maximum of 10 maximal expiratory efforts from Total Lung Capacity (TLC), whilst seated and wearing a nose clip. Each effort was undertaken 1 minute apart. Subjects were asked to maintain maximal pressures for at least 2-3 seconds, so that maximum pressure sustained for 1 second could be recorded. Care was taken to ensure that there was an adequate seal around the mouthpiece, and the highest pressure from 3 manoeuvres that varied by less than 5% was noted.

2.2.2.4.4 Methodological issues around Respiratory Muscle Testing

A number of methodological issues have been noted in the literature respiratory muscle pressure testing, the principal ones being:

- Nose-clip or no nose-clip?
- Measurement of Inspiratory Pressures from RV or FRC
- Measurement of Expiratory Pressures from TLC or FRC

The first of these issues centres on whether or not a nose-clip should be worn when carrying out mouth pressures measurements (MIP and MEP). The reference data available in children were derived with nose-clip in place (Stefanutti and Fitting 1999, Rafferty *et al.* 2000), although the 2002 American Thoracic Society/European Respiratory Society statement on respiratory muscle testing concludes that ‘noseclips are not required’ when testing static mouth pressures (ATS/ERS 2002).

The pressure generated during respiratory muscle pressure manoeuvres reflects the pressure developed by the respiratory muscles (P_{mus}), plus the passive elastic recoil pressure of the respiratory system including the lungs and the chest wall (P_{rs}). At FRC, the elastic recoil is zero, so that mouth pressure is equal to P_{mus} . However, at residual volume (RV), the P_{rs} may be as much as $-30\text{cm H}_2\text{O}$, and thus may contribute significantly to MIP values. Similarly at TLC, the P_{rs} can be up to $+40\text{cm H}_2\text{O}$, thus affecting the MEP values. Since it is difficult to estimate and subtract elastic recoil values, measures of inspiratory pressures were made from FRC. Ideally, MEP would also have been measured from FRC. However, in a group of children used to performing forced expiratory manoeuvres (spirometry) from TLC, MEP readings were measured from TLC.

2.2.2.4.5 Respiratory Muscle Pressure Reference Data

The paediatric reference data of Stefanutti and Fitting (1999) was used, where age and gender-specific means and standard deviations for SnIP, MIP(FRC) and MEP(TLC) are quoted. SnIP, MIP and MEP values from our study subjects were converted to standard deviation scores (SDS) using this validated reference data (Stefanutti and Fitting 1999).

2.2.2.5 Exhaled Breath Condensate (EBC)

2.2.2.5.1 Exhaled Breath Condensate Collection

Samples were collected with the subject in a seated position using a commercially available system (ECoScreen, Jaeger, Hoechberg, Germany) with a one-way inspiratory valve, cooling chamber and collection system (Figure 2.7).

Figure 2.7

Collection of EBC



An oral inhalation,-oral exhalation method was used and a nose-clip was worn in accordance with the ATS/ERS recommendations (Horvath *et al.* 2005). Collection time used was 20 minutes, during which the subject undertook normal tidal breathing. After this time, between 2 and 4mls of exhaled water vapour were collected.

Exhaled breath collection was carried out prior to any forced expiratory manoeuvres being undertaken, and also prior to exercise. The design of the mouthpiece for the Ecoscreen device is such that the condenser is at a higher level than that of the mouthpiece, minimising the risk of salivary contamination. Following collection, the samples were immediately, transferred to the Immunobiology laboratory, Institute of Child Health, eluted into cryovials (Nalge-Nunc International, Roskilde, Denmark) and stored at -80°C .

2.2.2.5.2 Exhaled Breath Condensate Cytokine analysis

Wide discrepancy in reported in the levels of detectable IL-8 in EBC is reported. Cunningham and colleagues studied 21 CF patients and detected IL-8 in EBC in 33% with median concentrations of 49 pg.mL⁻¹ (Cunningham *et al.* 2000). This contrasts with work reported by an Italian group (Bodini *et al.* 2005) who detected IL-8 in 90% CF patients and controls but with concentrations that are all <1 pg.mL⁻¹ i.e. 50-fold lower than Cunningham. In view of the discrepancies in levels of detectable cytokines found in EBC, two methods of analysis were chosen. First, the same ultrasensitive IL-8 enzyme-linked immunosorbent assay – ELISA kit (KHC0084, BioSource International Inc., Camarillo, California) used by Bodini and colleagues was used (Bodini *et al.* 2005). This kit detects IL-8 levels within the range of 0.39 pg.mL⁻¹ to 25 pg.mL⁻¹. Secondly, a 10-plex cytokine analysis system which detects 10 different cytokines – IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, Interferon gamma (IFN- γ), granulocyte and macrophage colony stimulating factor (GM-CSF) and TNF α , (Human 10-plex cytokine assay, BioSource International Inc., Camarillo, California) was used. For IL-8, the standard curve is such that IL-8 levels within the range of 16.5-4000 pg.mL⁻¹ can be detected. i.e. should be able to detect IL-8 levels similar to those reported by Cunningham and colleagues (Cunningham *et al.* 2000).

2.2.2.5.2.1 Ultrasensitive enzyme-linked immunosorbent assay (ELISA) for interleukin-8

(ELISA) were used to measure the concentration of IL-8 in exhaled breath. An ultrasensitive IL-8 (KHC0084, BioSource International Inc., Camarillo, California) kit was used, which detects IL-8 over a range from 0.39 pg.mL⁻¹ to 25 pg.mL⁻¹. The protocol was based on manufacturer's instructions.

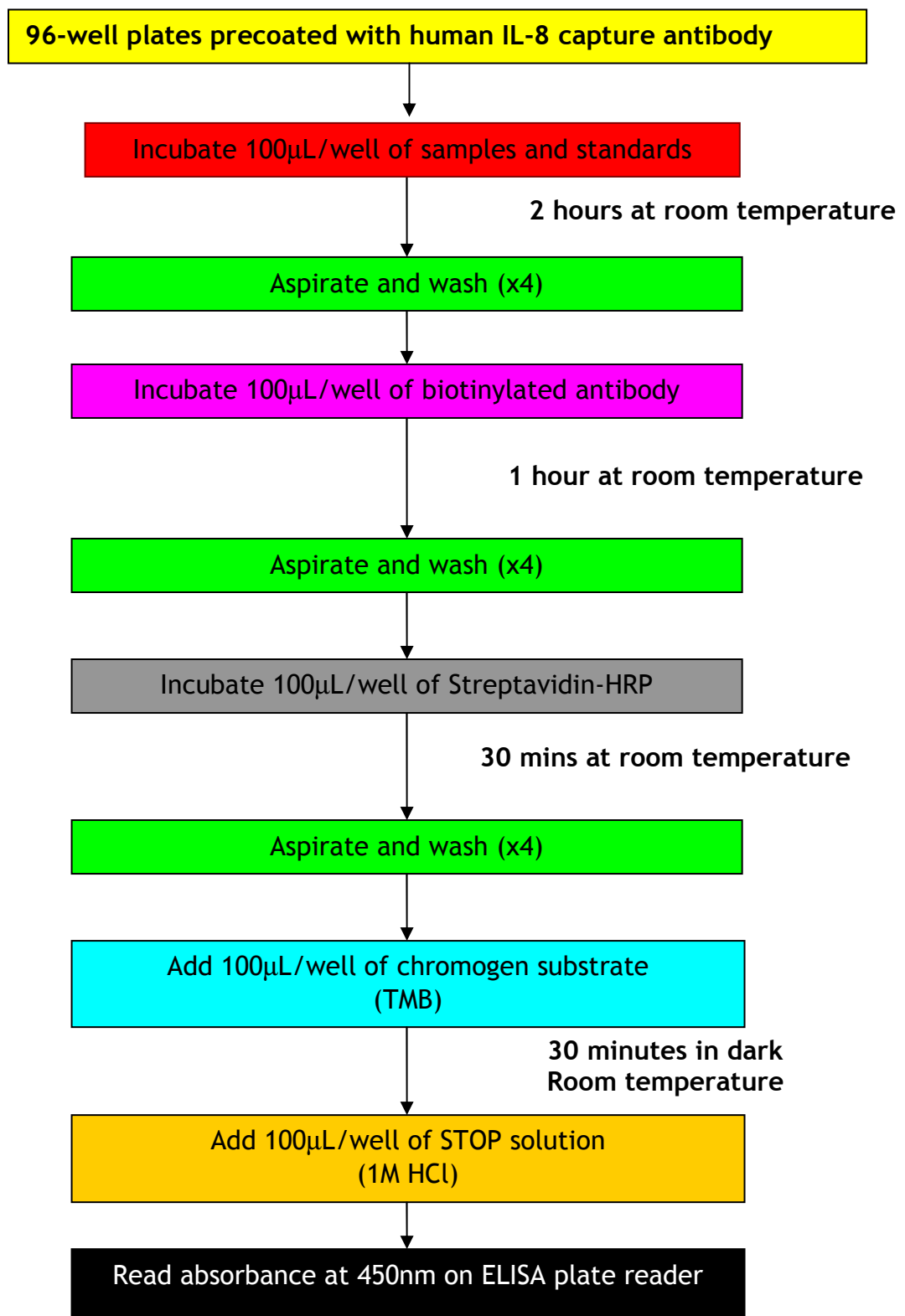
A high-binding 96-well plate had already been pre-coated with capture antibody. Prior to the first incubation, samples in duplicate and serially-diluted standards were added at 100 μ L/well. Additionally, a patient EBC sample was spiked with a known concentration of IL-8, and a blank sample was run in duplicate. Standards were generated by 1:2 serial dilutions from 25 pg.mL⁻¹ down to 0.39 pg.mL⁻¹. The first incubation consisted of 2 hours at room temperature, during which IL-8 antigen binds to the capture antibody on one site.

Wells were then aspirated and washed 4 times with 200µL wash buffer per well, following which a biotinylated antibody specific for human IL-8 was added at 100µL/well. During this second incubation (1 hour at room temperature), this antibody binds to the immobilised human IL-8 captured during the first incubation.

Wells were again aspirated and washed 4 times with 200µL wash buffer per well. After removal of excess of the second antibody, Streptavidin-horseradish peroxidase enzyme (Streptavidin-HRP) is added. This binds to the biotinylated antibody to complete a 'four-member' sandwich. After a third (30 minute, room temperature) incubation, washing (x4) is again carried out, to remove all of the unbound enzyme. Thereafter, tetramethylbenzidine (TMB) was added (100µL/well). This is a substrate which is acted upon by the bound enzyme to produce colour. Plates were protected from light and incubated at room temperature for 30 minutes. The intensity of the coloured product is directly proportional to the concentration of human IL-8 present in the original sample. The colour reaction was stopped by the addition of a stop solution (1M hydrochloric acid) which was added at 100µL/well.

Optical densities of each well were then read using an ELISA plate reader (Revelation 4.02, Dynex Technology, Chantilly, VA, USA) at 450nm (reference filter 650nm). Cytokine concentrations in the unknown samples were read from the curve generated from a known standard included on each plate. This technique allowed confidence to detect IL-8 within the range of 0.39 to 25 pg.mL⁻¹. The highest coefficient of variation quoted for this kit for replication of responses on the same plate (intra-plate precision) is ≤6%, and coefficient of variation for responses between plates is ≤8% (inter-plate precision).

Figure 2.8
Methodology for human interleukin-8 ELISA



2.2.2.5.2.2 Human 10-plex cytokine assay

A human 10-plex kit (BioSource International Inc., Camarillo, California) was used to assay levels of IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN- γ , GM-CSF and TNF- α . This is a multiplex analysis protein system utilising Luminex™ technology. The Luminex™ system uses uniformly sized microspheres which are internally labelled with graded proportions of a red and nearly red fluophore, 658nm and 712nm respectively, providing the capacity to interrogate and classify 100 discrete beads. This means that the system has the potential for measuring up to 100 analytes in a single sample. Essentially the procedure is an ELISA on a bead. Beads of a single identity are covalently coupled to a single capture antibody for the cytokine of interest. Samples and standards of known concentration are pipetted into the wells of a filter bottom 96-well plate and incubated for 2 hours. After washing the beads, cytokine-specific biotinylated detector antibodies are added to quantitate the amount of each cytokine captured on the bead. The detector antibody is incubated with the beads for 1 hour. After washing to remove excess biotinylated detector antibodies, streptavidin conjugated with a fluorescent protein, R-Phycoerythrin (Streptavidin-RPE) is added. A 30 minute incubation takes place, during which Streptavidin-RPE binds to the detector antibodies associated with the immune complexes on the beads. After a wash to remove any unbound beads, the beads are analysed using the Luminex xMAP™ system which incorporates the Luminex100™ system plate analysis (Luminexcorp, Austin, Texas, USA).

This system has been validated for the measurement of serum cytokines (Zhao *et al.* 2003), though no data for the analysis of EBC exist. Comparison with individual ELISAs has shown that Luminex™ technology is of equivalent accuracy for the majority of cytokines (du Pont *et al.* 2005). The lower limits of detection and inter-assay variations (BioSource International Inc., Camarillo, California) for each cytokine using this kit are tabulated below.

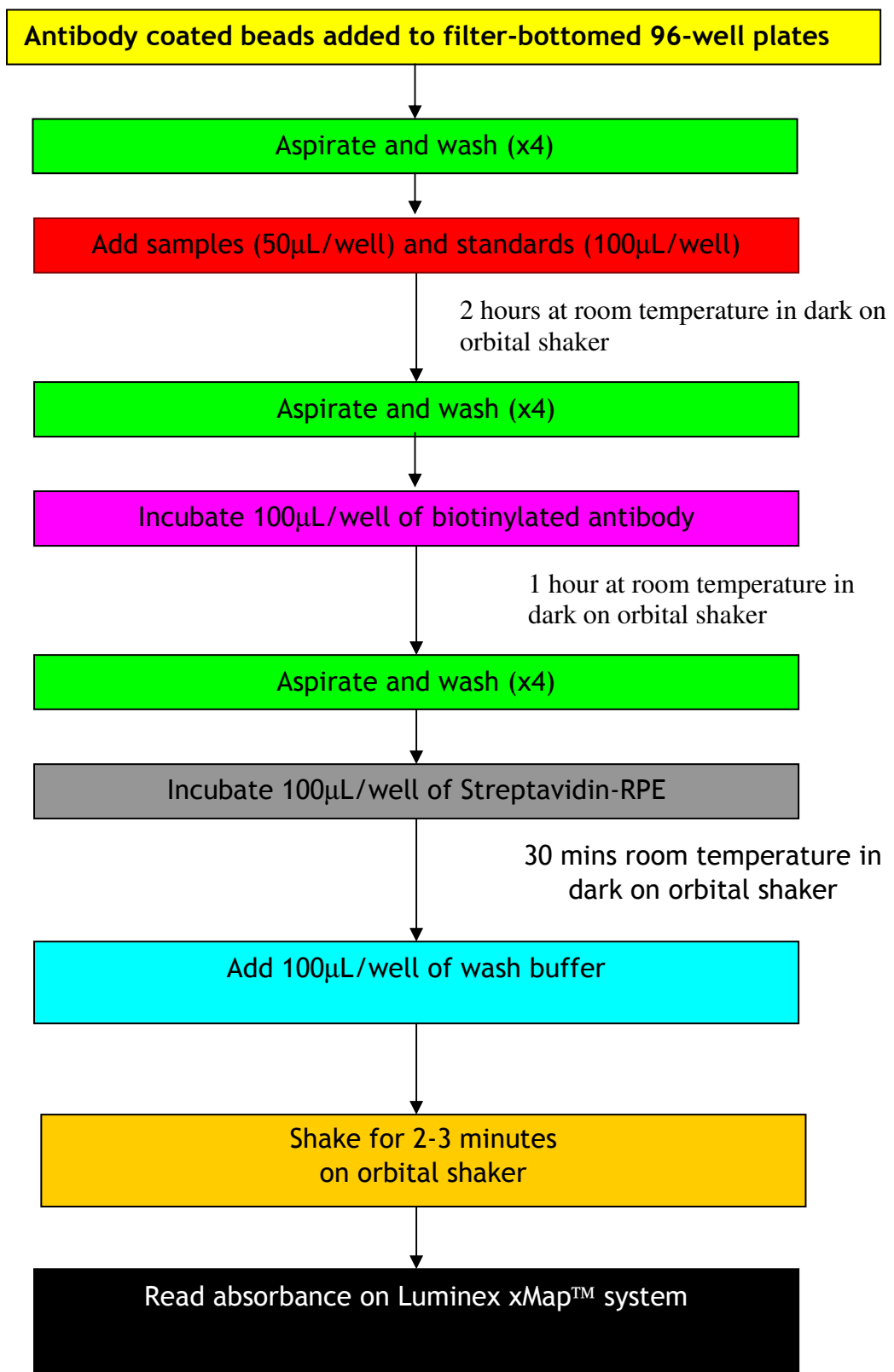
Table 2.1

Lower detection limits and interassay variability for human 10-plex cytokine kit

CYTOKINE	LOWER LIMIT OF DETECTION pg.mL⁻¹	INTERASSAY VARIATION
GM-CSF	15	9.1%
IFN- γ	5	9%
IL-1 β	15	8.8%
IL-2	6	9.5%
IL-4	5	8.7%
IL-5	3	7.5%
IL-6	3	7%
IL-8	3	9.8%
IL-10	5	9.8%
TNF- α	10	8.3%

The intra-assay variability is low, and the manufacturers quote correlation coefficients between 0.92 and 0.99 for the cytokines assayed. The schema for analysis is outlined below (Figure 2.9).

Figure 2.9
Methodology for human 10-plex cytokine analysis



2.2.2.6 Blood Tests

2.2.2.6.1 Sampling

Blood was taken either by peripheral venepuncture or sampled from a total implantable venous access device (TIVAD), such as a Port-a-cath™. The following samples were taken: 1ml Lithium-Heparin tube, 1ml ethylenediaminetetraacetic acid (EDTA) tube, 5mls plain tube. In subjects in whom Angiotensin Converting Enzyme (ACE) genotype had not previously been established, then a further 5ml EDTA tube was taken and stored. In all cases, blood samples were taken prior to exercise being undertaken.

2.2.2.6.2 Immediate sample processing

Once taken, blood was sent to the Great Ormond Street Hospital Chemical Pathology laboratory for analysis of C-reactive protein (CRP), and also to the Haematology laboratory for analysis of full blood count with white cell differential and also erythrocyte sedimentation rate (ESR).

The plain tube was spun in a centrifuge (Rotina 46R, Wolf Laboratories Limited, Pocklington, UK) at 3000 revolutions per minute for 7 minutes. Serum was then extracted and stored in cryovials (Nalge-Nunc International, Roskilde, Denmark) at -80°C for later cytokine analyses. In the event of deoxyribonucleic acid (DNA) being required to establish ACE genotype, a 5ml EDTA sample was stored at -80°C for future DNA extraction and ACE genotyping.

2.2.2.6.3 C-reactive protein

C-reactive protein was measured in the Chemical Pathology laboratory at Great Ormond Street Hospital for Children using the Ortho Diagnostics 5.1 chemistry analyser (Ortho-Clinical Diagnostics, High Wycombe, Buckinghamshire, UK) and an automated immune-rate immunoassay (Vitros CRP Slide, Ortho-Clinical Diagnostics, High Wycombe, Buckinghamshire, UK). This uses technology that is very similar to the ELISA technique described above (section 2.2.5.2.1).

The patient sample, when added will bind to beads coated with phosphorylcholine (capture beads). A monoclonal anti-CRP antibody labelled with horseradish peroxidase (HRP) acts as a signal generator for the assay. First, the patient sample is added to the slide. Secondly, CRP binds to the capture bead and also to the monoclonal anti-CRP

antibody/HRP complex. The addition of hydrogen peroxide results in enzyme mediated oxidation of dye, the optical density of which is measured at a wavelength of 670nm. The optical density is directly proportional to the CRP concentration of the sample. Calibration is regularly performed using the Vitros Chemistry Products calibration kit 7 in order that accuracy of measurement can be analysed against standards of known CRP concentration.

2.2.2.6.4 Full blood count

An automated full blood count was performed in the Haematology laboratory at Great Ormond Street Hospital for Children, along with measurement of differential white blood cells including a neutrophil count using the Sysmex XE 2100 analyser (Sysmex Corporation, Kobe, Japan). The analyser is factory-calibrated, and undergoes in-house quality control testing on a daily basis.

Paediatric tubes (EDTA) are manually introduced into the Sysmex XE 2100 machine via a sampler, from which blood is sampled and diluted. Blood then moves through a tube thin enough that cells pass by one at a time, so that cell characteristics can be assessed in five channels, by a combination of fluorescence flow cytometry and electrical impedance techniques. This series of measurements enables a full differential white cell analysis to be obtained.

2.2.2.6.5 Erythrocyte sedimentation rate (ESR)

This test was performed in the Haematology laboratory at Great Ormond Street Hospital for Children using a manual Westergren method (International Council for Standardization in Haematology Expert Panel on Blood Rheology *J Clin Pathol* 1993). In short, venous blood was mixed with an aqueous solution of sodium citrate and allowed to stand in an upright pipette tube, 200mm in length and filled to the zero mark. Erythrocyte sedimentation rate (ESR) was measured as the observed fall of the red blood cells, in millimetres over 1 hour.

2.2.2.6.6 Serum cytokine analyses

Enzyme-linked immunosorbent assays (ELISA) were used to measure serum IL-8 concentration, using an ultrasensitive IL-8 kit (KHC0084, BioSource International Inc., Camarillo, California), and identical methodology to section 2.2.2.5.2.1.

2.2.2.6.7 Angiotensin Converting Enzyme (ACE) genotyping

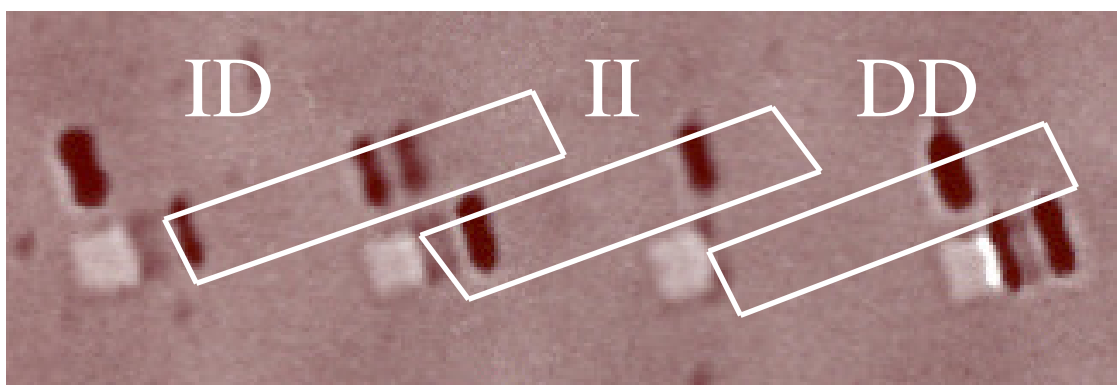
Dr Christina Hubbart (Research Assistant, Department of Cardiovascular Genetics, Rayne Institute, University College London) undertook DNA extraction, and carried out angiotensin converting enzyme (ACE) genotyping on my behalf.

DNA was extracted using a 'salting-out' protocol for extracting DNA from whole blood (Miller *et al.* 1988).

ACE genotype was determined by using a three-primer technique that has been previously described (O'Dell *et al.* 1995). Primer ratios corresponded to 50pmol of *ACE1* (D-specific oligonucleotide) and *ACE3* (common oligonucleotide) and 10pmol of *ACE2* (I-specific oligonucleotide) in a 20µL reaction. This yielded amplification products of 84bp for the D allele and 65bp for the I allele. Amplification products were visualised using ethidium bromide staining on 7.5% polyacrylamide 'MADGE' (Multiple Array Diagonal Gel Electrophoresis) gels. Accuracy was ensured by using replica PCRs set up without the I-specific primer (*ACE2*) to confirm the presence of the D allele.

Figure 2.10

PCR for ACE genotype using 3-primer system



2.2.2.7 Echocardiography

Trans-thoracic echocardiography was undertaken on all study patients, using a commercially available cardiovascular ultrasound system (GE Vivid System 7, GE Healthcare, UK). The timing of the echocardiogram was such that it was carried out in the resting state, and prior to cardiopulmonary exercise testing. M-mode, two-dimensional and Doppler echocardiographic images were acquired from the standard parasternal, apical and subcostal views. Echocardiogram was carried out with the subject either supine, or in the left lateral position, by experienced paediatric sonographers (Gill Riley, Katie Maslin, and Anna Barlow) from the Department of Cardiology, Great Ormond Street Hospital for Children, London, UK.

Cardiac structure and morphology were assessed before moving on to carry out measures as laid out in the proforma of echocardiographic measures in CF devised for this study. The proforma is similar to that used in a study of cardiac function in adult CF patients (Fraser *et al.* 1999), and was agreed independently by two Consultant Paediatric Cardiologists at Great Ormond Street (Dr Graham Derrick and Dr Jan Marek). Standard M-mode measurements at end diastole were made measuring left ventricular wall thickness at both interventricular septum and posterior wall (IVSd and LVPWd), as well as right ventricular wall thickness (RVWd). Left ventricular dimensions were measured in both systole and diastole (LVDs and LVDd). These measures were taken from the parasternal long axis view as recommended by the American Society of echocardiography (Sahn *et al.* 1978).

The left ventricle fractional shortening (%) was assessed by M-mode measures of fractional shortening using the following equation (Quinones *et al.* 1978):

$$\text{LV fractional shortening (\%)} = \frac{\text{LVD (end diastole)} - \text{LVD (end-systole)}}{\text{LVD (end-diastole)}} \times 100$$

LV ejection fraction (%) was derived from the M-mode measures of ventricular dimensions as follows (Feigenbaum *et al.* 1972):

$$\text{LV ejection fraction (\%)} = \frac{[\text{LVD (end diastole)}]^3 - [\text{LVD (end-systole)}]^3}{[\text{LVD (end-diastole)}]^3} \times 100$$

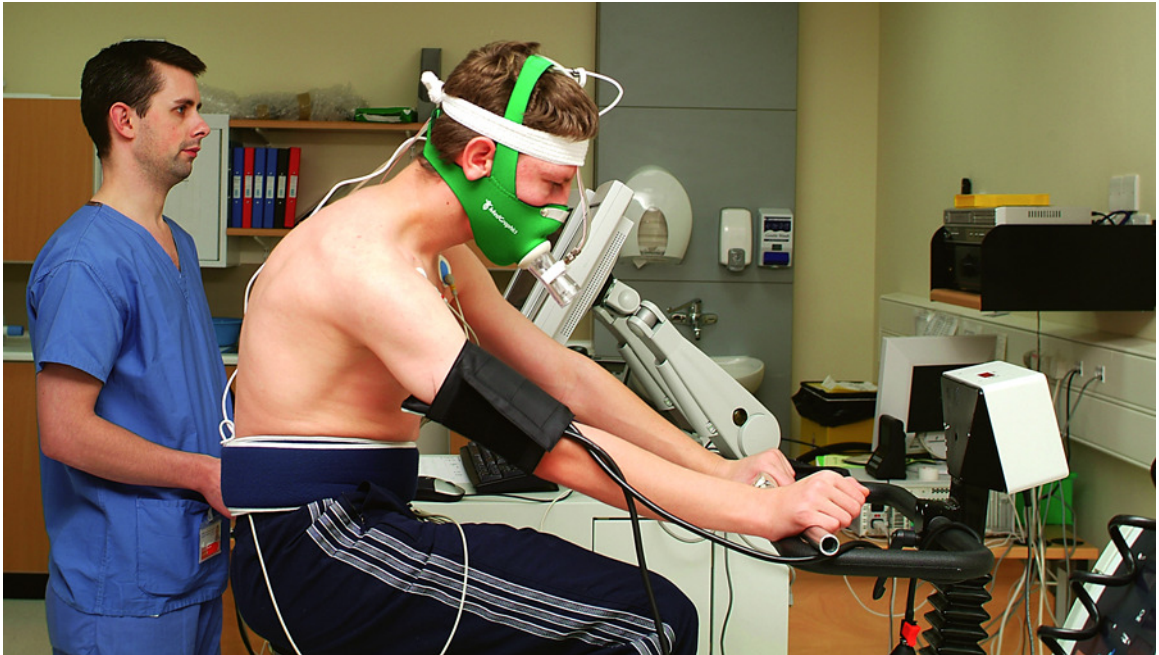
Systolic pulmonary artery pressure (or sPAP) was measured from the continuous Doppler signal of the tricuspid regurgitation (TR) gradient (if any TR present) using the modified Bernoulli equation described by Fraser *et al.* (1999) : $Dp=4v^2$ where Dp is the calculated peak pressure difference between RV and right atrium (RA), and v is the measured peak flow velocity of tricuspid regurgitant jet. The Dp value is added to RA pressure which is assumed to be 5mmHg in children (Du *et al.* 2004).

2.2.2.8 Cardiopulmonary Exercise Testing

A cardiopulmonary exercise testing protocol was already in place for children attending the exercise laboratories at the Institute of Child Health. Cardiopulmonary exercise testing was carried out with breath by breath ventilatory gas analysis using a commercially available metabolic cart (MedGraphics, USA). Prior to each test, the mass flow sensor was calibrated using a standard 3 litre syringe. Two-point sensor gas calibration (Zirconium oxide fuel cell and infrared CO₂ analyser) was performed prior to each test using standard calibration gases (16% O₂:4% CO₂:balance nitrogen and 24% O₂:balance nitrogen, Scott Medical Gases, Breda, Holland). 12-lead electrocardiography (ECG) was recorded before and during all tests (CardioControl Workstation, Welch Allyn, Delft, Holland). The subjects were fitted with nose-clips and wore a full face mask (MedGraphics, USA), connected to an expiratory limb pneumotachograph. The pneumotachograph measured flow, and a sampling line monitored mouth tidal pCO₂ and pO₂. This protocol can be used to calculate maximal oxygen consumption (VO₂max) during maximal exercise, and also peak oxygen consumption (VO₂peak) and anaerobic threshold (AT) for patients with CF undergoing near-maximal or submaximal exercise.

Subjects were exercised on an electronically-braked cycle ergometer. The choice of ergometer was dependent upon the height of the child. For children between 128 and 135cm. tall, the Ergoline 900 (Ergoline, Blitz, Germany) was used, and for those >135cm. tall, the Lode Excalibur (Lode, Groningen, Holland) was chosen. Transcutaneous oxygen saturations (SpO₂) and heart rate were continuously recorded during exercise using a pulse oximeter (Nonin, USA) which was placed over the right supraorbital artery fixed with a bandanna, this site being the least prone to exercise artefact. Oxygen uptake (VO₂), carbon dioxide production (VCO₂), end-tidal CO₂ (etCO₂), tidal volume (V_T) and respiratory rate were measured.

Figure 2.11
Subject undergoing cardiopulmonary exercise testing (CPET)



2.2.2.8.1 Cardiopulmonary Exercise Testing (CPET) Inclusion Criteria

Inclusion Criteria

Height >128cm (needed to reach pedals)

Age >8 years [Cadence maintenance difficult below this age]

Exclusion Criteria

Aged <8years

Height<128cm

Contagious illness or colonisation
i.e. Methicillin-resistant *Staphylococcus aureus* (MRSA)

Mental or behavioural deficiency preventing use of ergometer

Gross motor or neurological deficit preventing use of ergometer

2.2.2.8.2 Cardiopulmonary Exercise Testing (CPET) Protocol

Subjects were advised to refrain from eating for at least 2 hours before the test, and to avoid strenuous exercise for 24 hours before CPET. Although clear fluids were allowed, caffeine-containing drinks were avoided on the day of testing. All subjects underwent a cardiopulmonary physical examination. Resting heart rate (HR) and SpO₂ were recorded using a pulse oximeter (Nonin, USA), which was placed over the right supraorbital artery.

Direct estimation of maximal voluntary ventilation (MVV), measured by maximally deep and rapid respiration over a 12-second period was performed. As well as a direct measurement of MVV, a prediction was made using childhood CF-specific reference equations for calculating MVV (Stein *et al.* 2003).

The exercise testing protocol was adjusted to each individual child, such that a ramping protocol of increasing workload was chosen to achieve 8 to 12 minutes of incremental exercise testing. The expected achievable workload of 3W/kg was adjusted dependent upon the fitness quartile into which the child places themselves, and the pre-test spirometry values to attain a suitable ramping protocol.

Data will be collected over 4 phases of exercise:

- a. Resting data [3 to 5 minutes]
- b. Loadless cycling [3 minutes]
- c. Incremental exercise test [8 to 12 minutes]
- d. Recovery phase [4 minutes]

a. Resting data [3-5 minutes]

The subject was seated on the ergometer, and monitored during tidal breathing, until respirations became regular and gas-exchange measures stabilised.

b. Loadless cycling [3 minutes]

The subject cycled with no resistance (ergometer set to zero watts), whilst maintaining a steady cadence at 60-80rpm. This allowed muscles to warm up, and steady state gas exchange was achieved at the end of the warm-up phase.

c. Incremental exercise test [8-12 minutes]

Using a predetermined ramping protocol, exercise was undertaken with a steadily increasing workload, whilst maintaining a cadence at 60-80rpm. The aim was to reach maximal exercise capacity in an 8-12 minute period.

d. Recovery phase [4 minutes]

The subject was monitored in the recovery period. Resistance was removed from the ergometer, and gentle pedalling (20-30rpm) was encouraged to prevent syncope. Gas exchange data was recorded for 4 minutes, and the ECG recording continues until heart rate returned to near-baseline.

A summary of the full CPET protocol is provided in appendix 4.

2.2.2.8.3 Cardiopulmonary Exercise Testing (CPET) Measured Parameters

The following parameters were measured:

- Heart Rate
- Blood Pressure (checked manually every 1-2 minutes during CPET)
- Oxygen utilisation (VO_2) - Measured by indirect calorimetry
- Carbon dioxide elimination (VCO_2) - Measured by indirect calorimetry
- Work Rate (Watts)
- Respiratory Rate
- Tidal volumes
- Minute ventilation
- End-tidal CO_2 (etCO_2)
- Oxygen saturations (SpO_2)

Clinically important outcome measures include:

- **VO₂ peak**

The peak oxygen consumption level during the test – i.e. level of oxygen utilisation at maximal exercise.

- **(VO₂ max)**

Although often quoted as a primary outcome measure in exercise-related research, the VO₂ max is of limited usefulness in children for two reasons. Firstly, such volitional tests may be submaximal, and secondly, the plateau of oxygen uptake at maximal exercise occurs only in a proportion of childhood tests.

- **Minute ventilation (V_E)**

The product of tidal volume and respiratory rate, the minute ventilation on exercise represents the ventilatory adaptation to exercise. Using the measured or predicted MVV, an assessment of breathing reserve at the end of exercise can be made (V_Emax – MVV).

- **Anaerobic Threshold**

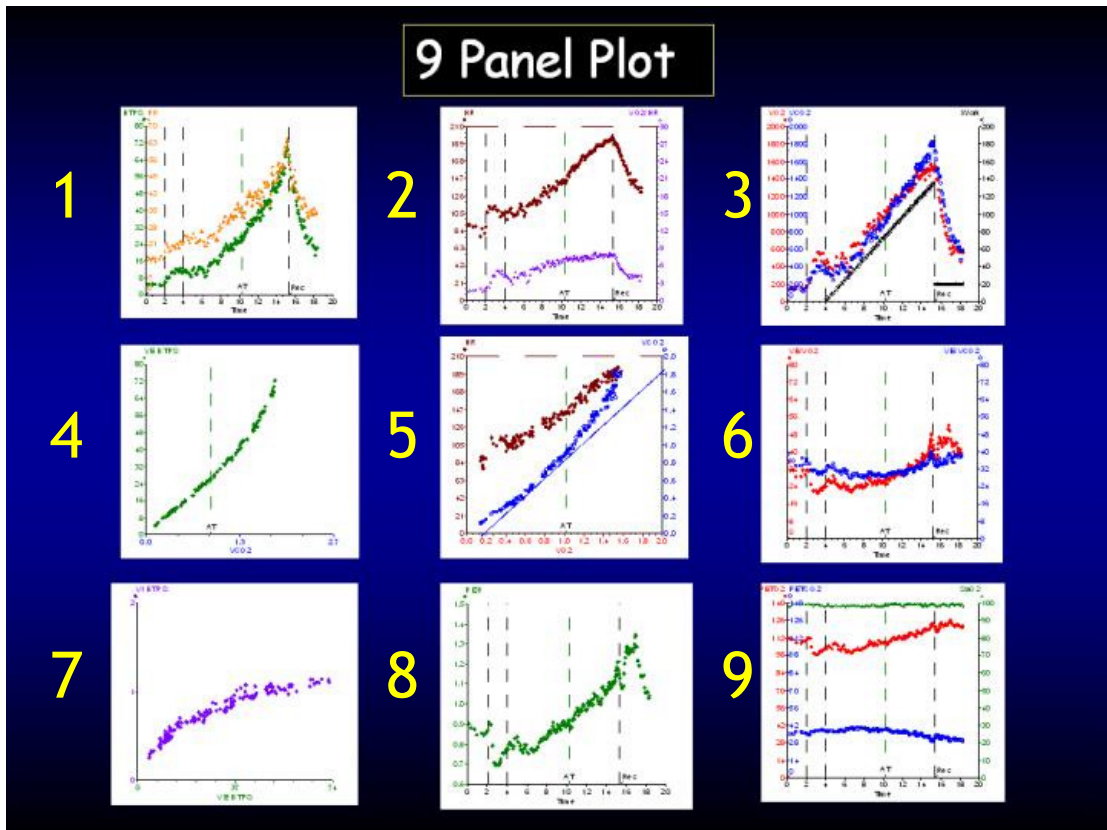
Anaerobic threshold is calculated as the level of exercise at which aerobic energy is supplemented by anaerobic mechanisms. The increasing lactic acid burden is buffered by bicarbonate, and the resultant carbon dioxide load removed through an increase in ventilation. The cycle test can determine the point at which this happens using the V-slope method (explained below) to ascertain the point at which carbon dioxide elimination increases. This is seen to be a reproducible measure of Anaerobic Threshold (Thin *et al* 2002).

Anaerobic Threshold has advantages over VO₂max as a primary outcome as it is more easily reproducible between occasions and between patients. The reason for this is because VO₂max requires the patient to get up to maximal exercise capacity (perhaps a tall order for 8-16 year old CF patients), whereas AT is a sub-maximal measure. AT is quoted as the level of oxygen uptake at which significant metabolic supplementation through anaerobic metabolism takes place.

2.2.2.8.4 Cardiopulmonary Exercise Testing (CPET) Data Generation

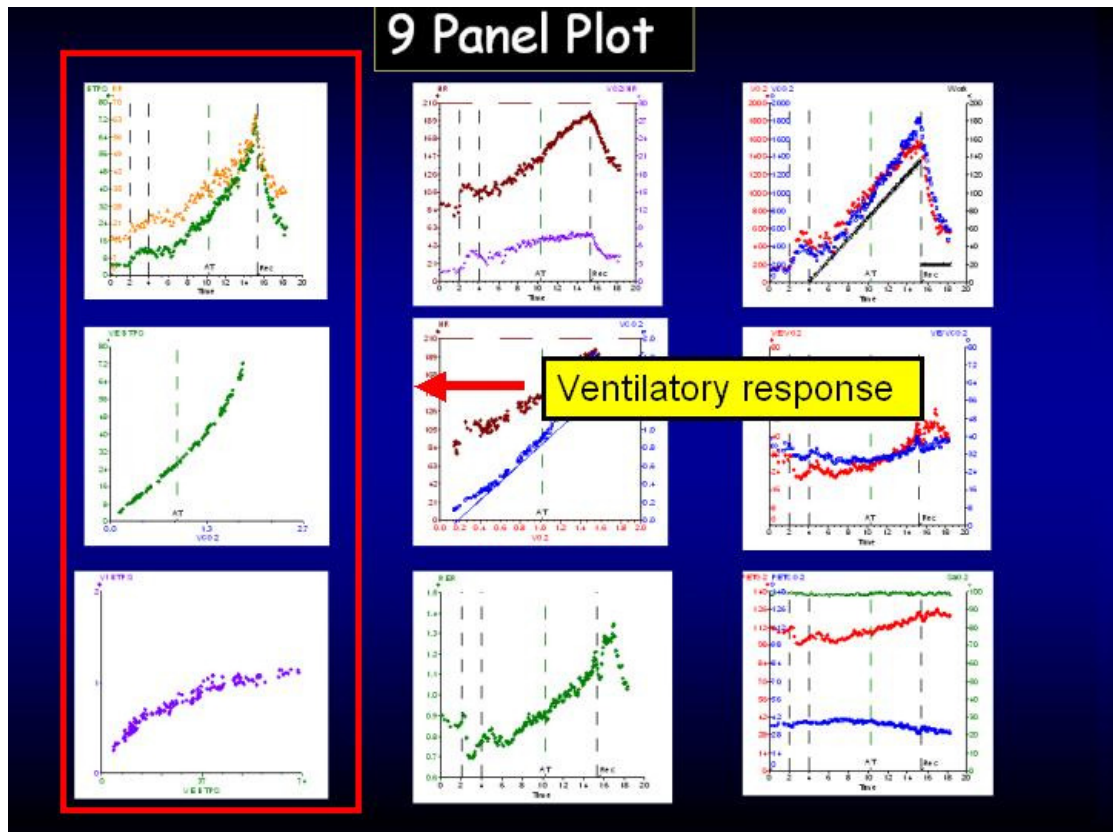
Following completion of a cardiopulmonary exercise test (CPET), a nine-panel plot of test data is generated. An example of such a plot taken from a healthy control subject is displayed below (Figure 2.12):

Figure 2.12
9-panel CPET testing plot from healthy subject



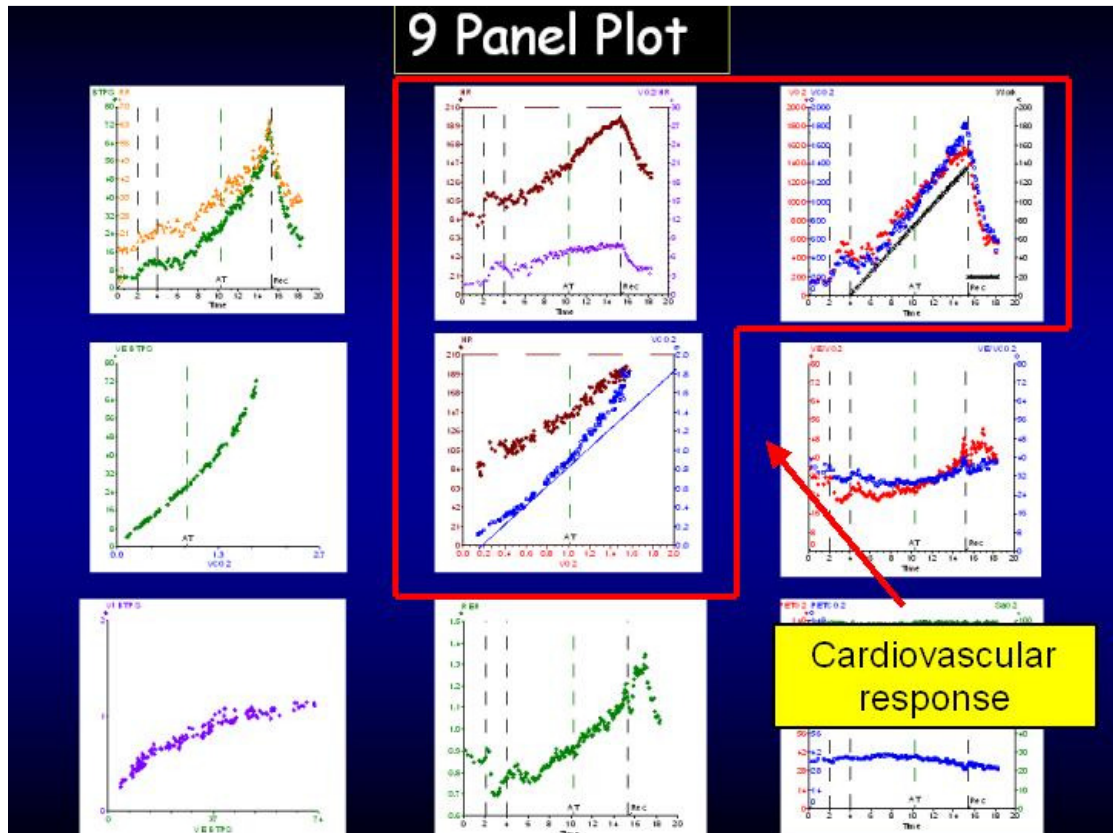
Each of the nine panels provides different information regarding the exercise test.

The ventilatory responses are best assessed in panels 1, 4 and 7.



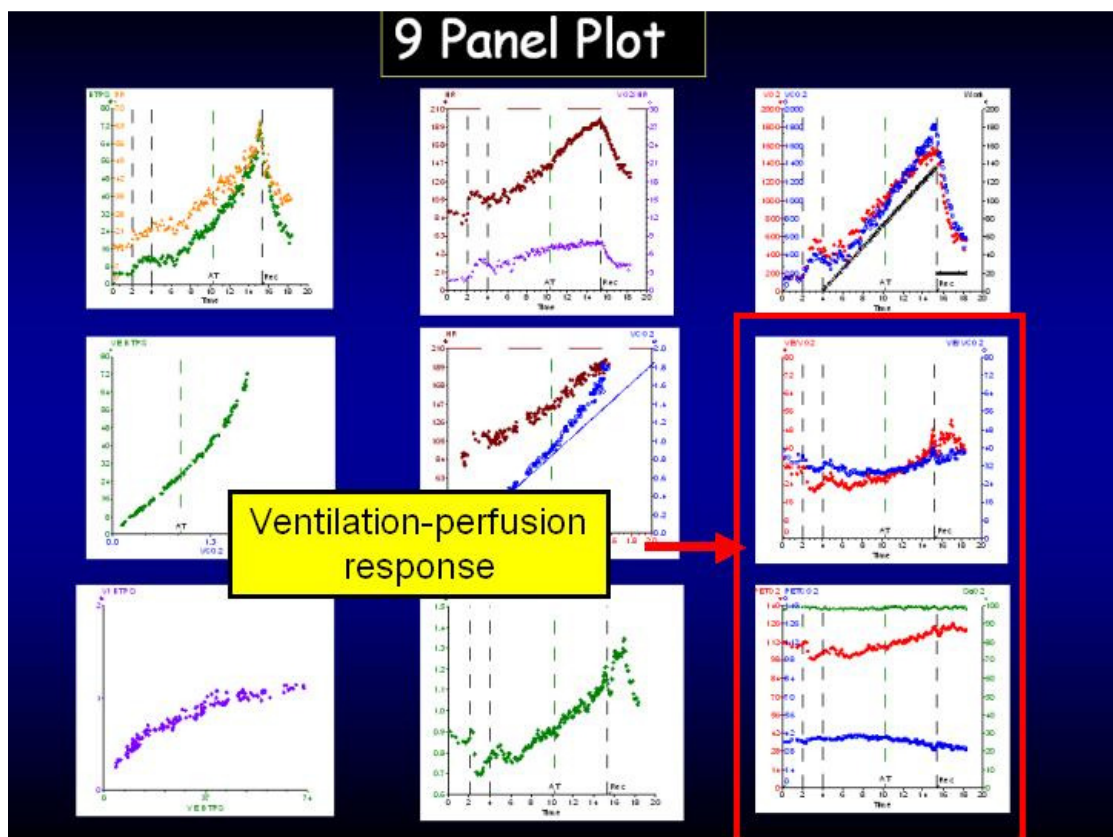
Panel 1 is a plot of minute ventilation (V_E , green) and respiratory rate (RR, orange) against time. Panel 4 is a plot of minute ventilation versus carbon dioxide elimination (VCO_2), and panel 7 is a plot of V_E against tidal volume (V_T).

Cardiac indices are best assessed using panels 2, 3 and 5.



Panel 2 shows heart rate (HR, brown) and oxygen uptake per heart beat (VO_2/HR – the so-called ‘oxygen pulse’, purple) against time. The oxygen pulse provides an indirect measure of the contribution made by stroke volume to cardiac output. Panel 3 shows oxygen uptake (VO_2 , red), carbon dioxide elimination (VCO_2 , blue), and workload (black) plotted against time. Panel 5 illustrates HR (brown) and VCO_2 (blue) against time. The point at which an upkick in CO_2 production occurs can be assessed from this plot – the so-called V-slope method - and it is likely that this is the point at which anaerobic threshold (AT) has been reached. The buffering of lactate results in an obligatory increase in VCO_2 relative to VO_2 at AT due to increased CO_2 production as HCO_3^- buffers lactate. In addition to V-slope analysis, the point at which AT occurs requires confirmation in panels 6 and 9.

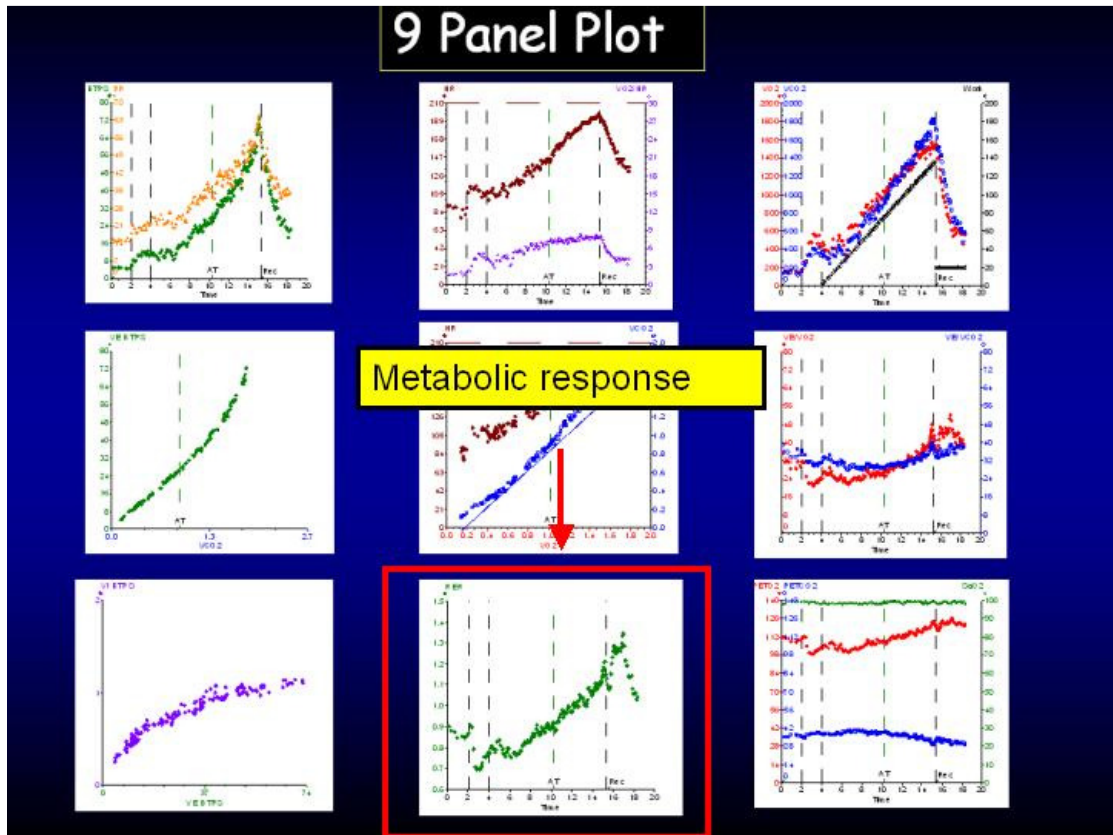
Panel 6 and 9 reflect the responses of the pulmonary vasculature during exercise.



Panel 6 shows the ventilatory equivalents for oxygen (V_E/VO_2 , red) and carbon dioxide (V_E/VCO_2 , blue) plotted against time, whereas panel 9 illustrates oxygen saturations (SpO_2 , green), and end-tidal concentrations of oxygen (etO_2 , red) and carbon dioxide ($etCO_2$, blue) against time.

Anaerobic threshold (AT) occurs at a point where V_E/VO_2 begins to rise whilst V_E/VCO_2 is either falling or has reached a plateau (panel 6). i.e. hyperventilation occurs with respect to O_2 but not CO_2 at the point where AT occurs due to a period of isocapnic buffering. This period is so-called because there is a lack of ventilatory compensation for the evolving metabolic acidosis (Wasserman 1978). During this period, circulating HCO_3^- buffers the metabolic acid produced, and the period lasts for around 1-2 minutes, after which ventilatory compensation is required leading to an increase in the ventilatory equivalent for CO_2 ($\uparrow V_E/VCO_2$). On panel 9, the point at which AT occurs is marked by a rise in end-tidal oxygen (etO_2) levels as a result of hyperventilation., though $etCO_2$ remains constant due to the phenomenon of isocapnic buffering described above.

Panel 8 is an assessment of the metabolic response to exercise and is a plot of respiratory exchange ratio (RER, green) against time.



2.2.2.9 Assessment of skeletal muscle function

Measures of both proximal (quadriceps maximal isometric voluntary strength), and distal (hand-grip strength dynamometry) muscle strength were made on each subject.

2.2.2.9.1 Quadriceps maximal isometric voluntary contraction

To quantify quadriceps muscle strength, maximal voluntary isometric contractions were measured using a custom-built isometric dynamometer (University College [Royal Free Campus] Medical School Physiology Department). Briefly, the subject was sat in an upright position with knee flexed to 90° . A clamp placed just above the ankle transmitted knee extensor force via an inextensible chain to a strain gauge bar. A secure strap was placed around the hips to prevent hip extension. The subject was instructed to try to extend their knee and generate as much force as possible (Figure 2.13). Subjects were requested to maintain the maximal contraction for 2-3 seconds and then relax. Forces from the strain gauge bar were amplified and displayed on a chart recorder. The strain gauge was calibrated with a set of known masses, in order to derive a regression equation for force measurement. Regular 3-point calibrations with 5 and 20kg masses were undertaken during the duration of the study.



Figure 2.13
Quadriceps muscle strength testing

2.2.2.9.2 Quadriceps force reference data

The reference data of Backman *et al.* (1989) was used, where age and gender-specific means and standard deviations for maximal quadriceps contraction are given. Maximal forces achieved by voluntary quadriceps contraction in our study subjects were converted to standard deviation scores (SDS) using this validated reference data (Backman *et al.* 1989).

2.2.2.9.3 Handgrip Strength Dynamometry

An estimation of distal skeletal muscle strength was made using peak force measurements made using a Jamar hydraulic hand dynamometer (Sammons Preston, Bolingbrook, IL, USA). Prior to testing, each child was instructed on how to use the Jamar dynamometer. Testing was carried out with the child standing straight with their back against a wall, and arm held in 90 degrees of elbow flexion (Figure 2.14).

Grip strength was tested with the dynamometer in the second position, as used in the collection of the quoted reference data (Mathiowetz *et al.* 1986). Alternate measures of dominant and non-dominant hand grip strength were made one minute apart, to a total of three measures on each side. The maximal force generated was recorded.

Figure 2.14

Child performing handgrip strength dynamometry



2.2.2.9.4 Handgrip Strength Reference Data

The paediatric reference data of Mathiowetz *et al.* (1986) was used, where age and gender-specific means and standard deviations for dominant and non-dominant hand grip strength are given. Maximal handgrip strength values from our study subjects were converted to standard deviation scores (SDS) using this validated reference data (Mathiowetz *et al.* 1986).

2.2.2.10 Bone densitometry

Bone mineral content (BMC) was assessed in 8 to 12 year-old CF subjects using dual X-ray absorptiometry (DXA) scanning of the lumbar spine (Lunar Prodigy, GE Medical Systems) – Figure 2.15. This was used to estimate bone mineral density (BMD) for a given bone area: $BMD = BMC / \text{Bone area}$. BMD was expressed as a standard deviation score related to an age-matched population. However, it is known that a BMD z score measure will underestimate BMD in a small subject, and overestimate in tall subjects (Fewtrell *et al.* 2003). Therefore, a volumetric assessment – the bone mineral apparent density (BMAD) was undertaken to adjust for calculated bone volume rather than bone area.

Figure 2.15

Child undergoing a DXA scan



2.2.2.11 Quality of life assessment: Cystic Fibrosis Questionnaire [CFQ]

Psychological well-being was assessed using the Cystic Fibrosis Questionnaire (CFQ). The CFQ is a quality of life assessment that was developed in France (Henry *et al* 1997). It has since been adapted for use in a number of countries (Rozov *et al.* 2006) including Spain, Germany (Henry *et al* 1998), USA (Quittner *et al* 2000), Holland (Klijn *et al* 2004), as well as current adaptation for UK use (Bryon 2004). In each of these translations, both backward and forward translations were completed and re-validation of the questionnaire was carried out. A CD-ROM package that accompanies the questionnaire enables nine quality of life (QOL) dimensions to be assessed (Table 2.2).

Table 2.2

Quality of life dimensions that are assessed by the UK-CFQ

1	Physical functioning
2	Energy/well-being
3	Emotions
4	Social limitations
5	Role
6	Embarrassment
7	Body image
8	Eating disturbances
9	Treatment burden

This study used the UK-translated CFQ (Bryon 2004 – Personal communication). The CFQ-14+ was used for children aged over 14 years, and is a self-assessed questionnaire that takes around 10 minutes to complete. A self-reporting format for 12 and 13 year-olds was used for this age group. The CFQ child P is a parent-proxy evaluation used for children aged 6 to 13 and was used for the younger children in our study. An interviewer format questionnaire for children aged 6-11 year-olds is also available but was not used in this study. Statistical analyses were carried out to assess overall differences in quality of life, or in individual QOL dimensions with respect to hypoxia. Copies of the various CFQ questionnaires used are attached in Appendix 3.

2.3 *In vitro* Study Methods

2.3.1 Tissue Culture – Cell lines and incubator conditions

Tissue culture was carried out using immortalised cell lines (Dieter Gruenert, University of Vermont, USA). Firstly, a non-CF airway epithelial cell line was chosen - 1HAEO⁻, a transformed human airway epithelial cells which form tight junctions (Cozens *et al.* 1994). Secondly a Cystic Fibrosis airway epithelial cell line was chosen - CFBE41o⁻, a transformed cystic fibrosis bronchial epithelial cell line that maintains cell polarity.

The cells were grown in 25ml flasks (T25, Nalge-Nunc International, Roskilde, Denmark), in a standard tissue culture incubator (Galaxy-R, Wolf Laboratories Limited, Pocklington, UK) maintained at 37⁰C and with a CO₂ level of 5%. Humidity was maintained by keeping a filled tray with a water-copper sulphate mixture at the base of the incubator.

Each 500ml of tissue culture media (SIGMA M7278 minimum essential medium) was enriched with foetal calf serum (50mls), L-glutamine (5mls) and a procaine penicillin/Streptomycin (PenStrep) mixture (5mls). Media was changed every 2-3 days, and passaging of cells was carried out on a twice weekly basis with cells being split 1 in 5.

2.3.2 Tissue Culture – Hypoxic conditions

2.3.2.1 Hypoxia as single variable

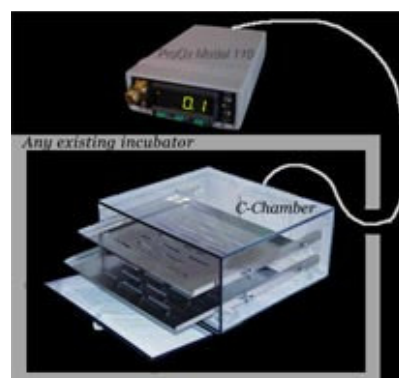
Hypoxia was controlled by a Pro-Ox controller (Biospherix Limited, Redfield, NY13437, USA), regulating oxygen concentration within a two-shelf tissue culture chamber (Biospherix Limited), housed in a standard incubator (Galaxy-R, Wolf Laboratories Limited, Pocklington, UK) as illustrated below (Figure 2.16). Carbon dioxide within the cell culture chamber was maintained at 5% using a Pro-CO₂ controller (Biospherix Limited, Redfield, NY13437, USA).

Figure 2.16

- a) Cell culture chamber and Pro-Ox controller during hypoxia experiments
- b) How controller supplies gas to chamber



a

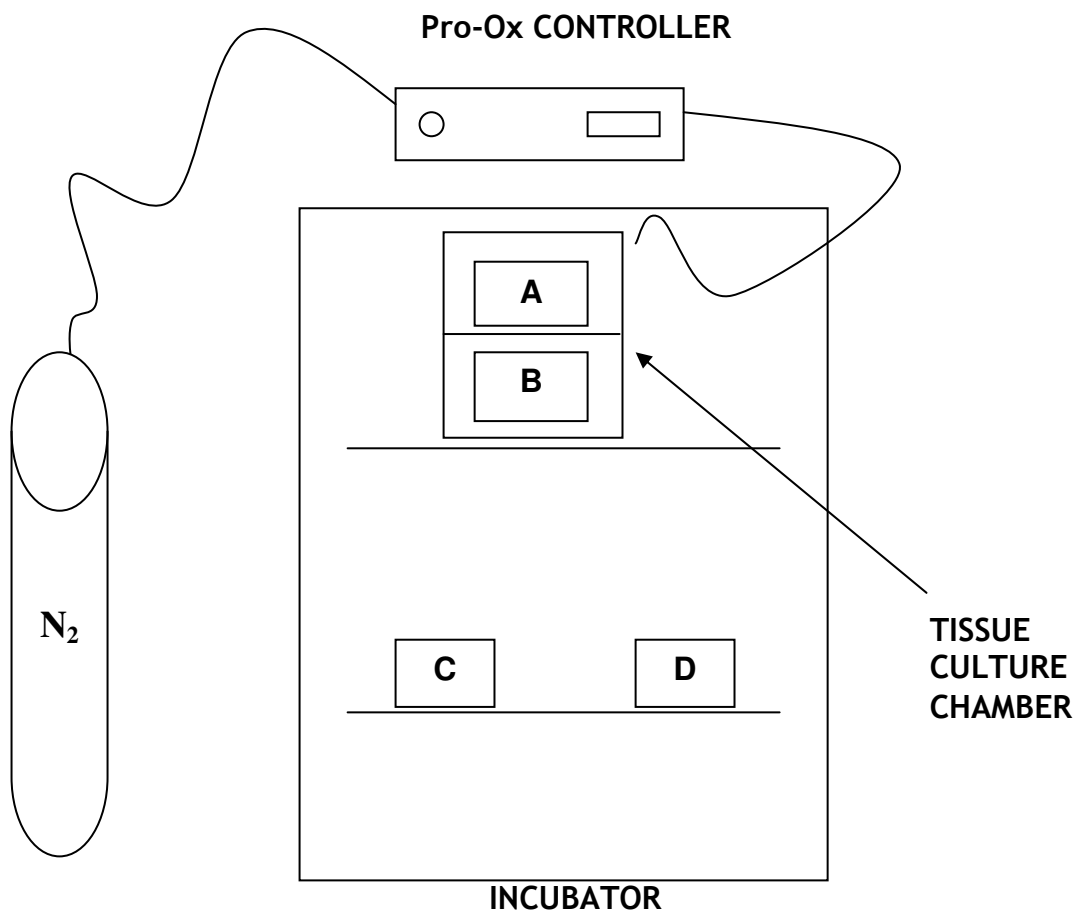


b

In order to maintain a control environment, cells were incubated simultaneously in normoxic conditions (incubator) alongside hypoxic conditions (tissue culture chamber) as shown below (Figure 2.17). For the purpose of these experiments, cells were seeded in 25ml flasks (T25, Nalge-Nunc International, Roskilde, Denmark) at a seeding density of 0.7×10^6 per flask in 5mls media. Flasks for time zero, 8 hours, 24 hours and 48 hours were each placed in the chamber/incubator for each cell line and environmental condition. i.e. Samples for each cell line and under hypoxic and normoxic conditions with a separate flask for each time-point.

Figure 2.17

Regulation of hypoxia using Nitrogen flow via Pro-Ox controller



CELL CULTURE:

- A - HAE in hypoxic conditions
- B - CFBE in hypoxic conditions
- C - HAE in air (normoxia)
- D - CFBE in air (normoxia)

Simultaneous incubation of air versus hypoxia for each of the cell lines was carried out over a 48 hour period. Sampling of cell supernatant was carried out at time zero, and then at 8, 24 and 48 hours thereafter.

The following conditions were studied:

<u>TC Chamber</u>	<u>Incubator</u>
Normoxia 21%	Normoxia (Control)
Hypoxia 10%	Normoxia (Control)
Hypoxia 5%	Normoxia (Control)
Hypoxia 1%	Normoxia (Control)
Hypoxia 0.1%	Normoxia (Control)

2.3.2.2 Hypoxia and lipopolysaccharide stimulation of cells

As above, hypoxia within the tissue culture chamber was controlled using a Pro-Ox controller, and a Pro-CO₂ controller was used to maintain CO₂ at a level of 5%. Tissue culture media were enriched with lipopolysaccharide (LPS) derived from *Pseudomonas aeruginosa* (L9143 Sigma-Aldrich, St Louis, MO, USA), with either high (50µg.mL⁻¹ media), or low (10µg.mL⁻¹) levels of LPS added, or none.

Cells were seeded in 25ml flasks (T25, Nalge-Nunc International, Roskilde, Denmark) and incubated simultaneously in normoxic conditions (incubator) alongside hypoxic conditions (tissue culture chamber). Seeding density was 0.7x10⁶ cells per flask in 5mls media. Flasks for time zero, 24 hours and 48 hours were each placed in the chamber/incubator for each cell line and environmental condition. i.e. High, low and no LPS samples for each cell line and under hypoxic and normoxic conditions with a separate flask for each time-point.

Cells were studied at 10%, 1% and 0.1% levels of hypoxia and compared to normoxic cells that had been grown simultaneously.

2.3.2.3 Hypoxia and LPS prolonged time course experiment

The previous time-course experiments described ran for 48 hours. A further experiment was carried out comparing extreme hypoxia (0.1%) with normoxia over a 4-day period with sampling carried out at 0, 24, 48, 72 and 96 hours.

Flasks had either high-dose LPS ($50\mu\text{g}\cdot\text{mL}^{-1}$ media) added or no LPS at all. This enabled comparison of cell lines with and without LPS under extreme hypoxic and normoxic conditions at each timepoint.

2.3.3 Sampling of cell supernatant

At each timepoint in the experiments, cell supernatant was sampled from the T25 flask, and stored in cryovials (Nalge-Nunc International, Roskilde, Denmark) at -80°C for later cytokine analyses.

2.3.4 Cell counting

Following aspiration of supernatant, the remaining cells within the flask were washed with phosphate-buffered saline (PBS). 2mls of Trypsin-EDTA solution was then added and the flask was replaced in the incubator for a further 5 minutes to ensure that cells had separated and were floating. 5mls of tissue culture media was then added to the flask to inactivate the trypsin. The resulting solution was placed in a 15ml tube, and centrifuged at 1200 rpm for 5 minutes. Following this, a pellet representing the cells was found at the bottom of the centrifuge tube. Media was aspirated and the pellet was re-suspended in 5mls medium and thoroughly mixed using a vortex mixer.

16 μL of Trypan blue solution (T8154 Sigma-Aldrich, St Louis, MO, USA) was added to 16 μL of the cell suspension, and cells were counted using a haemocytometer and microscope (Carl-Zeiss, Jena, Germany). Cells are selective in the compounds that pass through the membrane, and in a viable cell Trypan blue is not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue colour under a microscope. Live cells or tissues with intact cell membranes are not coloured. Since live cells are excluded from staining, this staining method is also described as a dye exclusion method.

2.3.5 Enzyme-linked immunosorbent assay (ELISA) for interleukin-8 (IL-8)

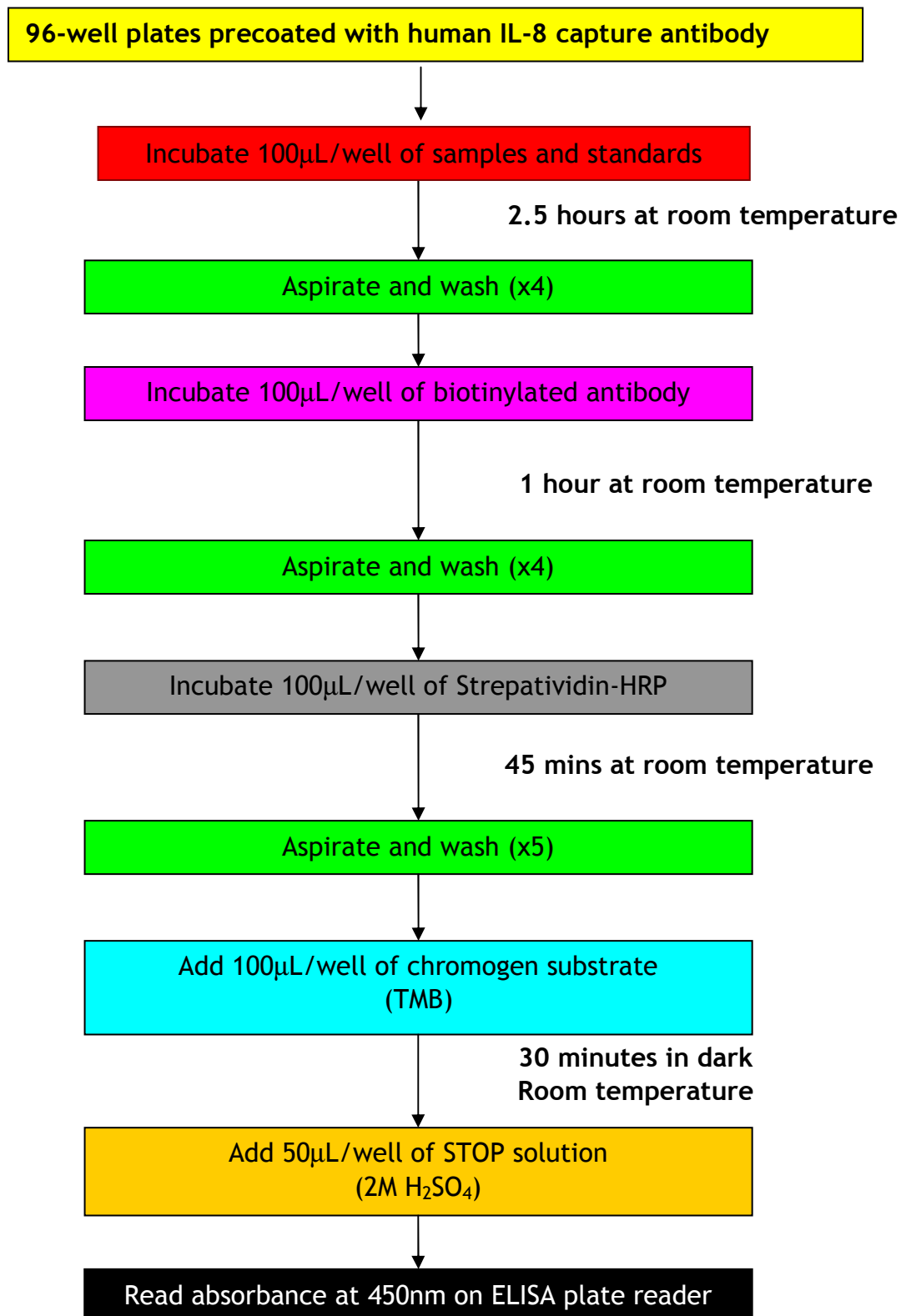
Enzyme-linked immunosorbent assay (ELISA) was used to measure the concentration of IL-8 in tissue culture cell supernatant for both human airway epithelial cells and CF bronchial epithelial cells. A human IL-8 (ELH-IL8-001, RayBiotech Inc., Norcross, GA, USA) ELISA kit was used, which detects IL-8 levels in the range of 5 pg.mL⁻¹ to 2000 pg.mL⁻¹. In order for IL-8 levels to be detected in our cell supernatant samples, the samples were centrifuged (Rotina 46-R, Wolf Laboratories Limited, Pocklington, UK) to allow cell debris to sink to the bottom of the tube, and then diluted to 1 in 100 using an assay diluent provided with the ELISA kit. The protocol was based on manufacturer's instructions. The procedure is very similar to that described in section 2B 2.4.2.1, utilising a 96-well plate that had already been pre-coated with capture antibody. Standards were generated by 1:3 serial dilutions from 2000 pg.mL⁻¹ down to a lowest of 2.74 pg.mL⁻¹. Some differences are noted between this method and that used in section 2.2 2.5.2.1, and these are summarised below (Figure 2.18).

Optical densities of each well were read using an ELISA plate reader (Revelation 4.02, Dynex Technology, Chantilly, VA, USA) at 450nm (reference filter 650nm). Cytokine concentrations in the unknown samples were read from the curve generated from a known standard included on each plate. This technique allowed confidence to detect IL-8 within the range of 2.74 to 2000 pg.mL⁻¹.

The highest coefficient of variation quoted for this kit for replication of responses on the same plate (intra-plate precision) is <10%, and coefficient of variation for responses between plates is <12% (inter-plate precision).

Figure 2.18

Methodology for human interleukin-8 ELISA (RayBiotech)



2.4 Statistical Methods

2.4.1 Data Storage

The results of the clinical examination, lung function tests, skeletal and respiratory muscle strength measures, sleep and exercise study data, measures of inflammation and also responses to the Cystic Fibrosis questionnaires were entered into an Excel spreadsheet (Microsoft Office 2000, Seattle, USA). When all the data was collected, the Excel spreadsheet was copied into a single database in the statistics package, Statistical Package for the Social Sciences (SPSS) for windows v13.0 (SPSS, Chicago USA). It was from this SPSS database that all of the results were analysed.

2.4.2 Data Analysis

In order to select an optimal definition of clinically-important hypoxia in CF, receiver-operator (ROC) curves were used to quantify (or directly observe) the sensitivity and specificity characteristics of a number of definitions of hypoxia (both existing and *de novo*) for a variety of outcome measures pertaining to CF inflammation.

The index study group were studied as a whole, as well as dichotomised into hypoxic and normoxic subgroups that were compared and contrasted with each other. The data were analysed in this format to establish specifically whether any differences noted between groups can be attributed to hypoxia. Comparisons between the groups were done using Mann-Whitney analyses. Gene frequencies for ACE genotype were analysed for Hardy-Weinberg equilibrium using chi-squared statistics. The raw data for this calculation is added in Appendix 5.

Assessments of association between parameters were made using Spearman rank correlation co-efficients, expressed as r^2 values. Limits of agreement between measures were assessed using the methodology of Bland and Altman (Bland and Altman 1986).

Repeatability of *in vitro* data was assessed using repeated measures analysis of variance (ANOVA) tests. Bonferroni corrections for multiple contrasts not assuming equal variances were used.

CHAPTER 3:

Investigation of arterial oxygen saturations during sleep, at rest and during exercise in children with cystic fibrosis

3.1 Introduction

Episodes of hypoxia may occur at times of physiological stress in CF, namely during sleep, exercise, air travel, and infective CF chest exacerbations. Repeated hypoxic insults (such as those during sleep and exercise) may exacerbate lung inflammation, and impair the pulmonary circulation and quality of life (see Chapter 1). However, there is no consensus as to how hypoxia should be defined in CF, and no universal definitions of hypoxia exist in CF regardless of disease state.

The lack of definition of nocturnal hypoxia hampers the description of the prevalence of CF hypoxia in clinical practice, and perhaps has led to both under-recognition and under-treatment of this potentially important clinical entity.

Although arterial oxygen sampling is carried out in adult practice, this is not routine in children as it is painful. Pulse oximetry remains the main tool by which arterial oxygen saturation (SpO_2) is assessed in children with CF. Traditionally, resting awake values are recorded in clinic, with most centres also undertaking some form of annual exercise test during which SpO_2 will be measured.

The definition of exercise-induced arterial hypoxia (EIAH) is also controversial and different definitions are used in clinical exercise testing. EIAH has been defined as a fall in SpO_2 of $\geq 4\%$ from baseline in CF (Narang *et al.* 2003), and also in healthy children (Nourry *et al.* 2004). If this represents a fall in SpO_2 from 93% to 89%, one could deem this likely to represent a significant physiological drop in PaO_2 from 70 to 60mmHg. However the definition holds less well in a very fit child whose SpO_2 fall from 99% to 95% during exercise (PaO_2 fall from 100 to 80mmHg).

3.2 Aims

The aim of this chapter is to delineate the definition of hypoxia which has the best sensitivity/specificity characteristics in the detection of elevated markers of inflammation, in order to select an optimal definition of hypoxia in children with CF.

3.3 Methods

3.3.1 Assessment and definition of hypoxia in CF

Existing sleep hypoxia definitions used in paediatric practice and in the CF literature (see Section 3.3.1.2), as well as several new definitions, were applied to various markers of inflammation measured in our study population. Receiver-operator (ROC) curves were used to quantify (or directly observe) the hypoxia cut-off with the best trade-off between sensitivity and specificity for each given measure of inflammation. From these analyses, a definition of hypoxia can be obtained that best associates with inflammation (the purported effector mechanism by which hypoxia may contribute to lung disease), and this can then be used to dichotomise the study population. The inter-relationship of hypoxia and clinical status can then be explored (Chapter 4).

3.3.1.1 Resting Hypoxia in CF

Resting SpO₂ was obtained in all subjects using a supraorbital probe (Nonin, USA) - Section 2.2.2.2. Resting hypoxia in CF has been previously defined as an awake SpO₂ < 95% (Coffey *et al.* 1991).

3.3.1.2 Nocturnal Hypoxia in CF

Briefly, overnight pulse oximetry recording was carried out in the subjects' home using a Minolta Pulsox-3i oximeter (Konica Minolta Holdings inc., Singapore) – see section 2.2.1.1. Data were downloaded using the Download 2001 software package (Stowood Scientific Instruments, Oxford, UK) as per section 2.2.1.1. The various methods of defining nocturnal hypoxia in CF are discussed in detail in chapter 1, and include:

1. Specified time with low saturations:

- a. SpO₂ <90% for >5% night (Frangolias *et al.* 1999)
- b. >25% sleep time with SpO₂ below 93%
- Alder Hey definition (Southern, *Personal Communication* 2004)
- c. Greater than 10 minutes of continuous sleep recording with SpO₂ <92%
- CHoP definition (Narang, *Personal Communication* 2007)

2. Minimum sleep SpO₂: (Tepper *et al.* 1983, Bradley *et al.* 1999)

3a. Measures of mean sleep SpO₂: (Coffey *et al.* 1991)

3b. Lowest hourly mean sleep SpO₂ below 90%: (Versteegh *et al.* 1990)

3.3.1.3 Exercise Induced Hypoxia in CF

Transcutaneous oxygen saturations (SpO₂) and heart rate were continuously recorded during exercise using a pulse oximeter (Nonin, USA) placed over the right supraorbital artery, and fixed with a bandanna (Section 2.2.2.8).

Exercise-induced arterial hypoxia (EIAH) has been classified as a 4% drop in SpO₂ from baseline in both CF (Narang *et al.* 2003) and non-CF (Nourry *et al.* 2004) children.

3.3.2 Measures of inflammation

3.3.2.1 C-Reactive Protein (CRP)

CRP was analysed in the Chemical Pathology department, GOS Hospital, London, using an Ortho Diagnostics 5.1 chemistry analyser (as per section 2.2.2.6.3). Elevated CRP was taken as >7mg.L⁻¹, for although the published upper limit of normal CRP is 3.1 mg.L⁻¹ (Kratz *et al.* 2004), the analyser could not accurately quantify values below 7mg.L⁻¹.

3.3.2.2 Full blood count and Erythrocyte Sedimentation Rate (ESR)

FBC and differential, along with ESR were analysed in the department of Haematology, GOS Hospital, London (as per sections 2.2.2.6.4 and 2.2.2.6.5). An elevated white cell count was taken as >11x10⁹.mL⁻¹, and elevated peripheral blood neutrophil count as >8 x10⁹.mL⁻¹ in keeping with published upper limits (Kratz *et al.* 2004).

3.3.2.3 Serum cytokine analysis

An ultrasensitive ELISA assay for quantification of serum interleukin-8 (IL-8) levels was undertaken, as described in section 2.2.2.6.3. Elevated IL-8 was classified as a level >10pg.mL⁻¹ (Zeigenhagen *et al.* 1999).

3.3.2.4 Number of intravenous antibiotic courses in year preceding study

Data on the number of intravenous antibiotic courses that the patient had received in the year prior to the study were obtained from the GOS hospital CF database.

3.4 Results

3.4.1 Resting daytime Hypoxia

None of our subjects had resting SpO₂ below 95%. Median recorded resting SpO₂ was 99% (range 95% to 100%).

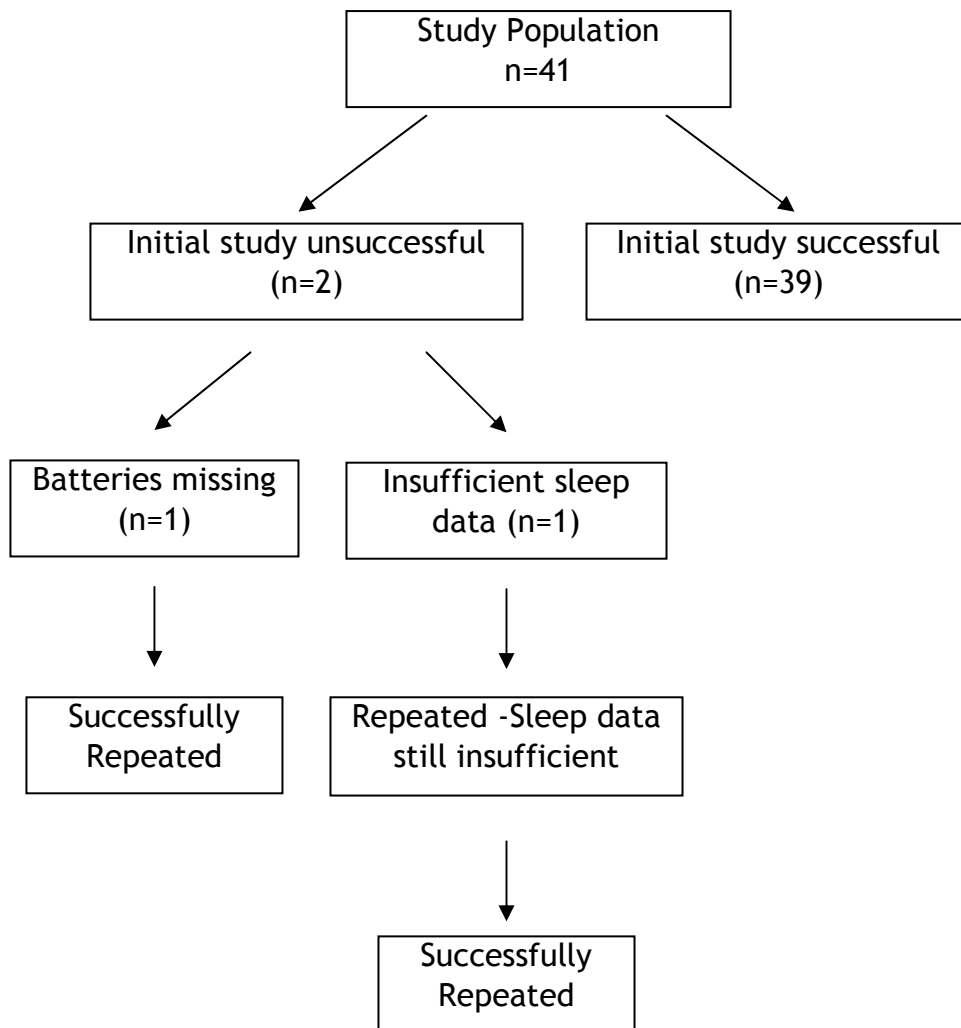
3.4.2 Nocturnal Hypoxia

3.4.2.1 Demographic data

Overnight oximetry was successfully performed in all forty-one study subjects (Figure 3.1).

Figure 3.1

Oximetry data regarding success of recordings



The study demographics are shown below (Table 3.1).

Table 3.1
Distribution of nocturnal SpO₂ in our study population

		Median	Range	p-value
Age (years)		12.7	8-16.2	
Mean sleep SpO₂ (%)		95.7	90.6-97.9	
Mean sleep SpO₂ (%) by Sex	20 Male	96.6	91-97.8	p=0.02 *
	21Female	94.8	90.6-97	
Mean sleep SpO₂ (%) by CFTR Genotype				
- ΔF508/ΔF508	25	96.1	91-97.8	p=0.19**
- ΔF508/Other	13	94.6	90.6-97.2	
- Other/Other	3	95.5	95.4-97.3	
Mean sleep SpO₂ (%) by <i>Pseudomonas aeruginosa</i> status				
- Previous Infection	37	95.6	90.6-97.8	p=0.54*
- Never infected	4	96.2	94.1-97	
Mean sleep SpO₂ (%) by <i>Staphylococcus aureus</i> status				
- Previous Infection	33	95.7	90.6-97.8	p=0.49*
- Never infected	8	95.5	91.7-96.6	

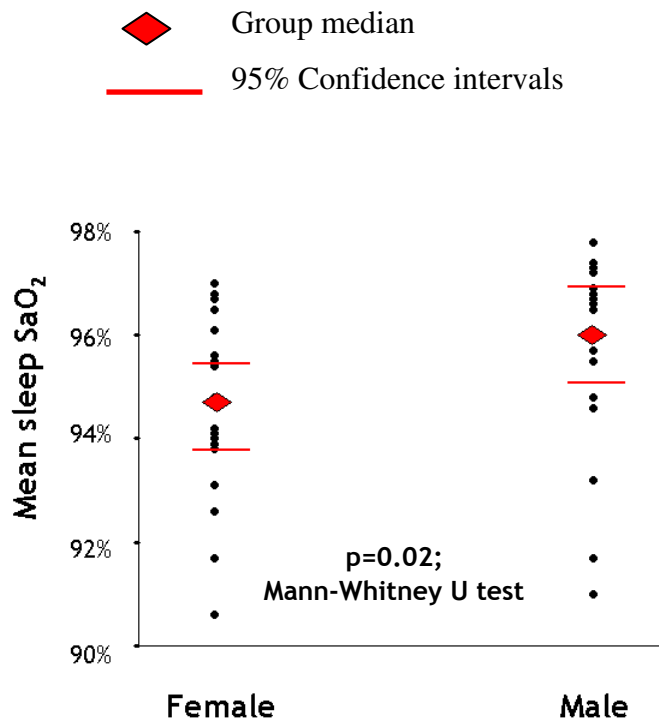
* Mann-Whitney U-test

** Kruskal-Wallis test

These data suggest that female patients have a statistically lower mean sleep SpO₂ than do their male counterparts. This is displayed graphically below (Figure 3.2) showing individual mean sleep SpO₂ plots for boys and girls along with group medians and 95% confidence interval data.

Figure 3.2

Distribution of nocturnal mean sleep SpO₂ in boys (n=20) and girls (n=21) with CF



This difference would be in keeping with previous observations of a gender gap in CF mortality (Rosenfeld *et al.* 1997). There is a spread of values however, and numbers are small.

The small numbers in our study precluded meaningful multivariate analysis to adjust for potential covariates such as age, genotype differences, lung function, infective status, exercise capacity and physical activity levels (Selvadurai *et al.* 2004).

3.4.2.2 Classification of nocturnal hypoxia

As outlined above in chapter 1 and also in section 3.3.1.2, a number of definitions for sleep hypoxia in CF and means of quantifying hypoxia have been proposed. Each of these definitions was then applied to our study population.

3.4.2.2.1 Nocturnal Hypoxia defined as SpO₂ <90% for >5% night

This definition was applied to a Canadian adult CF population aged 17-53 years (Frangolias et al 1999). Applying this definition to our study population, 3 subjects (8%) meet criteria for nocturnal hypoxia (Table 3.2).

Table 3.2

Demographics of study patients with nocturnal hypoxia using Frangolias' definition

	Median (IQR) Age	Sex	Mean (sd) Sleep SpO ₂	Median (IQR) time spent <93%
Hypoxic Group [SpO ₂ <90% for >5% sleep time] (n=3)	15.3 (14.5,15.5)	2F/1M	91.6 (1.3)	81% (33,92)
Normoxic Group (n=38)	12.4 (10, 14.2)	19F/19M	95.5 (1.6)	0.1% (0)

Applying a definition of SpO₂ below 90% for >10% sleep time (Smith *et al.*1994) detected the same three patients as the Frangolias definition of SpO₂ below 90% for >5% sleep time (3.3.2.2.1).

3.4.2.2.2 Alder Hey definition

On applying a definition of SpO₂ <93% for >25% study (Southern, *Personal Communication* 2004) to our study population, 6 subjects (15%) from our study population met criteria for nocturnal hypoxia (Table 3.3).

Table 3.3

Demographics of study patients with nocturnal hypoxia using Alder Hey definition

	Median (IQR) Age	Sex	Mean (sd) Sleep SpO ₂	Median (IQR) time spent <93%
Hypoxic Group [SpO ₂ <93% for >25% sleep time] (n=6)	14.9 (12.5-15.7)	2M : 4F	91.8 (0.9)	75.2% (45.5-83.7)
Normoxic Group (n=35)	12.3 (10-14.2)	19M : 17F	95.8 (1.3)	0.1% (0-2.5)

3.4.2.2.3 Children’s Hospital of Philadelphia (CHoP) definition

The CHoP definition (SpO₂ below 92% for >10 minutes of continuous sleep recording, in the absence of obstructive sleep apnoea) was applied to our study population (Table 3.4). Although an assumption is made that the desaturation in CF is not due to obstruction, the same six individuals were identified as with the application of the Alder Hey definition (Section 3.4.2.2.2) to our study population.

Table 3.4

Demographics of study patients with nocturnal hypoxia using CHoP definition

	Median (IQR) Age	Sex	Mean (sd) Sleep SpO ₂	Median (IQR) time spent <92%
Hypoxic Group [SpO ₂ <92% for 10 continuous minutes of sleep] (n=6)	14.9 (12.5-15.7)	2M : 4F	91.8 (0.9)	163 (82-265) minutes
Normoxic Group (n=35)	12.3 (10-14.2)	19M : 17F	95.8 (1.3)	0 (0-4) minutes

3.4.2.2.4 Lowest hourly mean sleep SpO₂ below 90%

The software package used in the methodology was unable to calculate lowest hourly mean SpO₂, preventing this methodology being applied to our study population.

3.4.2.2.5 Minimum sleep SpO₂

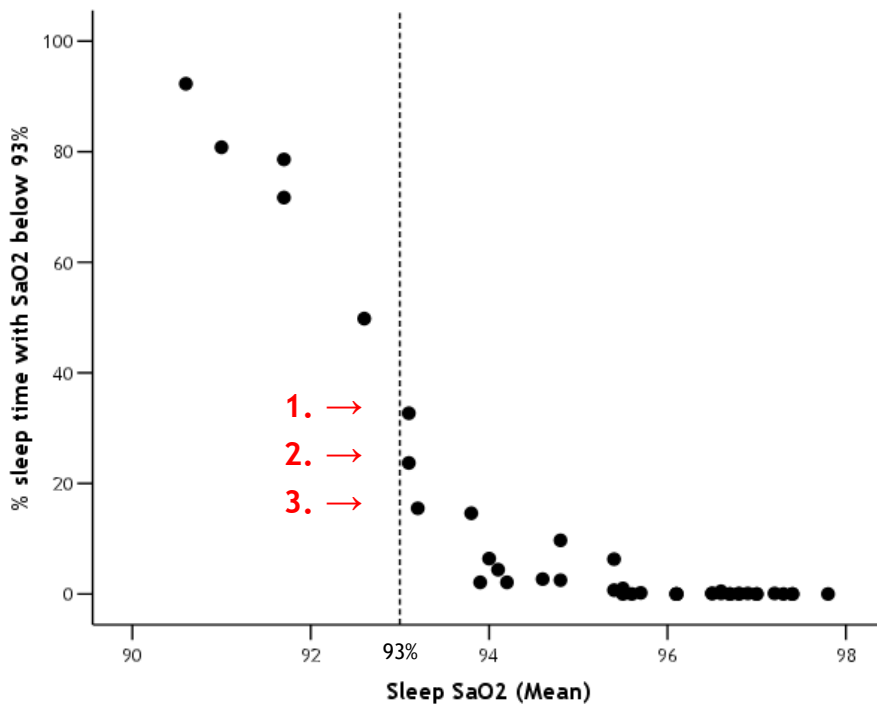
Data on minimum sleep SpO₂ in CF have been previously reported [Tepper *et al.* 1983, Bradley *et al.* 1999]. However, no definition of the extent of desaturation required to constitute sleep hypoxia was given in either study.

3.4.2.2.6 Mean sleep SpO₂

Although mean sleep SpO₂ has been reported by previous authors, no definition of the mean sleep SpO₂ below which hypoxia exists is given. Data collected were used to investigate associations with other clinical parameters. A relationship appears to exist between mean SpO₂ and the time spent with SpO₂ below 93%. This is illustrated below (Figure 3.3).

Figure 3.3

Mean sleep SpO₂ versus % time spent with SpO₂ below 93%



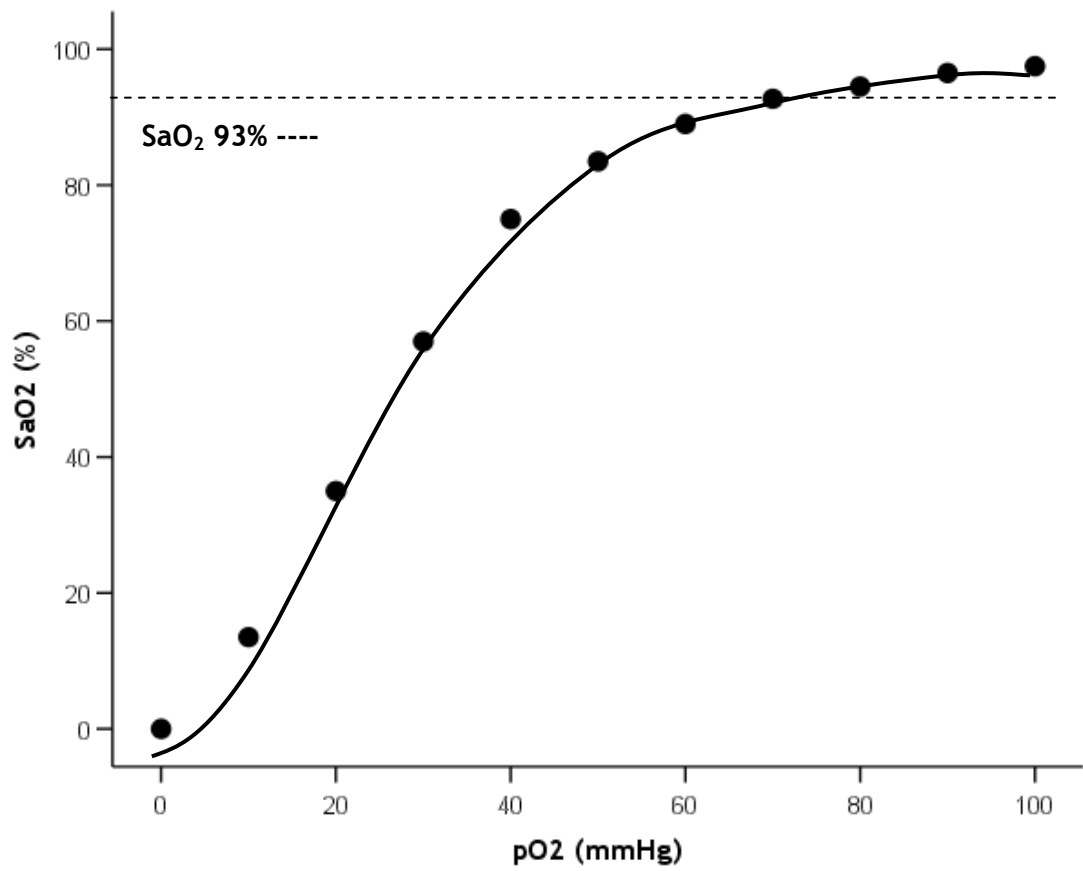
However, mean sleep SpO₂ are a poor indicator of oxygenation during sleep. SpO₂ are not normally distributed about a mean. Rather, periods of low saturation occur during REM sleep due to lower tidal volumes as a result of skeletal muscle inactivity, with higher SpO₂ in non-REM phases, resulting in negative skew of Gaussian distribution. This is illustrated clearly in Figure 3.3 above, whereby subjects 1, 2 and 3 all have mean SpO₂ around 93% but spend approximately 35, 25 and 15% of the night respectively with SpO₂ below 93%.

A cut-off value taken where hypoxia is considered to be important (such as time below 93%) does therefore appear a more useful measure. A cut-off of 93% is taken from the point on the oxygen dissociation curve at which small changes in pO₂ result in exponential decreases in % SaO₂. Extrapolation from the following graph (Figure 3.4) suggests that a SaO₂ value of 93% equates to a pO₂ of around 70mmHg (9kPa).

Figure 3.4

The oxygen-haemoglobin dissociation curve

[Source: Ganong WF, Review of Medical Physiology (14th Edition), 1989]



3.5 Development of optimal definition for nocturnal hypoxia

3.5.1 Application of existing and *de novo* hypoxia definitions to the detection of clinically-important adverse outcomes

One of the main objectives of this study was to define a level of sleep hypoxia that is clinically relevant to patient well-being in children with CF. Existing sleep hypoxia definitions were taken and compared to newly-derived cut-offs for hypoxia in CF to assess their sensitivity and specificity in the detection of adverse outcomes with regard to CF inflammation.

For the reasons defined in section 3.3.2.2.6 above, time spent with SpO₂ below 93% was considered to be potentially the most important ‘cut-off’ point at which the deleterious effects of hypoxia might be seen. The percentage of sleep time with SpO₂ below 93% at which sensitivity and specificity for predicting occurrence of adverse effects is optimal remains to be established. Proportions of acceptable quality sleep time spent below 93% were considered thus, along with existing definitions:

- 30% sleep time spent with SpO₂ below 93%
- 25% sleep time spent with SpO₂ below 93% (Alder Hey definition)
- 20% sleep time spent with SpO₂ below 93%
- 15% sleep time spent with SpO₂ below 93%
- 10% sleep time spent with SpO₂ below 93%
- 5% sleep time spent with SpO₂ below 93%
- 5% sleep time spent with SpO₂ below 90% (Frangolias)
- 10 continuous minutes of sleep with SpO₂ below 92% (CHoP definition)

The adverse clinical outcomes considered were:

- Elevated C-reactive protein
- Elevated Erythrocyte Sedimentation Rate
- Elevated white blood cell counts
- Elevated neutrophil counts
- Elevated serum interleukin-8 levels
- Need for intravenous antibiotic therapy

In order to develop a *de novo* definition of clinically-important sleep hypoxia, these measures were used to assess sensitivity and specificity for each of the cut-offs considered. ROC curves were used to quantify (or directly observe) the hypoxia cut-off with the best trade-off between sensitivity and specificity for each given outcome measure.

Hypoxia is a stimulus of the NF- κ B pathway of inflammation, both directly and also has a potential indirect effect by inhibition of CFTR trafficking (Bebok *et al.* 2001, Guillebot *et al.* 2008) and facilitation of PA growth (Worlitzsch *et al.* 2002). Upregulation of NF κ B via direct or indirect (CFTR-mediated) pathways would promote IL-8 production, and neutrophil recruitment as well as increases in IL-6 - a precursor for CRP production (Heikkila *et al.* 2007), and TNF α .

- **C-reactive protein $>7\text{mg.L}^{-1}$**

Upregulation of the NF κ B inflammatory cascade may result in increases in IL-6 and consequent C-reactive protein (CRP) production. Elevated CRP was taken to be a level $>7\text{mg.L}^{-1}$. The upper limit of published normal laboratory reference values (Kratz *et al.* 2004) is 3.1mg.L^{-1} , but our laboratory was only able to measure with precision those CRP values $>7\text{mg.L}^{-1}$.

- **Elevated Erythrocyte Sedimentation Rate
[$>17\text{mm.hr}^{-1}$ (men) and $>25\text{mm.hr}^{-1}$ (women)]**

Erythrocyte sedimentation rate (ESR) is a non-specific marker for systemic inflammation. Elevated ESR was taken to be a level $> 17\text{mm.hr}^{-1}$ for men and $> 25\text{mm.hr}^{-1}$ for women in accordance with published normal laboratory reference values (Kratz *et al.* 2004).

- **Total white cell count $>11 \times 10^9.\text{mL}^{-1}$**

Upregulation of the NF κ B inflammatory cascade may result in increases in IL-8 production, and subsequent neutrophil chemoattraction. A total white cell count (WBC) $>11 \times 10^9.\text{mL}^{-1}$, was defined as abnormally high in accordance with published normal laboratory reference values (Kratz *et al.* 2004).

- **Peripheral blood neutrophil count $>8 \times 10^9 \cdot \text{mL}^{-1}$**

Upregulation of the NF κ B inflammatory cascade may result in increases in IL-8 production, and subsequent neutrophil chemoattraction. A peripheral blood neutrophil count (neutrophils) $>8 \times 10^9 \cdot \text{mL}^{-1}$, was defined as abnormally high in accordance with published normal laboratory reference values (Kratz *et al.* 2004).

- **Serum interleukin-8 (IL-8) $>10 \text{pg} \cdot \text{mL}^{-1}$**

Upregulation of the NF κ B inflammatory cascade may result in increases in IL-8 production. A serum IL-8 value $>10 \text{pg} \cdot \text{mL}^{-1}$ was taken to represent an elevated IL-8 level, for IL-8 levels in the sera and plasma of healthy individuals are reported to be $<10 \text{pg} \cdot \text{mL}^{-1}$ (Ziegenhagen *et al.* 1999).

- **Need (or absence of need) for intravenous antibiotic courses**

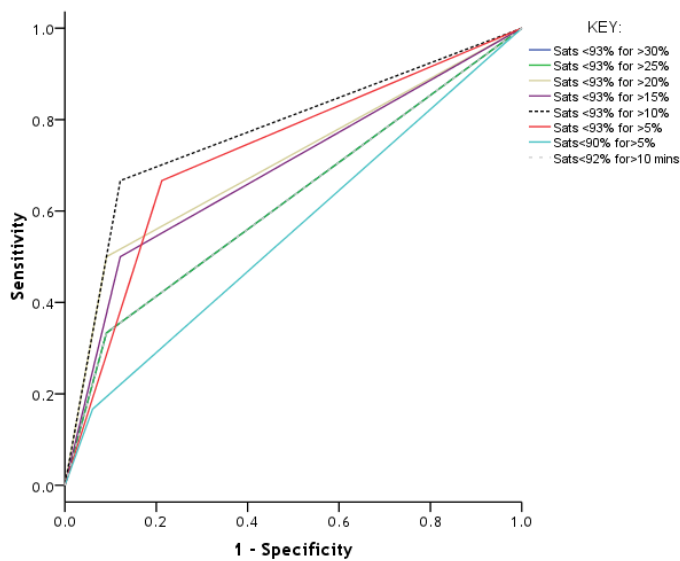
This was analysed in a binary fashion i.e. those requiring no intravenous antibiotic courses were compared with those requiring 1 or more courses.

3.5.1.1 Sleep Hypoxia and CRP

The ability of cut-offs for nocturnal hypoxia in detecting elevated CRP levels ($>7\text{mg.L}^{-1}$) was assessed, as shown in Figure 3.5. The full range of hypoxia definitions were assessed using Chi-square statistics (Fisher's exact test), with a summary of existing definitions and the most sensitive and specific de novo definition presented in Table 3.5.

Figure 3.5

ROC curve of performance of various cut-offs of nocturnal hypoxia in the identification of childhood CF subjects with $\text{CRP} \geq 7\text{mg.L}^{-1}$



Area under curve:

$\text{SpO}_2 <93\%$ for $>25\%$ study (Alder Hey definition) = 0.61

$\text{SpO}_2 <92\%$ for >10 continuous sleep minutes (CHoP definition) = 0.61

$\text{SpO}_2 <93\%$ for $>10\%$ study = 0.76

$\text{SpO}_2 <90\%$ for $>5\%$ study (Frangolias *et al.* 2001) = 0.55

The three existing definitions as well as the most sensitive and specific new definition are presented. The area under the curve is greatest for the definition of hypoxia as $\text{SpO}_2 <93\%$ for $>10\%$ study. This appears to be the most sensitive and specific cut-off of nocturnal hypoxia in identifying subjects with elevated CRP.

Table 3.5

Summary of identification of subjects with abnormal CRP (n=6) using different definitions of nocturnal hypoxia

	NEW SpO ₂ <93% for >10% study	ALDER HEY SpO ₂ <93% for >25% study	CHoP SpO ₂ <92% for 10 continuous study minutes	FRANGOLIAS SpO ₂ <90% for >5% study
CRP>7mg. L⁻¹	4 Hypoxic/ 2 Normoxic	2/4	2/4	1/5
CRP<7mg. L⁻¹	5/29	4/30	4/30	2/32
Chi-square statistic p-value (Fisher's exact test)	7.9 p=0.02	1.9 p=0.21	1.9 p=0.21	0.9 p=0.39
Kappa score p-value	0.43 p=0.05	0.22 p=0.17	0.22 p=0.17	0.13 p=0.36

* 40/41 subjects had a CRP measured

Kappa scores >0.41 are considered to show moderate agreement; >0.61, good agreement; and those >0.81, very good agreement (Altman 1992).

These data therefore suggest that there is moderate agreement between hypoxia as defined by SpO₂ <93% for >10% study and abnormal CRP defined as CRP>7mg.L⁻¹ i.e. by using nocturnal hypoxia as a marker for inflammation, we are able to achieve moderate agreement in identifying those patients at risk of an elevated CRP. The greatest level of agreement is reached by using a hypoxia definition of SpO₂ <93% for >10% study.

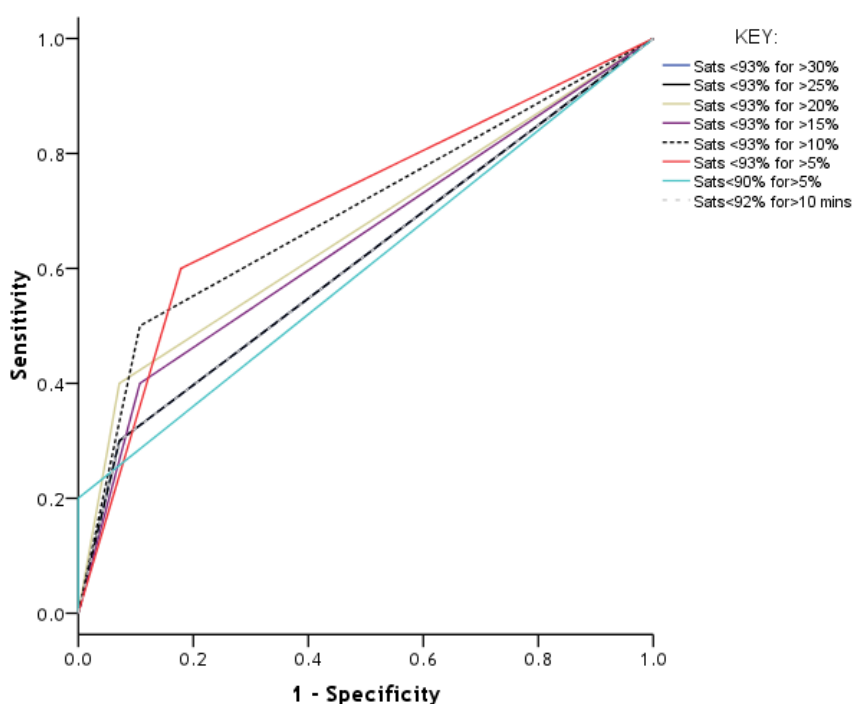
The cut-off of 7 mg.L⁻¹ for normal CRP may have been set too high, and the use of a kit to measure high sensitivity CRP (hsCRP) may have been useful. CRP has been used to assess cardiovascular risk, and the American Heart Association (AHA) and Center for Disease Control (CDC) joint statement suggests CRP cut-offs of <1 mg.L⁻¹ (low risk), 1-3 mg.L⁻¹ (average risk) and >3 mg.L⁻¹ (high-risk) for cardiovascular disease (Pearson *et al.* 2003). Therefore, if a pro-inflammatory state is suggested by hsCRP >3 mg.L⁻¹, valuable subject information may have been missed by using a less sensitive testing kit, and differences between hypoxic and normoxic groups may have been missed.

3.5.1.2 Sleep Hypoxia and ESR

The performances of the chosen potential definitions for nocturnal hypoxia in detecting abnormal ESR were also analysed (Figure 3.6). The full range of potential hypoxia definitions were analysed using Chi-Square statistics (Fisher's exact test), with a summary of existing definitions and the most sensitive and specific *de novo* definition given in Table 3.6.

Figure 3.6

ROC curve of performance of various cut-offs of nocturnal hypoxia in the identification of childhood CF subjects with elevated ESR ($> 17\text{mm.hr}^{-1}$ in males, $> 25\text{mm.hr}^{-1}$ in females)



Area under curve:

$\text{SpO}_2 <93\%$ for $>25\%$ study (Alder Hey definition) = 0.61

$\text{SpO}_2 <92\%$ for >10 continuous sleep minutes (CHoP definition) = 0.61

$\text{SpO}_2 <93\%$ for $>10\%$ study = 0.70

$\text{SpO}_2 <90\%$ for $>5\%$ study (Frangolias *et al.* 2001) = 0.60

The three existing definitions as well as the most sensitive and specific new definition are presented. The area under the curve is greatest for the definition of hypoxia as $\text{SpO}_2 <93\%$ for $>10\%$ study. This appears to be the most sensitive and specific cut-off of nocturnal hypoxia in identifying subjects with elevated ESR.

Table 3.6

Summary of identification of subjects with abnormal ESR (n=10) using different definitions of nocturnal hypoxia

	NEW SpO ₂ <93% for >10% study	ALDER HEY SpO ₂ <93% for >25% study	CHoP SpO ₂ <92% for 10 continuous study minutes	FRANGOLIAS SpO ₂ <90% for >5% study
Abnormal ESR	5 Hypoxic /5 Normoxic	3/7	3/7	2/8
Normal ESR	3/25	2/26	2/26	0/28
Chi-square statistic p-value (Fisher's exact test)	6.8 p=0.02	3.4 p=0.10	3.4 p=0.10	5.9 p=0.06
Kappa score p-value	0.42 p=0.01	0.27 p=0.07	0.27 p=0.07	0.27 p=0.02

* 38/41 subjects had an ESR measured

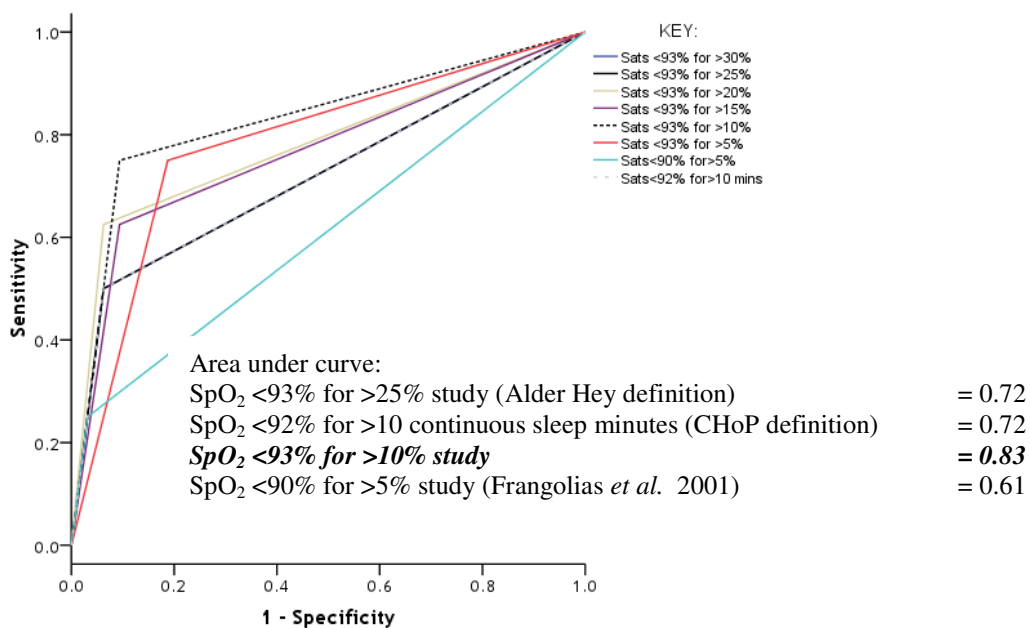
The hypoxia definition of SpO₂ <93% for >10% study has moderate agreement in detecting subjects with an abnormal ESR.

3.5.1.3 Sleep Hypoxia and White Cell Count

The ability of cut-offs for nocturnal hypoxia in detecting elevated white cell counts (>11 x10⁹.mL⁻¹) was assessed - Figure 3.7. The full range of hypoxia definitions were assessed using Chi-square statistics (Fisher's exact test), with a summary of existing definitions and the most sensitive and specific de novo definition presented in Table 3.7.

Figure 3.7

ROC curve of performance of various cut-offs of nocturnal hypoxia in the identification of childhood CF subjects with white blood cell count >11x10⁹.mL⁻¹



The area under the curve is greatest for the definition of hypoxia as SpO₂<93% for >10% study, which appears to be the most sensitive and specific cut-off of nocturnal hypoxia in identifying subjects with elevated white cell counts.

Table 3.7

Summary of identification of subjects with abnormal white cell count (n=8) using different definitions of nocturnal hypoxia

	NEW SpO ₂ <93% for >10% study	ALDER HEY SpO ₂ <93% for >25% study	CHoP SpO ₂ <92% for 10 continuous study minutes	FRANGOLIAS SpO ₂ <90% for >5% study
White Cell Count > 11x10 ⁹ mL ⁻¹	6 Hypoxic/ 2 Normoxic	4/4	4/4	2/6
White Cell Count ≤ 11x10 ⁹ mL ⁻¹	3/29	2/30	2/30	1/31
Chi-square statistic p-value (Fisher's exact test)	15.8 p=0.001	9.6 p=0.01	9.6 p=0.01	4.4 p=0.10
Kappa score p-value	0.63 p<0.001	0.48 p=0.002	0.48 p=0.002	0.29 p=0.04

* 40/41 subjects had a white cell count measured

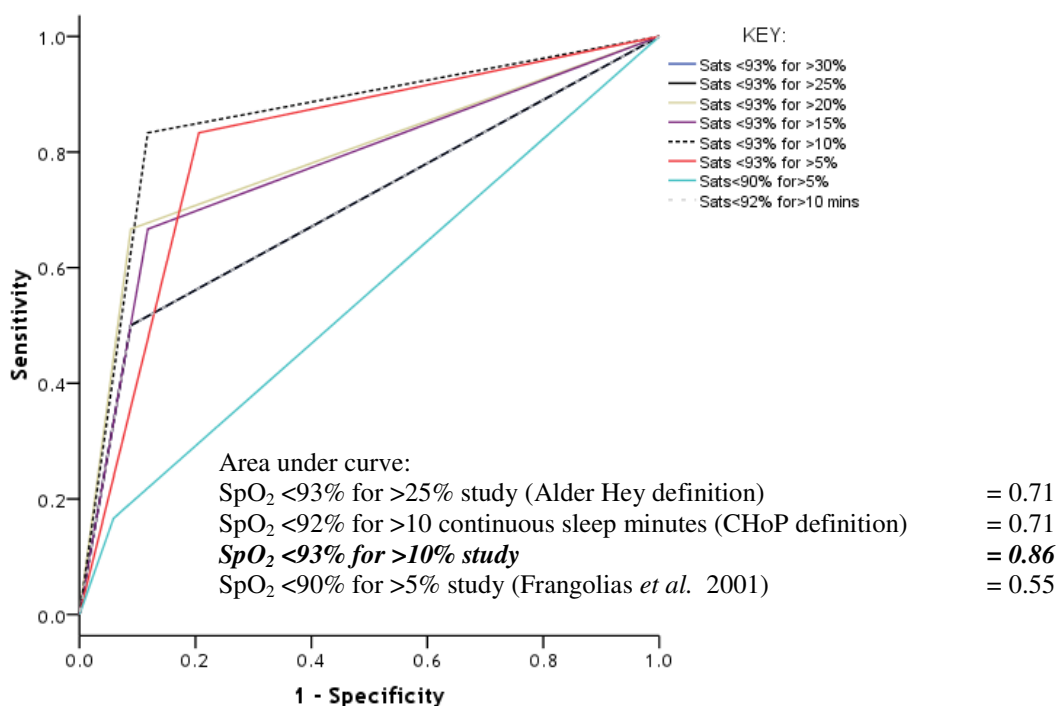
The hypoxia definition of SpO₂ <93% for >10% study has good agreement in detecting subjects with an abnormal white cell count.

3.5.1.4 Sleep Hypoxia and peripheral blood neutrophil counts

The performances of the chosen potential definitions for nocturnal hypoxia in detecting abnormal neutrophil counts were also analysed (Figure 3.8). The full range of potential hypoxia definitions were analysed using Chi-Square statistics (Fisher's exact test), with a summary of existing definitions and the most sensitive and specific *de novo* definition given in Table 3.8.

Figure 3.8

ROC curve of performance of various cut-offs of nocturnal hypoxia in the identification of elevated peripheral blood neutrophil counts (neutrophils $>8 \times 10^9 \text{ mL}^{-1}$)



The area under the curve is greatest for the definition of hypoxia as SpO₂ <93% for >10% study. This appears the most sensitive and specific cut-off of nocturnal hypoxia in identifying childhood CF subjects with a neutrophil count $>8 \times 10^9 \text{ mL}^{-1}$.

Table 3.8

Summary of identification of subjects with abnormal peripheral blood neutrophil counts (n=6) using different definitions of nocturnal hypoxia

	NEW SpO ₂ <93% for >10% study	ALDER HEY SpO ₂ <93% for >25% study	CHoP SpO ₂ <92% for 10 continuous study minutes	FRANGOLIAS SpO ₂ <90% for >5% study
Neutrophils > 8x10⁹ mL⁻¹	5 hypoxic / 1 normoxic	3/3	3/3	1/5
Neutrophils ≤ 8x10⁹ mL⁻¹	4 /30	3/31	3/31	2/32
Chi-square statistic p-value (Fisher's exact test)	15 p=0.001	6.8 p=0.03	6.8 p=0.03	0.86 p=0.4
Kappa score p-value	0.59 P<0.001	0.41 p=0.009	0.41 p=0.009	0.14 P=0.36

* 40/41 subjects had a neutrophil count measured

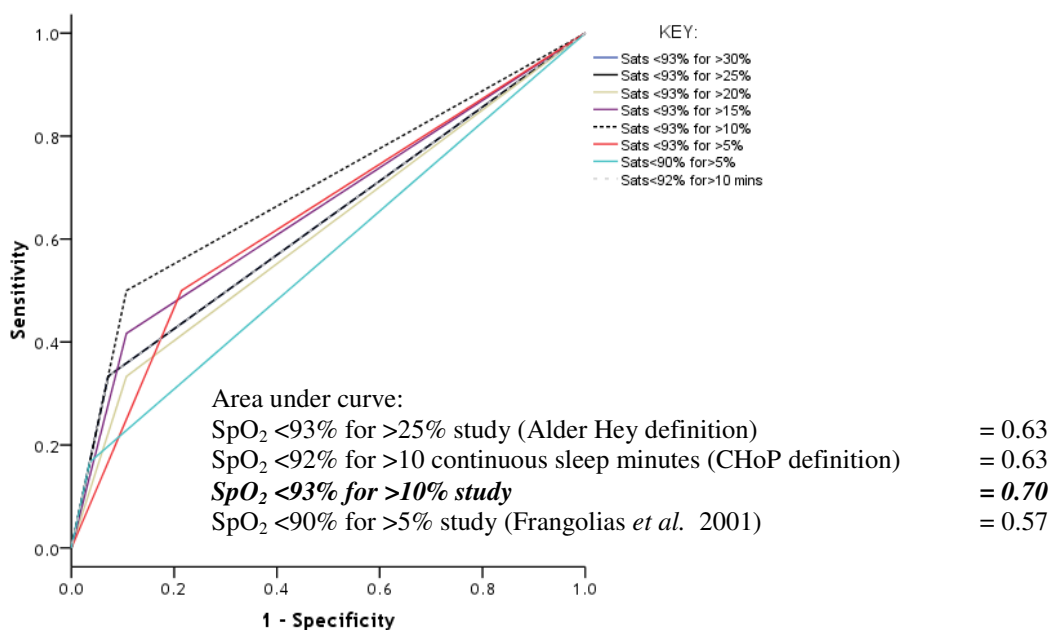
The hypoxia definition of SpO₂ <93% for >10% study has good agreement in detecting subjects with an abnormal neutrophil count.

3.5.1.5 Sleep Hypoxia and IL-8

The ability of cut-offs for nocturnal hypoxia in detecting elevated serum IL-8 levels ($>10 \text{ pg.mL}^{-1}$) was assessed - Figure 3.9. The full range of hypoxia definitions were assessed using Chi-square statistics (Fisher's exact test), with a summary of existing definitions and the most sensitive and specific *de novo* definition shown in Table 3.9.

Figure 3.9

ROC curve of performance of various cut-offs of nocturnal hypoxia in the identification of childhood CF subjects with serum interleukin 8 (IL-8) $\geq 10 \text{ pg.mL}^{-1}$



The area under the curve is greatest for the definition of hypoxia as SpO₂ <93% for >10% study. This appears the most sensitive and specific cut-off of nocturnal hypoxia in identifying childhood CF subjects with serum IL-8 levels $>10 \text{ pg.mL}^{-1}$.

Table 3.9

Summary of identification of subjects with abnormal serum IL-8 (n=12) using different definitions of nocturnal hypoxia

	NEW SpO ₂ <93% for >10% study	ALDER HEY SpO ₂ <93% for >25% study	CHoP SpO ₂ <92% for 10 continuous study minutes	FRANGOLIAS SpO ₂ <90% for >5% study
IL-8 $\geq 10 \text{ pg.mL}^{-1}$	6/6	4/8	4/8	2/10
IL-8 $< 10 \text{ pg.mL}^{-1}$	3/25	2/26	2/26	1/27
Chi-square statistic p-value (Fisher's exact test)	7.4 p=0.01	4.5 p=0.06	4.5 p=0.06	2.1 p=0.21
Kappa score p-value	0.42 p=0.01	0.31 p=0.03	0.31 p=0.03	0.17 p=0.15

* 40/41 subjects had a serum IL-8 measured

The hypoxia definition of SpO₂ <93% for >10% study has reasonable agreement in detecting subjects with an abnormal serum IL-8 level.

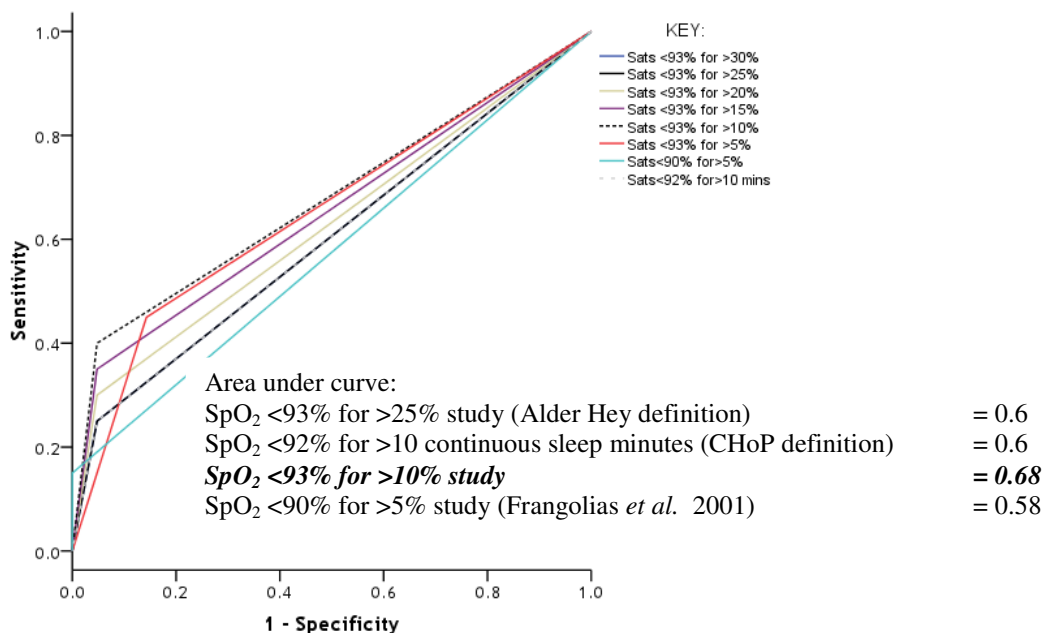
In non-CF models of childhood nocturnal hypoxia i.e. obstructive sleep apnoea (OSA), elevated IL-8 levels have been observed when compared with healthy controls (Tam *et al.* 2006), whilst adult OSA models also report increases in IL-8 when compared with controls (Alzoughaibi and Bahammam 2005). Other downstream cytokines of the NFκB inflammatory cascade, namely IL-6 and TNF-α have also been shown to be elevated in OSA (Alberti *et al.* 2003), whilst animal models of intermittent hypercapnic hypoxia mimicking OSA resulted in corresponding increases in IL-6 (Tam *et al.* 2007).

3.5.1.6 Sleep Hypoxia and need for intravenous antibiotics

Analysis of the chosen potential definitions for nocturnal hypoxia in the detection of those subjects who required at least one course of intravenous antibiotics in the preceding year (Figure 3.10), was also undertaken. The full range of potential hypoxia definitions was analysed using Chi-Square statistics (Fisher's exact test), with a summary of existing definitions and the most sensitive and specific *de novo* definition given in Table 3.10.

Figure 3.10

ROC curve of performance of various cut-offs of nocturnal hypoxia in the identification of children with CF requiring intravenous antibiotics



The area under the curve is greatest for the definition of hypoxia as SpO₂ <93% for >10% study. This appears the most sensitive and specific cut-off of nocturnal hypoxia in identifying childhood CF subjects in whom intravenous antibiotics were required.

Table 3.10

Summary of identification of subjects in need of intravenous antibiotics (≥ 1 course per year) (n=20), using different definitions of nocturnal hypoxia

	NEW SpO ₂ <93% for >10% study	ALDER HEY SpO ₂ <93% for >25% study	CHoP SpO ₂ <92% for 10 continuous study minutes	FRANGOLIAS SpO ₂ <90% for >5% study
≥ 1 intravenous antibiotic course in preceding year	8 hypoxic / 12 normoxic	5/15	5/15	3/17
Zero intravenous antibiotic course in preceding year	1/20	1/20	1/20	0/21
Chi-square statistic p-value (Fisher's exact test)	7.4 p=0.009	3.6 p=0.09	3.6 p=0.09	3.4 p=0.11
Kappa score p-value	0.36 p=0.006	0.21 p=0.07	0.21 p=0.07	0.15 P=0.07

**Antibiotic data was analysed for all 41 subjects*

These data therefore suggest poor agreement between measures of nocturnal hypoxia and requirement for intravenous antibiotic therapy. The greatest level of agreement is, however, again achieved using a hypoxia definition of SpO₂ <93% for >10% study.

The hypoxia definition of SpO₂ <93% for >10% study appears to be the definition of sleep hypoxia which best predicts NFκB mediated inflammation in the form of IL-8, CRP, and neutrophil counts. Furthermore, it also is the definition with best sensitivity and specificity trade-off in detecting abnormal white cell count and ESR, as well as those requiring intravenous antibiotics for CF lung disease.

3.6 Exercise Hypoxia

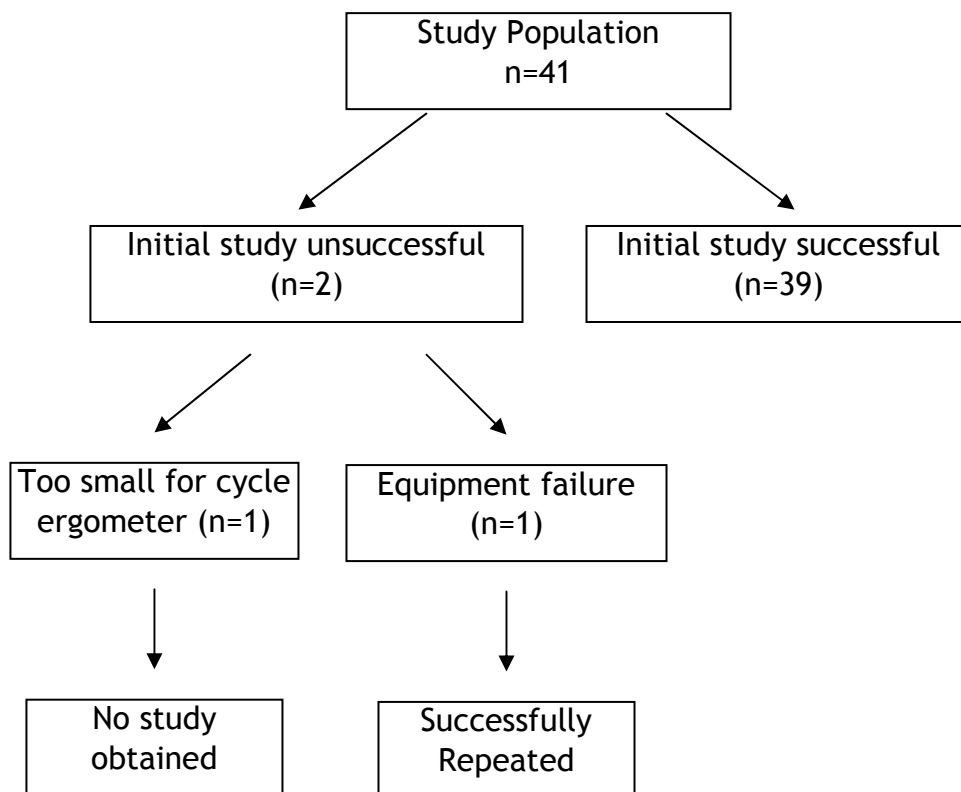
Having considered the various sleep hypoxia definitions in sections 3.4 and 3.5, the exercise data will now be reviewed, and the sensitivity and specificity characteristics of existing and de novo EIAH definitions will, be compared with the proposed new sleep hypoxia definition in the ability to detect clinically relevant outcome measures in children with CF.

3.6.1 Demographic data

Cardiopulmonary exercise testing and exercise SpO₂ recording (Section 2.2.2.8) was undertaken (Figure 3.11) in forty of the 41 study subjects.

Figure 3.11

Data regarding success of exercise SpO₂ recordings



The failure of patient number 1 (height 122.9cm.) due to their being too small for the cycle ergometer led to revision of the inclusion criteria for the study, with minimum height increasing from 120 to 128cm. The distribution of exercise SpO₂ data for the study population is shown below (Table 3.11).

Table 3.11Distribution of exercise SpO₂ in our study population

	Median	Range
Age (years)	12.9	8-16.2
Resting SpO ₂ (%)	99	95-100
Pre-exercise SpO ₂ (%)	19 Male 21 Female	99 99
Lowest Exercise SpO ₂ (%)	98	85-100
Change in SpO ₂ during exercise [ΔSpO ₂ (%)]	-1	-13 to +4

3.6.2 Exercise Hypoxia in CF study population

Exercise induced arterial hypoxia (EIAH) in CF is defined as a fall in SpO₂ during exercise of $\geq 4\%$ from baseline (Narang *et al.* 2003), and this is a definition that has also been used in healthy individuals (Nourry *et al.* 2004). Application of this definition to our study population identifies 8 subjects with evidence of EIAH (Table 3.12).

Table 3.12Subjects with $\geq 4\%$ fall in SpO₂ during exercise

Subject ID	Age	Sex	Baseline SpO ₂ (%)	Lowest exercise SpO ₂ (%)	Absolute fall in SpO ₂ (%)	Relative fall in SpO ₂ (%) $\frac{(\text{Baseline SpO}_2 - \text{Lowest SpO}_2)}{\text{Baseline SpO}_2}$
3	10.72	M	98	90	8	8.2
6	14.20	M	95	91	4	4.2
8	12.08	M	98	85	13	13.3
10	15.34	M	99	94	5	5.1
29	14.46	F	98	91	7	7.1
34	15.66	M	99	95	4	4
36	9.26	F	98	94	4	4.1
37	9.33	F	100	96	4	4

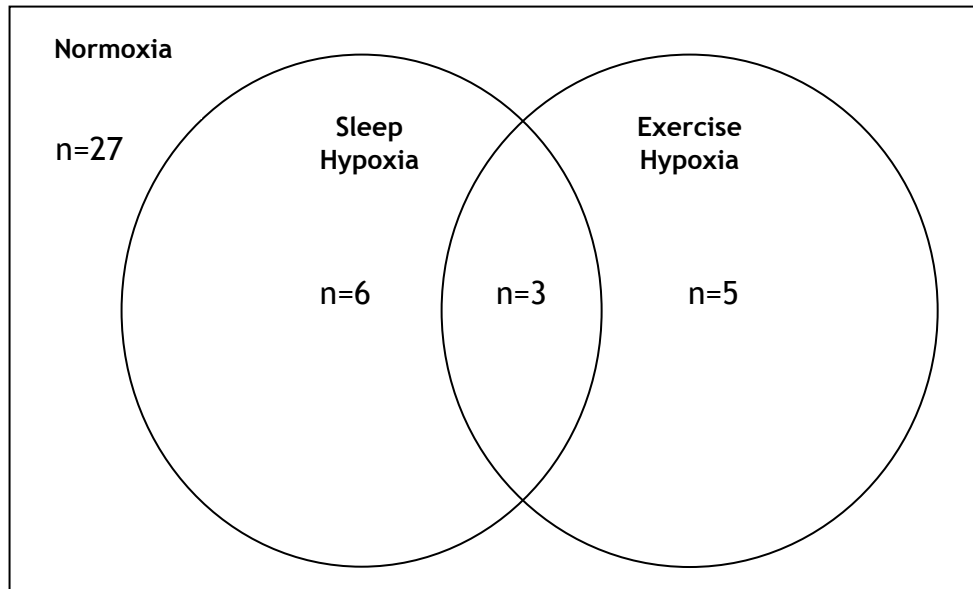
Comparing this to sleep hypoxia, the demographics of the study population are as follows (Figure 3.12):

Figure 3.12

Venn diagram illustrating overlap of exercise and sleep hypoxia in study population (n=41) using the following definitions:

Sleep hypoxia: $\text{SpO}_2 < 93\%$ for $>10\%$ sleep time

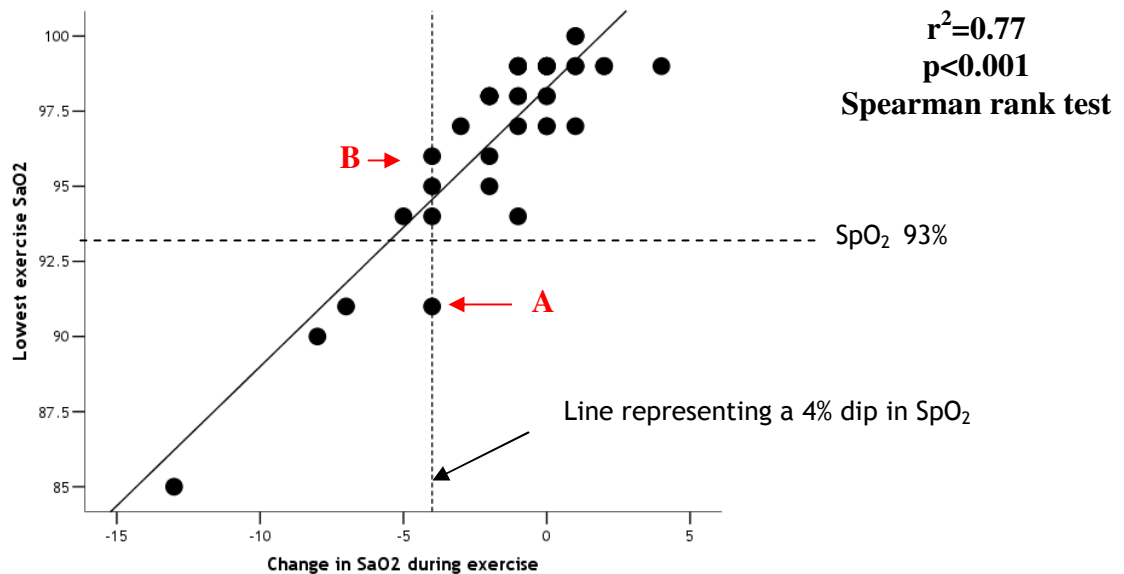
Exercise hypoxia: Fall in SpO_2 during exercise of $\geq 4\%$ from baseline



The definition of EIAH as a fall in SpO₂ during exercise of $\geq 4\%$ from baseline does, however, seem intrinsically flawed. Clearly, if this represents a fall in SpO₂ from 95% to 91% (Subject A), one could deem this to represent a likely significant physiological drop in PaO₂, however the definition holds less well for subject B, whose SpO₂ fall from 100% to 96% at the end of exercise (Figure 3.13).

Figure 3.13

Change in exercise SpO₂ versus lowest exercise SpO₂



Further work is needed to establish clinically significant parameters. If we use a cut-off of 93% (the point on the oxygen dissociation curve where small changes in pO₂ result in exponential decreases in % SpO₂), and categorise those in whom the nadir of exercise saturation is below 93% as hypoxic, the number of subjects with exercise hypoxia would fall to only 4 (Figure 3.14).

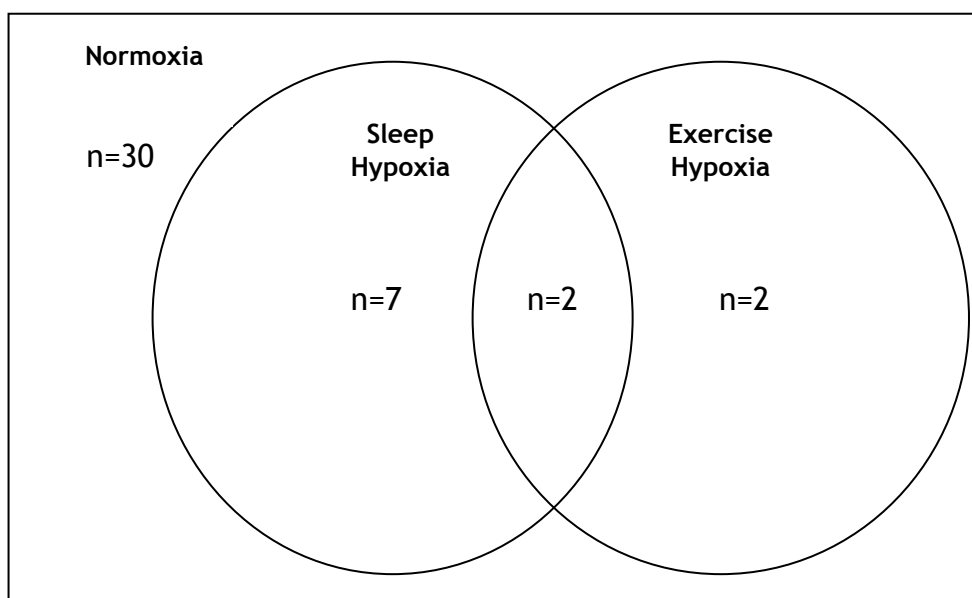
It would make physiological sense for a cut-off of 93% to be deemed a clinically important level of exercise desaturation. Furthermore, those with a nadir of SpO₂ below 93% also had the greater falls from baseline. In one subject a 13% dip in SpO₂ was recorded on exercise. Such exercise-related dips in SpO₂ below 93% may be regularly applied hypoxic insults to the patient with CF.

Figure 3.14

Venn diagram illustrating overlap of exercise and sleep hypoxia in study population (n=41) using the following definitions:

Sleep hypoxia: $\text{SpO}_2 < 93\%$ for $>10\%$ sleep time

Exercise hypoxia: $\downarrow\text{SpO}_2$ during exercise of $\geq 4\%$ and nadir of $\text{SpO}_2 < 93\%$



3.7 Development of optimal definition for EIAH

3.7.1 Application of existing and *de novo* hypoxia definitions to EIAH and comparison with optimal sleep definition

An optimal sleep definition having been identified, the performance of EIAH in detecting adverse clinical outcomes appears worthy of study.

Definitions considered therefore were:

- EIAH defined as $\geq 4\%$ dip in SpO_2 on exercise
- EIAH defined as $\geq 4\%$ dip in SpO_2 on exercise with lowest $\text{SpO}_2 < 93\%$
- 10% sleep time spent with SpO_2 below 93%

The adverse clinical outcomes considered were as above:

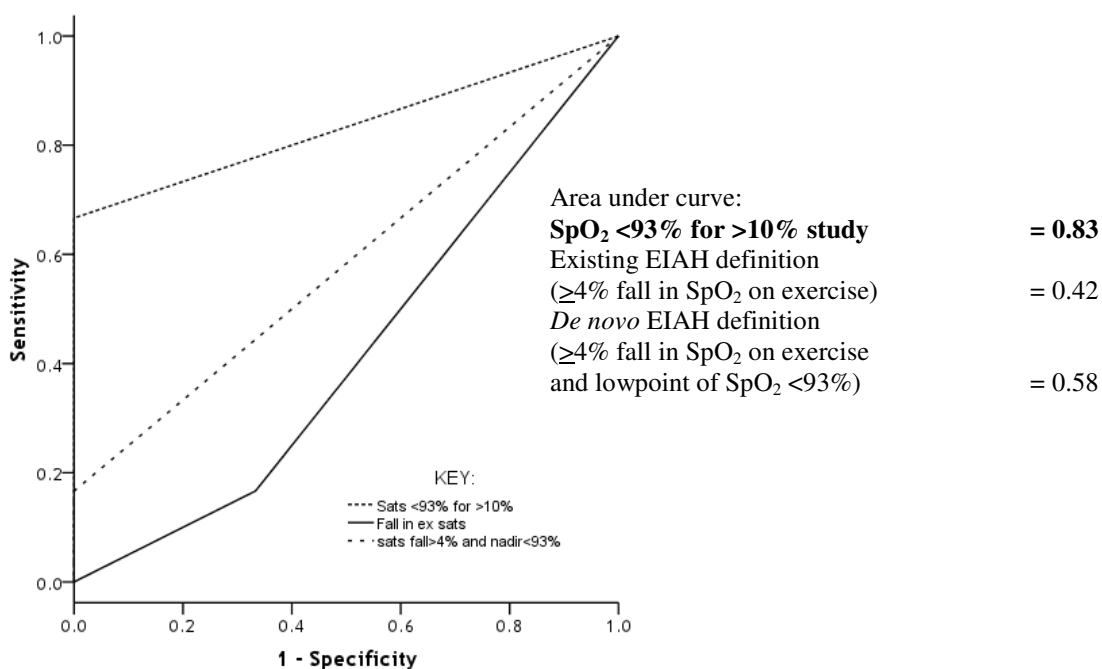
- Elevated C-reactive protein
- Elevated Erythrocyte Sedimentation Rate
- Elevated white blood cell counts
- Elevated neutrophil counts
- Elevated serum interleukin-8 levels
- Need for intravenous antibiotic therapy

3.7.1.1 EIAH, Sleep Hypoxia and CRP

ROC statistics were used to compare the sensitivity and specificity for measures of sleep and exercise hypoxia in the detection of abnormal CRP (Figure 3.15).

Figure 3.15

ROC curve of performance of sleep hypoxia and EIAH definitions in the identification of childhood CF subjects with $CRP \geq 7 \text{mg.mL}^{-1}$



The area under the curve is greatest for the definition of hypoxia as SpO₂ <93% for >10% study, suggesting that this is a more sensitive and specific definition than either of the EIAH definitions in identifying childhood CF subjects with $CRP \geq 8 \text{mg.mL}^{-1}$. This reiterated in Table 3.13.

Table 3.13

Summary of identification of abnormal CRP using sleep and exercise hypoxia definitions

	<i>De novo</i> SLEEP SpO ₂ <93% for >10% study	EIAH >4% fall in SpO ₂ on exercise	EIAH >4% fall in SpO ₂ on exercise and lowest exercise SpO ₂ <93%
$CRP > 7 \text{mg.mL}^{-1}$	4 Hypoxic/ 2 Normoxic	1/5	1/5
$CRP < 7 \text{mg.mL}^{-1}$	5/29	7/26	3/30
Chi-square statistic p-value (Fisher's exact test)	7.9 p=0.02	0.1 p=0.64	0.3 p=0.50
Kappa score p-value	0.43 p=0.05	-0.04 p=0.8	0.09 p=0.57

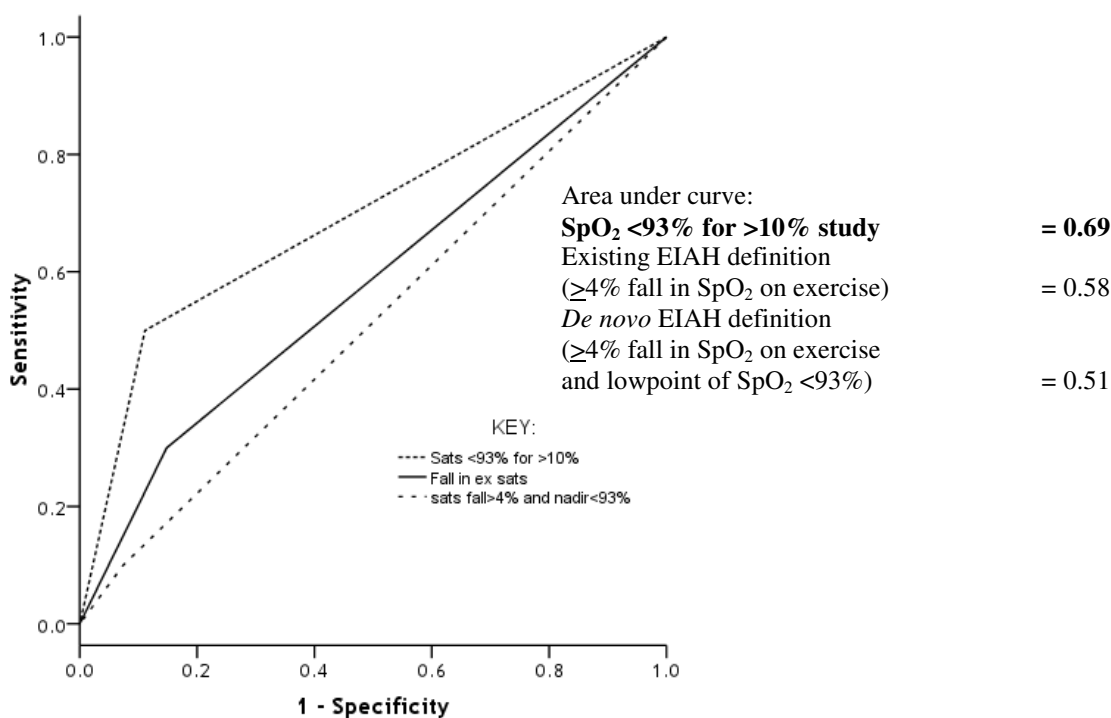
* 39/41 subjects had exercise data and a measured CRP

3.7.1.2 EIAH, Sleep Hypoxia and ESR

ROC statistics were used to compare the sensitivity and specificity for measures of sleep and exercise hypoxia in the detection of abnormal ESR - Figure 3.16.

Figure 3.16

ROC curve of performance of sleep hypoxia and EIAH definitions in the identification of childhood CF subjects with abnormal ESR



The area under the curve is greatest for the definition of hypoxia as SpO₂ <93% for >10% study, suggesting that this is a more sensitive and specific definition than either of the EIAH definitions in identifying childhood CF subjects abnormal ESR. This is reiterated in Table 3.14.

Table 3.14

Summary of identification of abnormal ESR using sleep and exercise hypoxia definitions

	<i>De novo</i> SLEEP SpO ₂ <93% for >10% study	EIAH >4% fall in SpO ₂ on exercise	EIAH >4% fall in SpO ₂ on exercise and lowest exercise SpO ₂ <93%
Abnormal ESR	5 Hypoxic/ 5 Normoxic	3/7	1/9
Normal ESR	3/25	4/23	2/25
Chi-square statistic p-value (Fisher's exact test)	6.8 p=0.02	1.1 p=0.27	0.1 p=0.62
Kappa score p-value	0.42 p=0.01	0.16 p=0.3	0.04 p=0.80

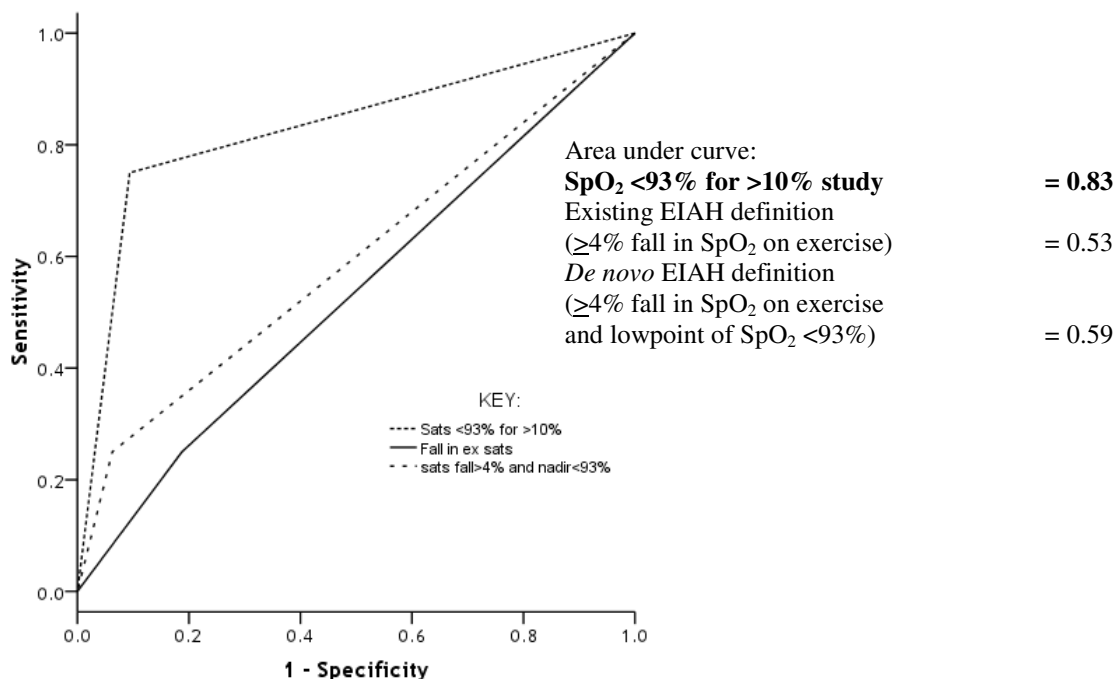
* 38/41 subjects had exercise data and a measured ESR

3.7.1.3 EIAH, Sleep Hypoxia and white cell counts

ROC statistics were used to compare the sensitivity and specificity for measures of sleep and exercise hypoxia in the detection of abnormal white cell counts - Figure 3.17.

Figure 3.17

ROC curve of performance of sleep hypoxia and EIAH definitions in the identification of childhood CF subjects with white cell counts $>11 \times 10^9 \text{.mL}^{-1}$



The area under the curve is greatest for the definition of hypoxia as SpO₂ <93% for >10% study, suggesting that this is a more sensitive and specific definition than either of the EIAH definitions in identifying childhood CF subjects abnormal white cell counts. This is further illustrated in Table 3.15.

Table 3.15

Summary of identification of abnormal white cell counts using sleep and exercise hypoxia definitions

	<i>De novo</i> SLEEP SpO ₂ <93% for >10% study	EIAH >4% fall in SpO ₂ on exercise	EIAH >4% fall in SpO ₂ on exercise and lowest exercise SpO ₂ <93%
White cell count > 11x10 ⁹ .mL ⁻¹	6/2	2/6	2/6
White cell count ≤ 11x10 ⁹ .mL ⁻¹	3/29	6/25	2/29
Chi-square statistic p-value (Fisher's exact test)	15.8 p=0.001	0.1 p=0.53	2.4 p=0.18
Kappa score p-value	0.63 p<0.001	0.06 p=0.72	0.23 p=0.12

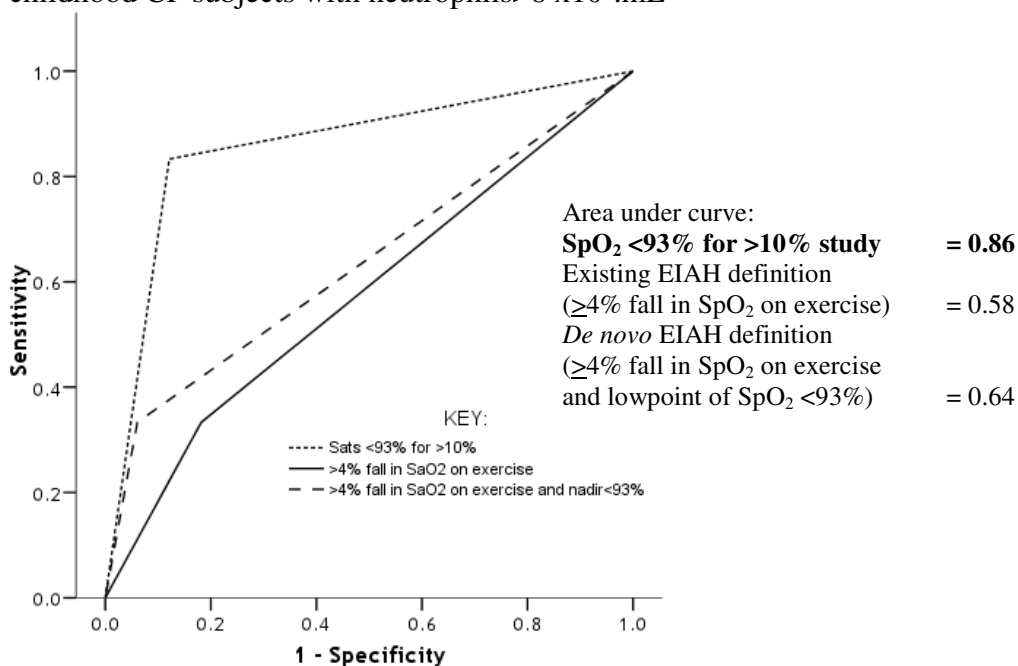
* 39/41 subjects had exercise data and a measured white cell count

3.7.1.4 EIAH, Sleep Hypoxia and peripheral blood neutrophil counts

ROC statistics were used to compare the sensitivity and specificity for measures of sleep and exercise hypoxia in the detection of abnormal peripheral blood neutrophil counts ($>8 \times 10^9 \text{.mL}^{-1}$) - Figure 3.18.

Figure 3.18

ROC curve of performance of sleep hypoxia and EIAH definitions in the identification of childhood CF subjects with neutrophils $>8 \times 10^9 \text{.mL}^{-1}$



The area under the curve is greatest for the definition of hypoxia as SpO₂ <93% for >10% study, suggesting that this is a more sensitive and specific definition than either of the EIAH definitions in identifying childhood CF subjects with elevated peripheral blood neutrophil counts. This is borne out by the associated statistics (Table 3.16).

Table 3.16

Summary of identification of abnormal peripheral blood neutrophil counts ($>8 \times 10^9 \text{.mL}^{-1}$) using sleep and exercise hypoxia definitions

	<i>De novo</i> SLEEP SpO ₂ <93% for >10% study	EIAH >4% fall in SpO ₂ on exercise	EIAH >4% fall in SpO ₂ on exercise and lowest exercise SpO ₂ <93%
Neutrophils > $8 \times 10^9 \text{.mL}^{-1}$	5 hypoxic / 1 normoxic	2/4	2/4
Neutrophils $\leq 8 \times 10^9 \text{.mL}^{-1}$	4 /30	6/27	2/31
Chi-square statistic p-value (Fisher's exact test)	15 p=0.001	0.72 p=0.58	4.1 p=0.10
Kappa score p-value	0.59 p<0.001	0.13 p=0.40	0.32 p=0.04

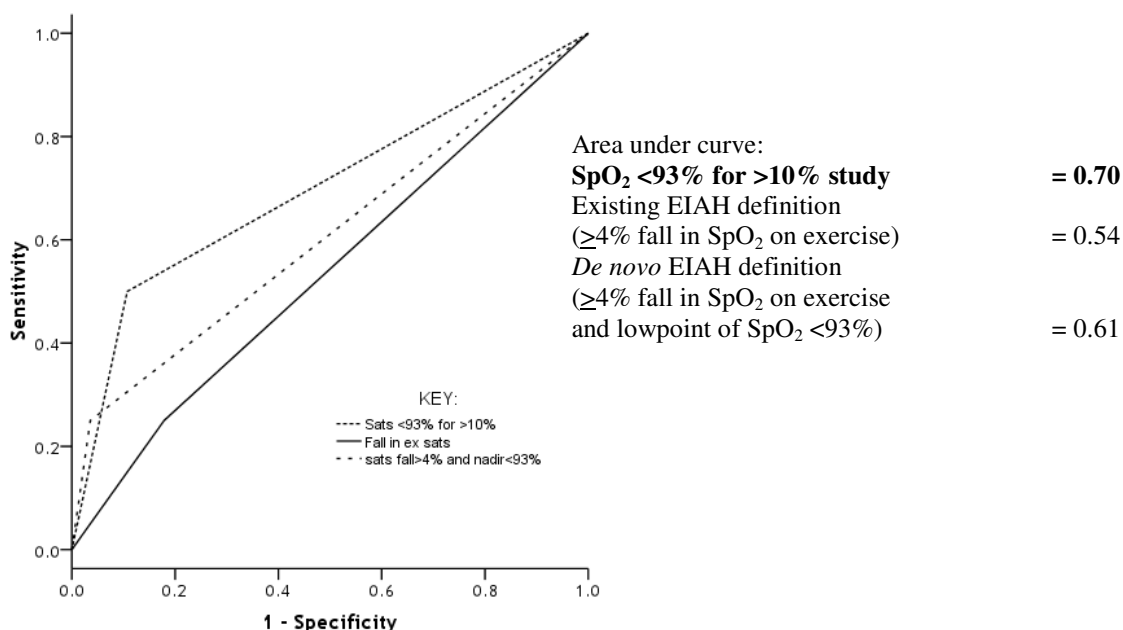
* 39/41 subjects had exercise data and a measured neutrophil count

3.7.1.4 EIAH, Sleep Hypoxia and serum IL-8 levels

ROC statistics were used to compare the sensitivity and specificity for measures of sleep and exercise hypoxia in the detection of abnormal serum IL-8 ($>10\text{pg.mL}^{-1}$) levels - Figure 3.19.

Figure 3.19

ROC curve of performance of sleep hypoxia and EIAH definitions in the identification of childhood CF subjects with serum IL-8 levels $>10\text{pg.mL}^{-1}$



The area under the curve is greatest for the definition of hypoxia as SpO₂ <93% for >10% study, suggesting that this is a more sensitive and specific definition than either of the EIAH definitions in identifying childhood CF subjects with elevated serum IL-8 levels. This is reiterated in Table 3.17.

Table 3.17

Summary of identification of abnormal serum IL-8 ($>10\text{pg.mL}^{-1}$) using sleep and exercise hypoxia definitions

	De novo SLEEP SpO₂ <93% for >10% study	EIAH >4% fall in SpO₂ on exercise	EIAH >4% fall in SpO₂ on exercise and lowest exercise SpO₂<93%
Interleukin-8 > 10 pg.mL⁻¹	6/6	3/9	3/9
Interleukin-8 ≤ 10 pg.mL⁻¹	3/25	5/23	1/27
Chi-square statistic p-value (Fisher's exact test)	7.4 p=0.01	0.1 p=0.53	4.3 p=0.08
Kappa score p-value	0.42 p=0.01	0.06 p=0.72	0.27 p=0.04

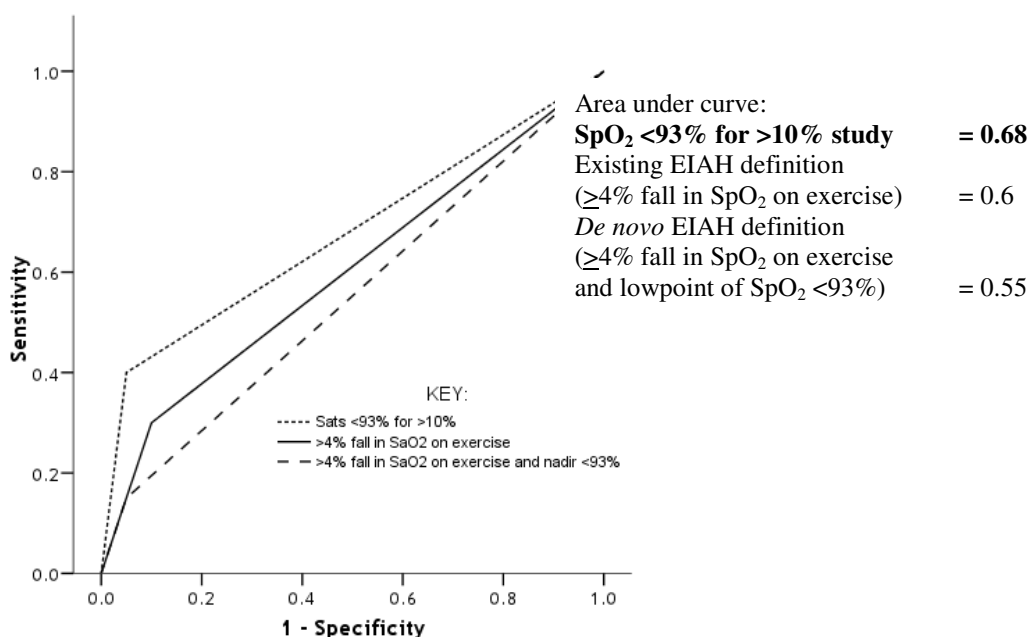
* 40/41 subjects had exercise data and a measured IL-8

3.7.1.5 EIAH, Sleep Hypoxia and the need for intravenous antibiotics

ROC statistics were used to compare the sensitivity and specificity for measures of sleep and exercise hypoxia in the detection of the need for intravenous antibiotics in the preceding 12 months - Figure 3.20.

Figure 3.20

ROC curve of performance of sleep hypoxia and EIAH definitions in the identification of children with CF requiring intravenous antibiotics



The area under the curve is greatest for the definition of hypoxia as SpO₂ <93% for >10% study, suggesting that this is a more sensitive and specific definition than either of the EIAH definitions in identifying children with CF who had required intravenous antibiotics in the preceding year. This is borne out by the statistics of Table 3.18.

Table 3.18

Summary of identification of the need for intravenous antibiotics (≥ 1 course per year) using sleep and exercise hypoxia definitions

	De novo SLEEP SpO₂ <93% for >10% study	EIAH >4% fall in SpO₂ on exercise	EIAH >4% fall in SpO₂ on exercise and lowest exercise SpO₂<93%
≥1 IV antibiotic course in preceding year	8 hypoxic / 12 normoxic	6/14	3/17
Zero IV antibiotic courses in preceding year	1/20	2/18	1/19
Chi-square statistic p-value (Fisher's exact test)	7.4 p=0.009	2.5 p=0.24	1.1 p=0.61
Kappa score p-value	0.36 p=0.006	0.20 p=0.11	0.10 p=0.29

* 40/41 subjects had exercise data. Data on antibiotic usage were available for all.

3.8 Discussion

3.8.1 SpO₂ in our CF study population compared with healthy controls

SpO₂ during sleep in our CF study population were lower than that reported in a non-CF population. A home oximetry study of 100 school-age children (Urschitz *et al* 2003), reported a median SpO₂ of 97%, and stated that desaturations below 92% were unusual.

3.8.2 Summary of performance of tested definitions of hypoxia in the detection of measures of inflammation

From the analyses presented, a *de novo* definition of sleep hypoxia (SpO₂ <93% for > 10% study time) appears to perform best in the detection of all measures of inflammation that were considered in this study, as assessed by comparison of ROC characteristics for a variety of measures of sleep hypoxia and EIAH.

The definition of SpO₂ <93% for > 10% study time appears to show the best ROC characteristics for detecting abnormal CRP and IL-8 levels, as well as elevated ESR, white cell and neutrophil counts, and also the need for intravenous antibiotics.

The high sensitivity and specificity of this hypoxia definition in the detection of elevations in CRP [for which IL-6 is a precursor (Heikkila *et al.* 2007)], as well as IL-8 and neutrophil counts would support the hypothesis that hypoxia is closely associated to NFκB-mediated inflammation.

3.8.3 Study Limitations

3.8.3.1 Study numbers

The study numbers are small (n=41), and frequencies of some adverse clinical outcomes within this study sample are too small to allow meaningful analysis. To confirm these observations in our study group and to further validate the adopted sleep hypoxia definition, the study should be repeated in a second population using similar outcome measures. However, while this is planned, it is beyond the scope of this MD thesis.

3.8.3.2 Lack of control subjects

A number of tests were undertaken on each of the study subjects that took a full day's testing protocol to achieve. Time constraints and the availability of historical control data for outcomes including spirometry, respiratory and skeletal muscle strength, and bone mineral density acted as justification for the absence of a healthy control group. The reliance on epidemiological control data is, however, an acknowledged limitation of this study.

3.8.3.3 Method of assessment of sleep hypoxia

By relying on time increments with low arterial oxygen saturations from oximetry measures alone as our gold standard, an assumption is made that the desaturation in CF is not due to obstruction. This assumption appears a reasonable one to make. Baseline SpO₂ are low, with little heart rate variability or obstructive (>4% fall in SpO₂) dips seen on analyses of the full studies of each of the hypoxic individuals; suggesting that hypoventilation is occurring throughout sleep (both REM and non-REM), although without EEG monitoring, the sleep phases are unknown. Indeed sleep state is unknown, and it is possible that the children were not asleep at all and that no desaturations were seen due to the subject being awake all night. A verbal report from parents suggests that all children were studied during sleep, although it is known that inaccuracies may exist in parental reporting of their children's sleep patterns (Minde *et al.* 1993).

The software package enabled calculation of the majority of desired study parameters, but the inability to calculate hourly mean SpO₂ measures was a potential limitation as this prevented us applying the existing nocturnal hypoxia criteria of Versteegh *et al.* (1990) to our study population. Oximetry-only studies can be associated with false negative results, for example oximetry alone may miss arousals related to obstruction.

Significant hypoxia is, however, either present or absent on the study, and oximetry appears both sensitive and specific in the detection of hypoxia. Indeed continuous documentation of SpO₂ over a minimum of 8 hours is the recommended first-line investigation of hypoxaemia in CF in the American Thoracic Society (ATS) recommendations for cardiopulmonary sleep studies in children (*Am J Respir Crit Care Med* 1996; **153**: 866-878), with full polysomnography (PSG) reserved for those CF patients in whom snoring or sleep disturbance is present.

A strength of the study however, is that oximetry was done in the patient's own home and they did not have to come into hospital and sleep in an artificial environment to undertake full polysomnography.

A further strength was the timing of the sleep study. All patients were studied at a time of clinical stability, and in each case the sleep study preceded the measurement of clinical and inflammatory outcomes.

3.8.4 Adoption of a new sleep hypoxia definition

Based on the ROC characteristics, and ability to detect measures of inflammation in a childhood CF population, SpO₂ <93% for > 10% study time is the definition of hypoxia to be used for assessing the inter-relationship of hypoxia and measured clinical outcomes. These inter-relationships will be explored in detail in Chapters 4 and 5. Study demographics using this new definition are displayed below (Table 3.19).

Table 3.19

Nocturnal hypoxia in our study population using new hypoxia definition

	Median (IQR) Age	Sex	Mean (sd) Sleep SpO₂	Median (IQR) time spent with SpO₂ <93%
Hypoxic Group [SpO ₂ <93% for >10% sleep time] (n=9)	14.9 (12.3-14.9)	3M:6F	92.6 (1.1)	49.8 (19.6-79.7)
Normoxic Group (n=32)	12.2 (10-13.7)	17M:15F	96.1 (1.1)	0.1 (0.1-1.8)

Validation of this definition in a second population using similar outcome measures is planned.

Hypoxia, defined as SpO₂ <93% for >10% sleep time –the most sensitive and specific definition in the detection of measures of inflammation was noted in nine of 41 children studied. Given that only 1-2% UK children with CF receive oxygen therapy (Balfour-Lynn *et al.* 2005, Douglass *et al.* 2008), the proportion of children (22%) identified as hypoxic suggests that a proportion of children with CF may be being under-treated for potentially clinically significant nocturnal hypoxia.

In 1990, a study of daytime SpO₂ in 100 children with CF aged 5-16 years, was carried out at Great Ormond Street (GOS) Hospital (Betancourt *et al.* 1991). The median SpO₂ of the CF population was 94% (range 75%-99%), compared with 97% in a group of 50 age-matched controls.

In the 2005/06 GOS cohort studied for this thesis, median daytime SpO₂ were 99% (range 95-100%). Although the study exclusion criteria included children already receiving oxygen therapy, only 2 children were excluded for this reason, and this would not skew the median resting SpO₂ data. A clear difference in median daytime SpO₂ in the GOS CF population is evident, with marked improvement noted over a 15-year period. It is, therefore, suggested that the prevalence of hypoxia in children with CF has been falling over the past two decades. This fall is likely to be due to the current cohort of CF patients having less severe lung disease than their predecessors, as reflected in the ongoing continued improvement in survival in UK CF patients (Dodge *et al.* 2007).

3.8.5 Implications of measures of exercise induced arterial hypoxia

Hypoxia in CF is reported to occur more frequently during sleep than on exercise (Bradley *et al.* 1999, Coffey *et al.* 1991). Our results (Figures 3.12 and 3.14) bear out this finding, regardless of how EIAH is quantified. The implications of this include the recommendation that a sleep study may be indicated for those with exercise-induced arterial hypoxaemia.

The mechanisms for sleep hypoxia and EIAH are thought to differ, and this is borne out by the identification of different individuals with sleep hypoxia when compared to EIAH. Exploration of mechanisms by which differences occur will be attempted in chapter 4. Further research directions may seek to better understand the effects of EIAH, and help to develop a clinically-relevant definition of EIAH that can be used in the CF population.

3.8.6 Consideration of a new EIAH definition

The definition of EIAH in CF appears flawed, as a 4% dip in SpO₂ from baseline on exercise may not result in exercise-induced hypoxia at all i.e. a dip from 100% to 96% would be encompassed by this definition, although clearly a SpO₂ of 96% (equating to a PaO₂ of 12kPa) would not be considered hypoxic. Such falls in SpO₂ are known in elite athletes, and postulated to be due to intrapulmonary shunting, diffusion limitation, and ventilation-perfusion mismatching (Prefaut *et al.* 2000).

It was therefore proposed that a more robust definition of EIAH may be that of a fall in SpO₂ during exercise of $\geq 4\%$ and nadir of SpO₂ below 93%. However, the ROC characteristics of either existing or *de novo* definitions of EIAH in the detection of adverse clinical outcomes (Figures 3.15 to 3.20) appear inferior to the *de novo* sleep hypoxia definition.

This suggests that the most clinically useful measure of hypoxia in children with CF is SpO₂ $\leq 93\%$ for $>10\%$ sleep time, and groups will be dichotomised on this basis for chapters 4 and 5.

3.9 Definition of clinically important hypoxia in childhood CF

The definition to be used for assessing the inter-relationship of hypoxia in CF and a variety of study outcomes in Chapters 4 and 5 is SpO₂ $<93\%$ for $>10\%$ sleep time.

Chapter 4 will explore the association between hypoxia in children with CF (as defined by SpO₂ $<93\%$ for $>10\%$ sleep time) and various clinical, radiological, physiological and psychological outcome measures, whilst Chapter 5 will explore the association between hypoxia and surrogate measures of inflammation.

CHAPTER 4: Association of hypoxia and clinical outcomes in children with cystic fibrosis

4.1 Introduction

Following on from Chapter 3, where a *de novo* definitions of hypoxia in CF has been proposed, we now seek to apply the suggested definition of hypoxia ($\text{SpO}_2 < 93\%$ for 10% sleep time) to our study population.

By dichotomising on the basis of hypoxia, we are able to explore the relationship between hypoxia in children with CF and various clinical, radiological, physiological and psychological outcome measures as outlined below. It is hypothesised that hypoxia may be associated with deleterious clinical outcomes, in particular a reduction in exercise capacity (Figure 1.5).

4.2 Aims

The aim of this chapter was to investigate the relationship between measures of hypoxia and clinical parameters, namely:

- Anthropometric data
- Spirometry and lung function data
- Radiological data (Chrispin-Norman X-ray scores)
- Respiratory muscle pressure measurements (SnIP, MIP and MEP)
- Cardiopulmonary exercise testing (CPET) data
- Quality of life (CFQ) data
- Echocardiographic data

The relationship between measures of hypoxia and ACE genotype, bone densitometry, skeletal muscle strength, and surrogate measures of inflammation including exhaled breath condensate measures will be investigated in Chapter 5.

4.3 Methods

Application of a definition of hypoxia in CF as declared in section 3.9 allows dichotomisation of our population into hypoxic and normoxic groups for statistical analyses.

4.3.1 Definition of Hypoxia

Following the use of ROC statistics to consider a number of definitions of both nocturnal hypoxia and EIAH, a single definition ($\text{SpO}_2 < 93\%$ for 10% sleep time) was chosen as the most clinically useful definition of hypoxia in our study population. This definition was used to dichotomise the study population and allows comparison between hypoxic and normoxic groups, in order to investigate the relationship between hypoxia and the various study outcome measures described above, and to validate this as a clinically useful definition.

4.3.2 Anthropometric data and demographics

Briefly, anthropometric measures of height were made using a stadiometer, and body mass measurements were undertaken on electronic scales in accordance with the methodology of section 2.2.2.1.

4.3.3 Spirometry

Briefly, incentive spirometry was performed (Jaeger Masterscreen 4.65) in accordance with the ATS/ERS recommendations (Miller *et al.* 2005), as described in section 2.2.2.3.

4.3.4 Plethysmographic data

Plethysmography (see section 2.2.2.3.3) had been undertaken as part of each CF patient's annual assessment in the 12 months preceding the study. Measures of both residual volume (RV) and total lung capacity (TLC) were estimated, and from these measures, an estimate of gas trapping can be made from the RV to TLC ratio.

4.3.5 Chrispin-Norman chest radiograph score

Chrispin-Norman scoring (CNS) of a chest radiograph had been undertaken as part of each CF patient's annual assessment in the 12 months preceding the study. The modified CNS scoring system (Benden *et al.* 2005) was used (section 2.2.2.3.4). The proforma used for CNS scoring is included in Appendix 2.

4.3.6 Respiratory muscle pressure measurements

Measures of respiratory muscle pressure were undertaken in accordance with the methods described in section 2.2.2.4. Briefly these were:

- a) Sniff Inspiratory Pressure (SnIP) – A maximal sniff obtained from FRC with the contralateral nostril occluded.
- b) Mouth Inspiratory Pressure (MIP)
– A maximal inspiratory effort from FRC whilst wearing a noseclip.
- c) Mouth Expiratory Pressure (MEP)
– A maximal expiratory effort from TLC whilst wearing a noseclip.

An accompanying software package (PUMA software - Micro Medical Ltd., Chatham, Kent, UK) allowed real-time quality control assessment for each manoeuvre, as well as analysis of pressure-time plots.

4.3.7 Cardiopulmonary exercise testing (CPET)

Incremental cycle ergometer exercise testing with breath by breath ventilatory gas analysis using a metabolic cart (MedGraphics, St Paul, Minnesota, USA) was undertaken using the methodology of section 2.2.2.8.

Briefly, the subject exercises on an investigator-determined ramp protocol that is geared to the size, age and fitness level of the child. Resting data is collected followed by a period of unloaded cycling. The incremental exercise test then takes place, and the subject is subsequently monitored during their recovery.

Information is collected on heart rate, oxygen uptake (VO_2), carbon dioxide elimination (VCO_2), work rate, respiratory rate and tidal volume, as well as the monitoring of end-tidal CO_2 (etCO_2), and arterial oxygen saturations (SpO_2) throughout exercise.

4.3.8 Quality of life assessment using the United Kingdom Cystic Fibrosis Questionnaire [CFQ-UK]

Quality of life was assessed using the CFQ-UK. A CD-ROM package that accompanies the questionnaire enables various quality of life dimensions to be assessed. The methods are described in full in section 2.2.2.10.

Briefly, there are several versions of the questionnaire:

- The CFQ-14+ was used for children aged over 14 years, and is a self-assessed questionnaire that takes around 10 minutes to complete.
- A self-reporting format was used for 12 and 13 year-olds.
- The CFQ child P is a parent-proxy evaluation used for children aged 6 - 13.

Copies of the various CFQ questionnaires used are attached in Appendix 3.

4.3.9 Echocardiographic measures

Trans-thoracic echocardiography was undertaken on all study patients, in accordance with the methods described in section 2.2.2.7. Cardiac structure and morphology were first assessed.

Measures of left ventricle (LV) wall thickness at both interventricular septum and posterior wall (IVSd and LVPWd), and right ventricle wall thickness (RVWd) were made. LV fractional shortening (%) was assessed, and LV ejection fraction (%) estimated using the methodology of section 2.2.2.7.

Systolic pulmonary artery pressure (sPAP) was estimated from the Doppler signal of tricuspid regurgitation (TR) gradient (if any TR present) using the modified Bernoulli equation, described in section 2.2.2.7.

4.4 Results

Nine out of 41 subjects were classified as hypoxic using the definition $\text{SpO}_2 < 93\%$ for $>10\%$ sleep time. Thus the study was adequately powered to detect differences in primary outcome – exercise capacity (section 4.4.6) in accordance with the *a priori* power calculation outlined in section 2.1.3.

4.4.1 Anthropometric data and demographics

4.4.1.1 Anthropometric and demographic data for study population

Table 4.1 displays the age, sex and physical characteristics of the study population including average z scores for height, weight and BMI. Also displayed are CFTR genotypes, pancreatic status and *Pseudomonas aeruginosa* and *Staphylococcus aureus* infection status.

Table 4.1

Demographic data for the study population (n=41)

Variable	Study population (n=41)	
	Range	Median (IQR)
Age	8 to 16.2	12.7 (10.4 to 14.6)
Sex	20M:21F	-
Height (cm)	123 to 184	147 (136 to 160)
Weight (kg)	22 to 64	40 (30 to 47)
BMI ($\text{kg}\cdot\text{m}^{-2}$)	14 to 23	18 (15.7 to 19)
Height SDS	-2.1 to +1.8	-0.5 (-1.1 to 0.2)
Weight SDS	-2.2 to +1.7	-0.7 (-1.4 to 0.1)
BMI SDS	-2.2 to +2	-0.4 to (-1.1 to 0.2)
CFTR genotype		
• $\Delta\text{F508}/\Delta\text{F508}$	25	-
• $\Delta\text{F508}/\text{Other}$	13	-
• $\text{Other}/\text{Other}$	3	-
Pancreatic status		
Pancreatic-insufficient (PI)	PI (n=39)	-
Pancreatic-sufficient (PS)	PS (n=2)	-
Lung function		
FEV ₁ SDS	-5 to +2	-1.9 (-3.2, -0.9)
FVC SDS	-4 to +1.6	-1 (-2.3, 0)
<i>Pseudomonas aeruginosa</i> infection	• Ever (n=37)	-
	• Never (n=4)	-
<i>Staphylococcus aureus</i> infection	• Ever (n=33)	-
	• Never (n=8)	-

4.4.1.2 Anthropometric and demographic data and hypoxia

Using the definition of nocturnal hypoxia in CF ($SpO_2 < 93\%$ for $>10\%$ sleep time) suggested in section 3.9, it is apparent that the hypoxic group are older and relatively taller (Figure 4.1) but with body mass indices that are significantly lower (Figure 4.2) than their normoxic peers (Table 4.2).

Table 4.2

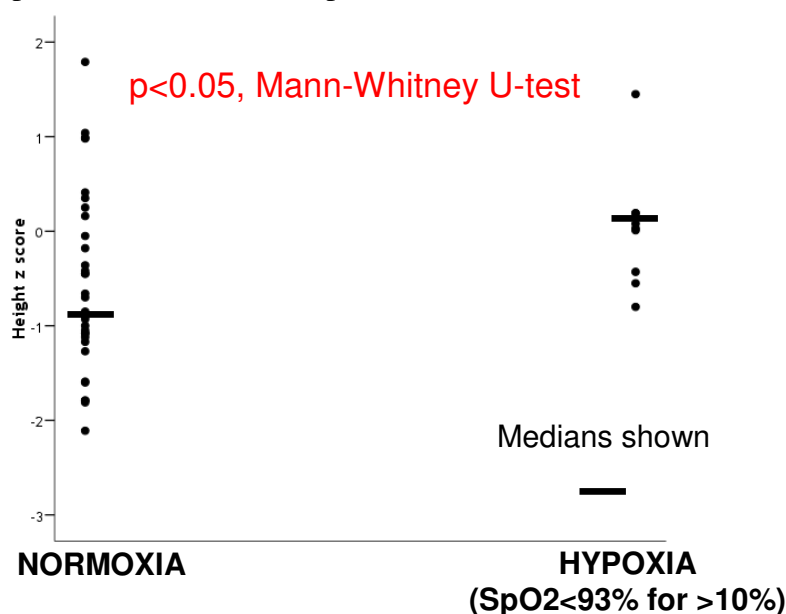
Comparison of demographic and anthropometric data between hypoxic ($SpO_2 < 93\%$ for $>10\%$ sleep time) and normoxic groups

Variable	Study population (n=41)		
	Hypoxic (n=9) ($SpO_2 < 93\%$ for $>10\%$ sleep time)	Normoxic (n=32)	p-value
Age (years)	14.9 (12.4, 15.6)	12.2 (10, 13.7)	<0.05*
Height SDS	0 (-0.5, +0.2)	-0.8 (-1.2, 0.1)	0.05*
Weight SDS	-0.8 (-1.4, -0.2)	-0.6 (-1.3, 0.1)	0.38*
BMI SDS	-1.1 (-2, -0.6)	-0.2 (-0.7, +0.3)	<0.01*

All values quoted are median (+/- IQR), *Mann-Whitney U-test

Figure 4.1

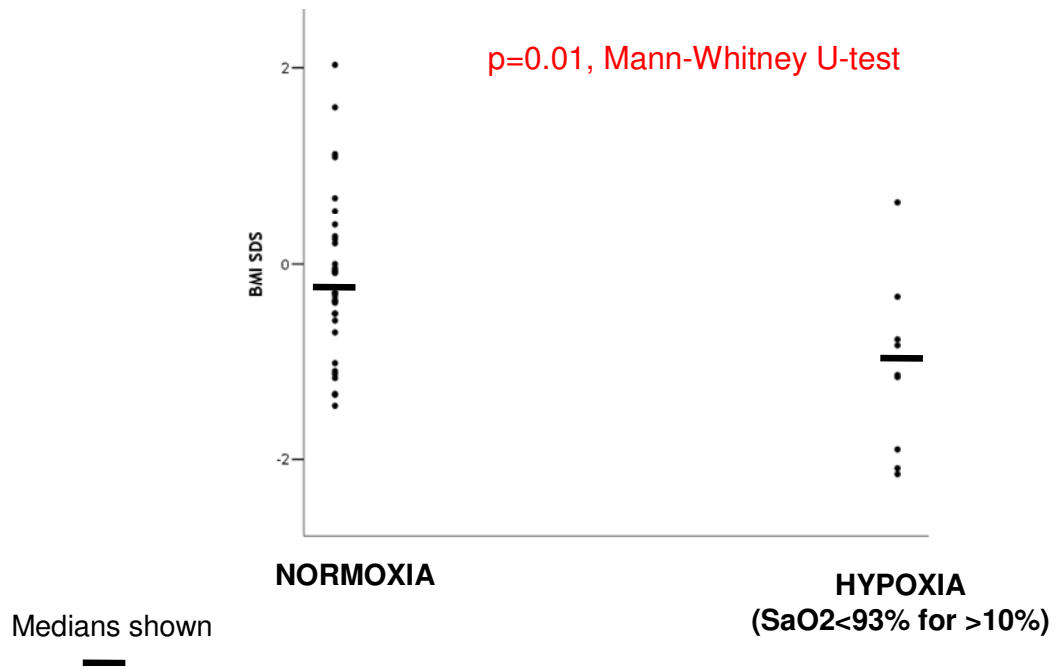
Distribution of height z scores in normoxic CF patients and those with hypoxia ($SpO_2 < 93\%$ for $>10\%$ sleep time)



Although statistical significance is achieved, there is considerable overlap between the heights of hypoxic and normoxic subjects. This may suggest an artefactual difference pertaining to small study numbers.

Figure 4.2

Distribution of BMI z scores in normoxic CF patients and those with hypoxia (SpO₂<93% for >10% sleep time)



BMI z scores appear to be significantly lower in the hypoxic group, although once again a degree of overlap exists. The catabolic effects of being in a pro-inflammatory state may impact negatively on nutritional status (Gan *et al.* 2004), providing a potential explanation for these observed differences.

4.4.2 Spirometry

4.4.2.1 Demographic data of study population

Spirometry was successfully undertaken in all 41 study subjects. Subjects ranged from 8 to 16.2 years of age, with a mean (sd) age of 12.5 (2.4) years, and a median age of 12.7 years. Twenty-one subjects were female, and 20 male. The distribution of spirometry measures is illustrated in Table 4.3.

Table 4.3

Spirometry of the study population

<i>Variable</i>	<i>Subjects (n=41)</i>	
	Range	Median (IQR)
FEV₁	-5.0 to +2.0 [35 to 128%]	-1.9 (-3.2 to -0.9) [74 (57 to 88)%]
FVC	-4.0 to +1.6 [51 to 123%]	-1.0 (-2.3 to 0) [88 (71 to 100)%]
FEF₅₀	-3.8 to 2.5 [9 to 159%]	-2.4 (-2.9 to -1.3) [51 (30-74)%]
FEF₇₅	-3.5 to 1.8 [7 to 156%]	-1.9 (-2.7 to -1.2) [33 (19 to 42)%]

All values are standard deviation score for lung function parameters.

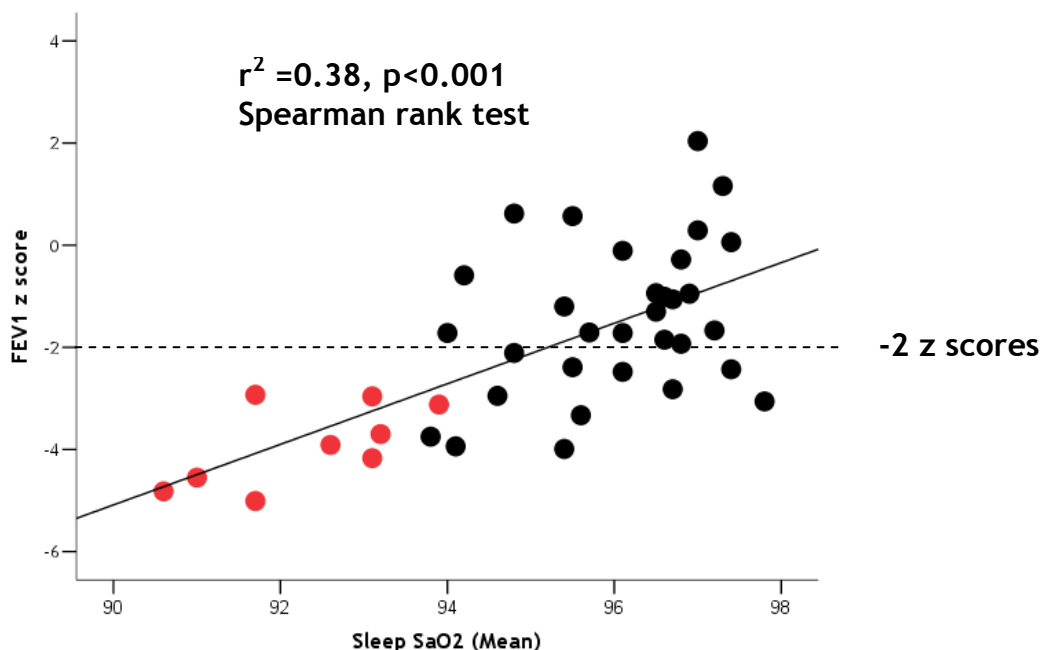
(%predicted values are given in parentheses [] thereafter).

4.4.2.2 Spirometric parameters and hypoxia

The relationship between FEV₁ and mean overnight SpO₂ was explored (Figure 4.3). Using z scores based on validated reference ranges (Rosenthal *et al.* 1993) Spearman rank correlation coefficient was $r^2=0.38$, $p<0.001$.

Figure 4.3

Correlation of FEV₁ SDS and mean overnight SpO₂



- Subject meeting criteria for hypoxia (SpO₂<93% for >10% sleep time)
- Normoxic subject

This graph illustrates that all of the hypoxic subjects had abnormal FEV₁.

Similar correlations were observed for comparison of mean sleep SpO₂ with FVC and mid-expiratory flow measures (Table 4.4).

Table 4.4

Correlation coefficients for mean sleep SpO₂ and spirometric parameters

	<i>FEV₁</i> <i>z score</i>	<i>FVC</i> <i>z score</i>	<i>FEF₅₀</i> <i>z score</i>	<i>FEF₇₅</i> <i>z score</i>
Mean sleep SpO₂	$r^2 = 0.38 *$	$r^2 = 0.25 **$	$r^2 = 0.24 **$	$r^2 = 0.38 *$

* p<0.001; ** p=0.001 (Spearman test)

Comparison of spirometric measures of lung function between those with nocturnal hypoxia and normoxic controls was carried out, using the newly proposed (SpO₂<93% for >10% sleep time) definition of hypoxia (Table 4.5).

Table 4.5

Comparison of spirometry data between hypoxic ($\text{SpO}_2 < 93\%$ for $>10\%$ sleep time) and normoxic groups

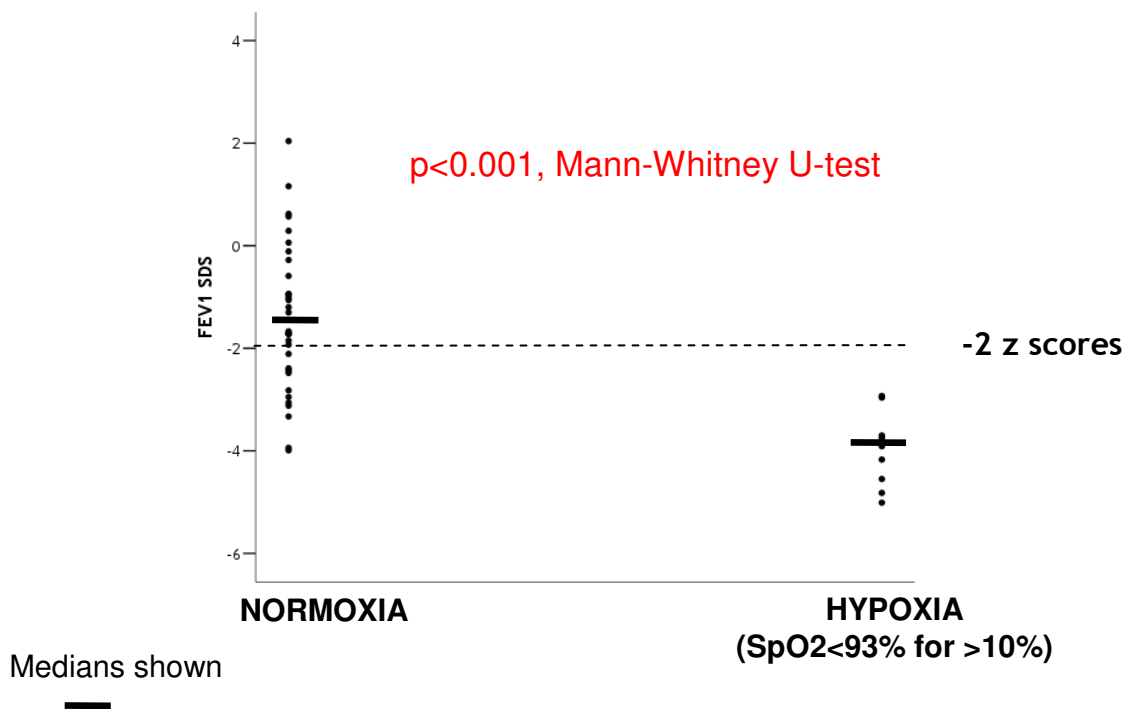
Variable	Study population (n=41)		
	Hypoxic (n=9) ($\text{SpO}_2 < 93\%$ for $>10\%$ sleep time)	Normoxic (n=32)	p-value
FEV ₁ SDS	-3.9 (-3.3, -4.7)	-1.7 (-2.5, -0.4)	<0.001*
FVC SDS	-2.6 (-3.8, -1.8)	-0.6 (-1.8, +0.1)	<0.001*
FEF ₅₀ SDS	-3.3 (-3.6, -2.8)	-1.8 (-2.5, -0.8)	<0.001*
FEF ₇₅ SDS	-2.9 (-3.2, -2.3)	-1.9 (-2.2, -1.0)	0.002*

Values are median (IQR) of lung function z scores, * Mann-Whitney U test.

All of the above spirometric indices were lower in the hypoxic group when compared to the remainder of the study population. Figure 4.4 illustrates the distribution of FEV₁ SDS values across hypoxic and normoxic groups.

Figure 4.4

Distribution of FEV₁ z scores in normoxic CF patients and those with hypoxia ($\text{SpO}_2 < 93\%$ for $>10\%$ sleep time)



It can be seen that if FEV₁ z score is within normal range (>-2 z scores), then no subject meets the criteria for hypoxia. Work to delineate the optimal level of lung function at which to consider a sleep study will be defined in section 4.5.3.

4.4.3 Plethysmographic data

Plethysmography was successfully undertaken in all 41 study subjects. Subjects ranged from 8 to 16.2 years of age, with a mean (sd) age of 12.5 (2.4) years, and a median age of 12.7 years. Twenty-one subjects were female, and 20 male. The measure considered to be most representative of gas trapping was RV/TLC Ratio. Across the entire study population, RV/TLC ratio had a median (IQR) value of 0.36 (0.27, 0.5).

4.4.3.1 Hypoxia and RV/TLC ratio

Hypoxic subjects ($SpO_2 < 93\%$ for $>10\%$ sleep time) had a median (IQR) ratio of RV/TLC of 0.5 (0.45, 0.64), compared with 0.32 (0.24, 0.45) in normoxic subjects ($p < 0.001$, Mann-Whitney U-test).

Therefore it is suggested that hypoxic CF subjects have a greater degree of gas-trapping than do their normoxic counterparts.

4.4.4 Radiographic data: Chrispin-Norman chest radiograph score (CNS)

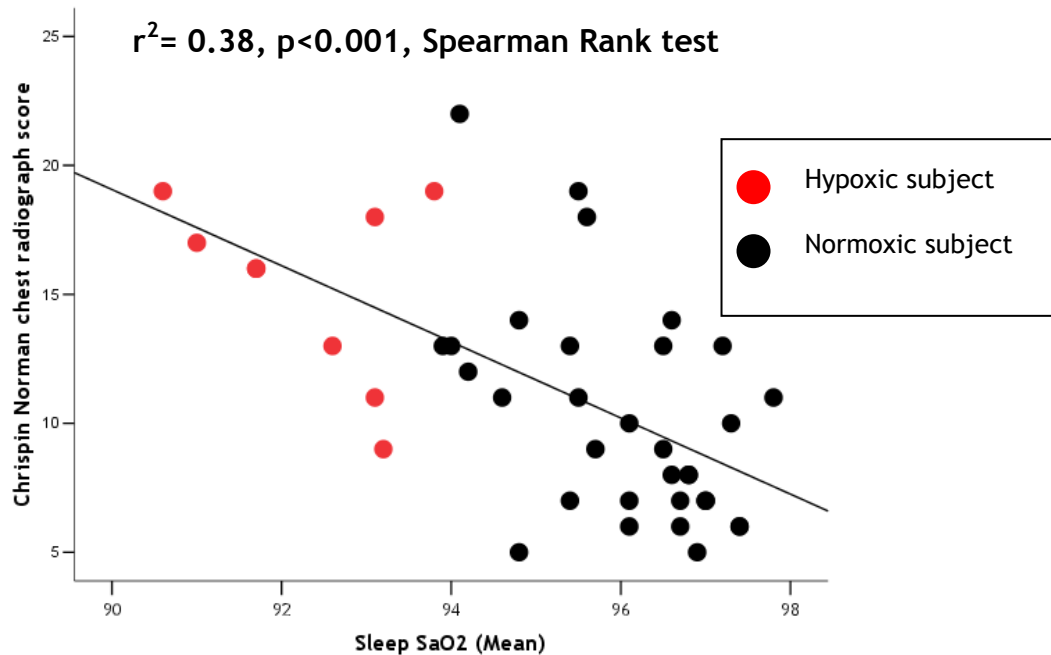
CNS was successfully undertaken in all 41 study subjects. Across the entire study population, median (IQR) CNS was 11 (7, 14). Given that baseline age differences exist between the groups when classified for nocturnal hypoxia (Tables 4.2 and 4.3), age-corrected CNS was also considered (CNS/Age). The CNS/age measure had a median value of 0.81 (0.65, 1.19).

4.4.4.1 Hypoxia and radiographic data

A relationship is observed between CNS and mean sleep SpO₂ (Figure 4.5), showing negative correlation ($r^2=0.38$, Spearman).

Figure 4.5

Relationship of mean sleep SpO₂ and Chrispin-Norman X-ray score



Statistically higher CNS were observed in the hypoxic (SpO₂<93% for >10% sleep time) group. These differences were maintained when the CNS were corrected for age (Table 4.6). These findings are of importance as they suggest that lung structure (as well as function) may be worse in the hypoxic group.

Table 4.6

Comparison of Chrispin-Norman chest radiograph scores between hypoxic (SpO₂<93% for >10% sleep time) and normoxic groups

Variable	Study population (n=41)		
	Hypoxic (n=9) (SpO ₂ <93% for >10% sleep time)	Normoxic (n=32)	p-value
CNS	16 (12,19)	10 (7,13)	0.002*
CNS/Age	1.1 (0.8,1.3)	0.8 (0.6,1)	0.04*

All values quoted are median (+/- IQR), *Mann-Whitney U-test

4.4.5 Respiratory muscle pressure measurements

4.4.5.1 Demographic data of study population

SnIP and MEP measurements were successfully undertaken in all 41 study subjects. Subjects ranged from 8 to 16.2 years of age, with a median age of 12.7 years. Twenty-one subjects were female, and 20 male. MIP measurements were successfully undertaken in forty of the 41 study subjects. One male subject was unable to perform MIP due to poor technique. He was at the lower end of the age range studied, at 9.02 years. Respiratory muscle pressure data for the entire study population is displayed below (Table 4.7)

Table 4.7
Demographics for SnIP/MIP and MEP

<i>Variable</i>	<i>Study population (n=41)</i>		
	Median (IQR) Pressure (cm H₂O)	Range (cm H₂O)	Median (IQR) SDS
SnIP (n=41)	89 (79-108)	29-207	-0.35 (-0.8 to 0)
MIP (n=40)	81 (65-104)	40-131	-0.12 (-0.7 to +1)
MEP (n=41)	90 (69-109)	38-149	-0.32 (-1.2 to +0.5)

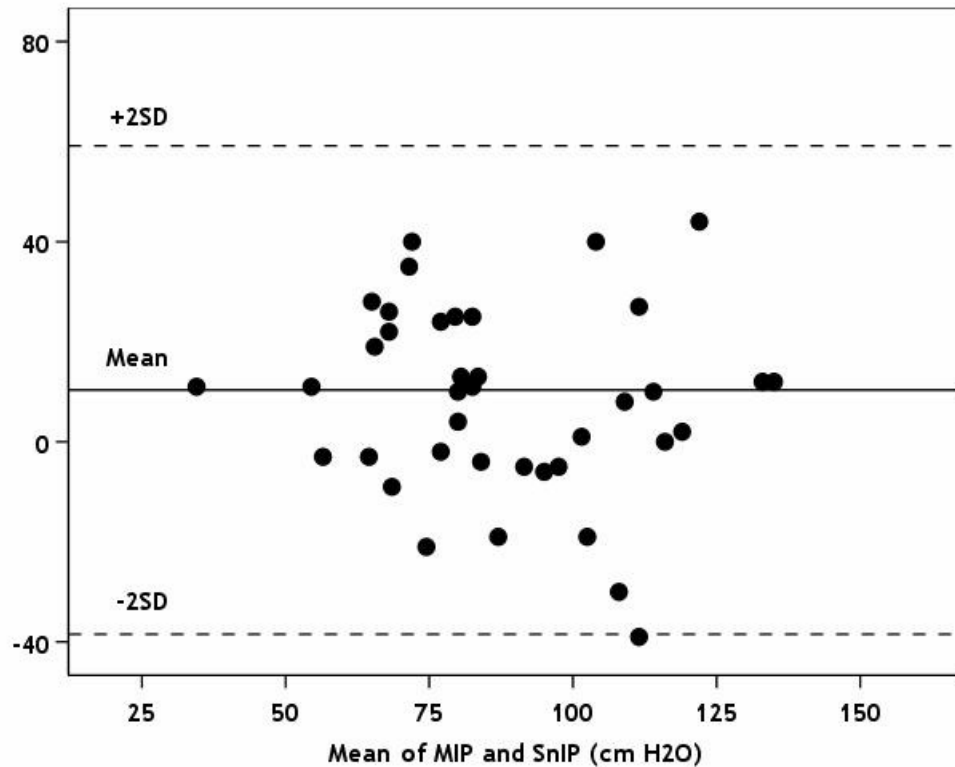
4.4.5.1.1 Relationship of SnIP and MIP in CF

Limits of agreement for SnIP and MIP were assessed using a Bland-Altman plot (Bland and Altman 1986). SnIP was, on average, higher than MIP with a mean (sd) difference of 10.4 (24.4) cm H₂O (Figure 4.6).

Figure 4.6

Limits of agreement for two methods of inspiratory pressure measurement (SnIP and MIP) using a Bland and Altman plot

SnIP-MIP difference
(cm H₂O)



These mean (sd) differences are similar to those reported in healthy children [13.5 (21.4) cm H₂O] (Rafferty *et al.* 2000).

4.4.5.2 Hypoxia and respiratory muscle pressure

Data were considered as absolute measures of muscle strength (cm H₂O), and also as z scores using age and sex-specific reference ranges (Stefanutti and Fitting 1999). Inter-group comparisons were carried out (Table 4.8), and no statistically significant differences were noted between hypoxic (SpO₂<93% for >10% sleep time) and normoxic subject groups.

Table 4.8

Effects of hypoxia ($\text{SpO}_2 < 93\%$ for $> 10\%$ sleep time) on respiratory muscle strength in children with CF

a) Absolute values			
<i>Variable</i>	<i>Study population (n=41)</i>		
	Hypoxic (n=9) ($\text{SpO}_2 < 93\%$ for $> 10\%$ sleep time)	Normoxic (n=32)	p-value
SnIP	92 (66,103)	89 (81,113)	0.57*
MIP	85 (54,103)	78 (66,109) [#]	0.55*
MEP	91 (83,120)	85 (67,109)	0.41*

b) z scores			
<i>Variable</i>	<i>Study population (n=41)</i>		
	Hypoxic (n=9) ($\text{SpO}_2 < 93\%$ for $> 10\%$ sleep time)	Normoxic (n=32)	p-value
SnIP	-0.2 (-1.3,-0.1)	-0.4 (-0.8, 0.2)	0.61*
MIP	-0.7 (-1.2, 1.2)	0.1 (-0.5, 1) [#]	0.41*
MEP	0.4 (-0.9,0.9)	-0.6 (-1.3,0.5)	0.47*

All values are median (IQR) *Mann-Whitney U-test, [#] n=31

A potential explanation for such a lack of effect may be that children with CF have a relative training effect on their respiratory muscles due to increased work of breathing, which allows preservation of muscle strength. This is supported by Figure 4.12 which illustrates similar minute ventilation at maximal exercise in both hypoxic and normoxic subjects, despite reduced lung volumes on forced expiratory manoeuvres in the hypoxic group.

4.4.6 Cardiopulmonary exercise testing (CPET) parameters

40 individuals successfully undertook CPET testing. A single individual was unable to undergo CPET testing, as he was too small for the exercise bike.

4.4.6.1 Demographic data of study population

Displayed below (Table 4.9) are the spread of selected CPET measures recorded in the study population across the phases of exercise.

Table 4.9

CPET data for the study population (n=40)

<i>Variable</i>	<i>Study population (n=40)</i>	
	Range	Median (IQR)
Age	8 to 16.2	12.9 (10.7, 14.7)
Sex	19M:21F	-
<u>Resting:</u>		
HR	66-127	92 (82, 108)
RR	11-45	23 (19, 27)
etCO ₂ (mm Hg)	24-42	34 (31, 36)
SpO ₂	95-100	99 (97, 100)
VO ₂ at rest (mls.m ⁻² .min ⁻¹)	111-269	184 (168, 216)
<u>AT:</u>		
VO ₂ at Anaerobic Threshold		
mls.kg ⁻¹ .min ⁻¹	11.3-37.3	19.6 (16.6,23)
mls.m ⁻² .min ⁻¹	357-978	606 (516, 696)
<u>Peak:</u>		
Peak VO ₂		
mls.kg ⁻¹ .min ⁻¹	21-55	35 (30, 42)
mls.m ⁻² .min ⁻¹	589-1685	1074 (917, 1277)
HR	144-208	180 (170, 191)
RR	26-86	58 (47, 63)
etCO ₂ (mm Hg)	23-47	35 (31, 38)
V _E (L)	22-96	56 (41, 66)
Workload (Watts.m ⁻²)	50-184	83 (68, 103)
Lowest exercise SpO ₂	85-100	98 (96, 99)
Change in SpO ₂ on exercise	-13 to +4	-1 (-2, 0)

4.4.6.2 Hypoxia and CPET testing

Dichotomisation by a definition of SpO₂<93% for >10% sleep time was used to investigate the effects of hypoxia on cardiopulmonary exercise (Table 4.10).

Table 4.10Effects of hypoxia ($\text{SpO}_2 < 93\%$ for $>10\%$ sleep time) on CPET in CF

<i>Variable</i>	<i>Study population (n=40)</i>		
	Hypoxic (n=9) ($\text{SpO}_2 < 93\%$ for $>10\%$ sleep time)	Normoxic (n=31)	p-value
<u>Resting:</u>			
HR	99 (93, 118)	89 (81, 101)	0.09*
RR	24 (20, 38)	23 (19, 27)	0.22*
etCO ₂ (mm Hg)	29 (28, 33)	35 (32, 36)	0.003*
SpO ₂	98 (97, 98)	99 (98, 100)	0.01*
VO ₂ at rest (mls.m ⁻² .min ⁻¹)	193 (175, 214)	184 (146, 216)	0.73*
VO₂ at AT			
mls.kg ⁻¹ .min ⁻¹	16.6 (15.4, 18.4)	20.6 (17.9, 23.8)	0.01*
mls.m ⁻² .min ⁻¹	503 (431, 587)	640 (559, 700)	0.01*
<u>Peak:</u>			
Peak VO ₂			
mls.kg ⁻¹ .min ⁻¹	28.8 (24, 34.2)	35.3 (33.3, 43.2)	0.01*
mls.m ⁻² .min ⁻¹	1138 (906, 1750)	1353 (1126, 1637)	0.04*
HR	170 (164, 178)	182 (174, 192)	0.02*
RR	61 (52, 69)	55 (47, 61)	0.11*
etCO ₂ (mm Hg)	36 (31, 38)	34 (31, 38)	0.92*
V _E (L)	57 (32, 73)	56 (41, 64)	1.0*
(V _E – predicted MVV) (L)	8.8 (-8.4, 15.3)	-10.2 (-26, -0.2)	<0.001*
Workload (Watts m ⁻²)	64 (57, 89)	86 (74, 106)	0.02*
Lowest exercise SpO₂	97 (93, 98)	98 (97, 99)	0.06*
Change in SpO₂ on exercise	-1 (-6, 1)	-1 (-2, 0)	0.75*

All values are median (IQR) *Mann-Whitney U-test

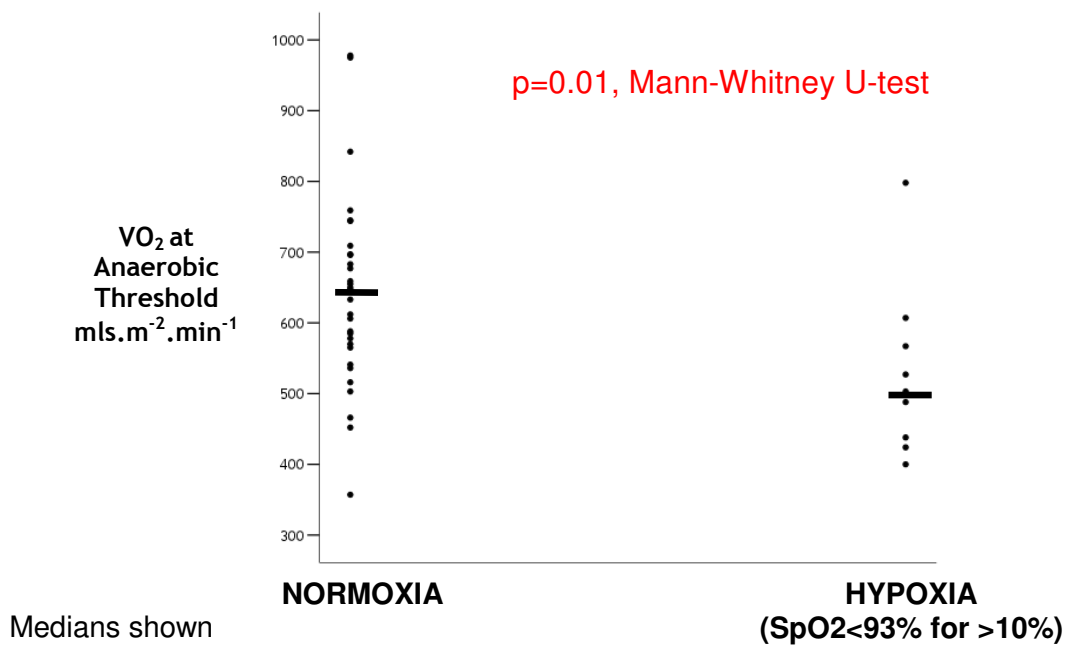
Resting end-tidal CO₂ measures were lower in the hypoxic group. The implication of this might be that hypoxic subjects were hyperventilating at rest relative to normoxic controls. Although no differences in median resting respiratory rates were noted, this hypothesis may be supported by the fact that the IQR for respiratory rate varied from 20-38 in hypoxic subjects compared with 19-28 in controls. Early work in exercise and CF (Godfrey and Mearns 1971) recognised the necessity for increased V_E in children with CF to maintain alveolar ventilation in the face of an increased physiological dead space.

4.4.6.2.1 Hypoxia and VO₂

It is noted that hypoxic subjects had reduced exercise capacity as measured by oxygen uptake (VO₂), at both anaerobic threshold (AT) (Figure 4.7) and peak exercise (Figure 4.8), as well as achieving a lower workload (Figure 4.9). A lower peak heart rate was reached in the hypoxic group, indicative of the fact that exercise was limited by ventilation with the presence of some cardiac reserve at the end of exercise.

Figure 4.7

Effects of hypoxia (SpO₂<93% for >10% sleep time) on oxygen uptake at anaerobic threshold in children with CF



A greater than 10% reduction in VO₂ at AT was seen in hypoxic subjects – the primary study outcome. VO₂ at AT is a more reproducible measure than peak oxygen uptake, for it is less likely to be influenced by the volitional aspect of exercise i.e. AT comes on considerably before peak exercise, and almost all if not every subject will reach AT with encouragement. This was the case in our study population where AT was attained in all.

The effect of the suggested increased height in the hypoxic group (Table 4.2) is controlled for by correcting the units for analysis of oxygen uptake for body surface area (mls.m⁻².min⁻¹) rather than weight (mls.kg⁻¹.min⁻¹) which is most commonly used for analysis.

Figure 4.8

Effects of hypoxia ($SpO_2 < 93\%$ for $>10\%$ sleep time) on peak oxygen uptake in children with CF

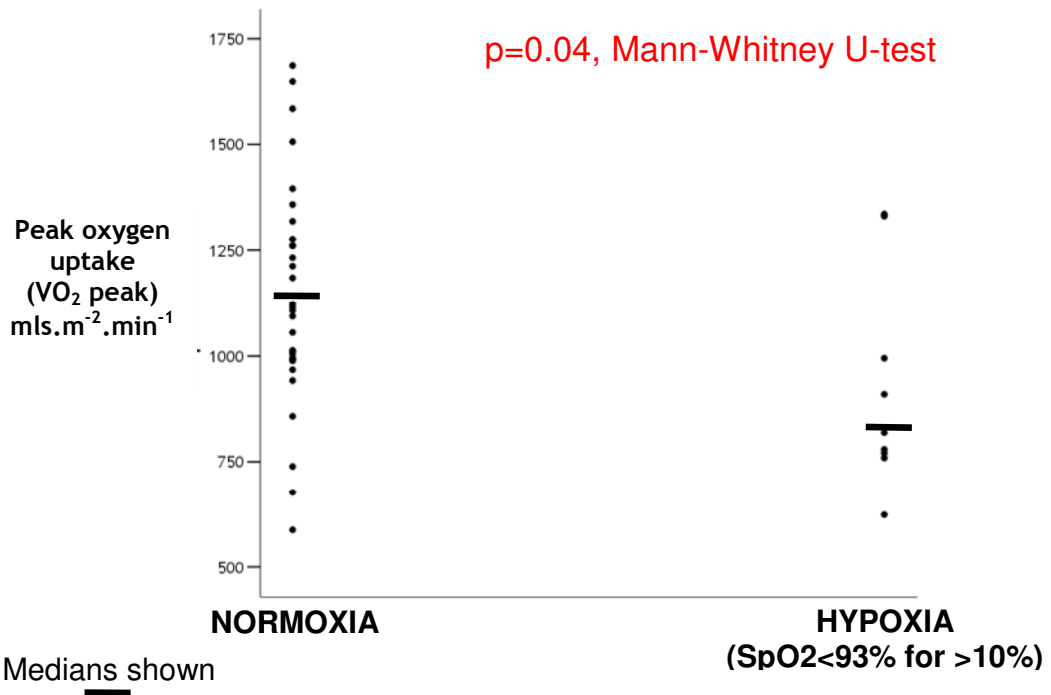
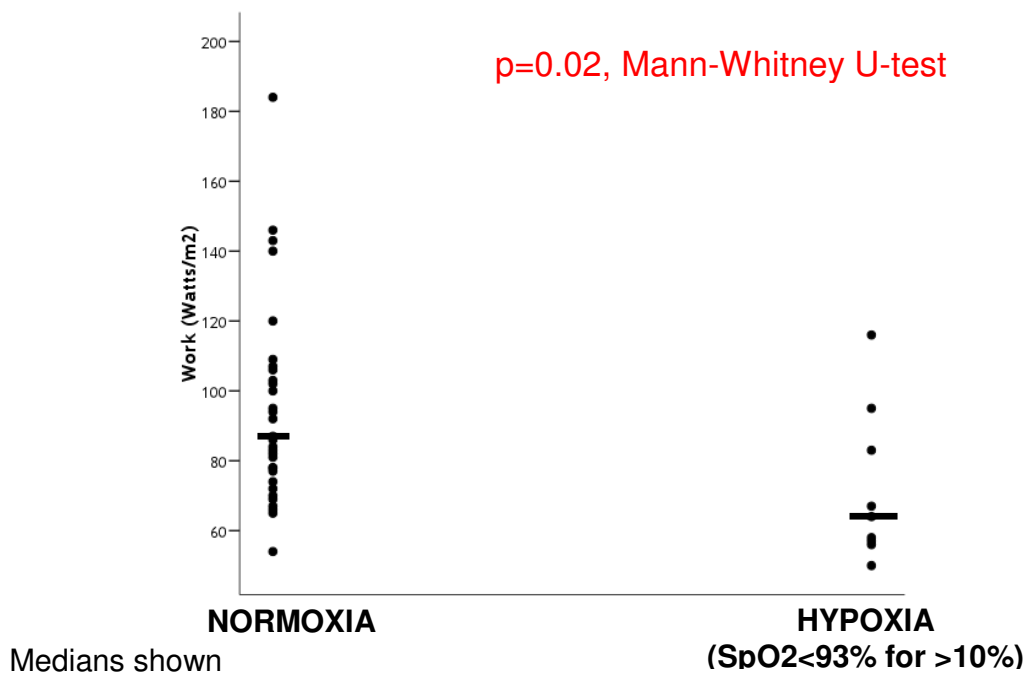


Figure 4.10 illustrates that hypoxic ($SpO_2 < 93\%$ for $>10\%$ sleep time) CF subjects attain lower peak oxygen uptake than normoxic ones.

Figure 4.9

Effects of hypoxia ($SpO_2 < 93\%$ for $>10\%$ sleep time) on peak workload achieved in children with CF



Workload (Watts.m^{-2}) achieved by hypoxic ($\text{SpO}_2 < 93\%$ for $>10\%$ sleep time) subjects was statistically lower in hypoxic subjects compared with normoxic controls (Figure 4.9).

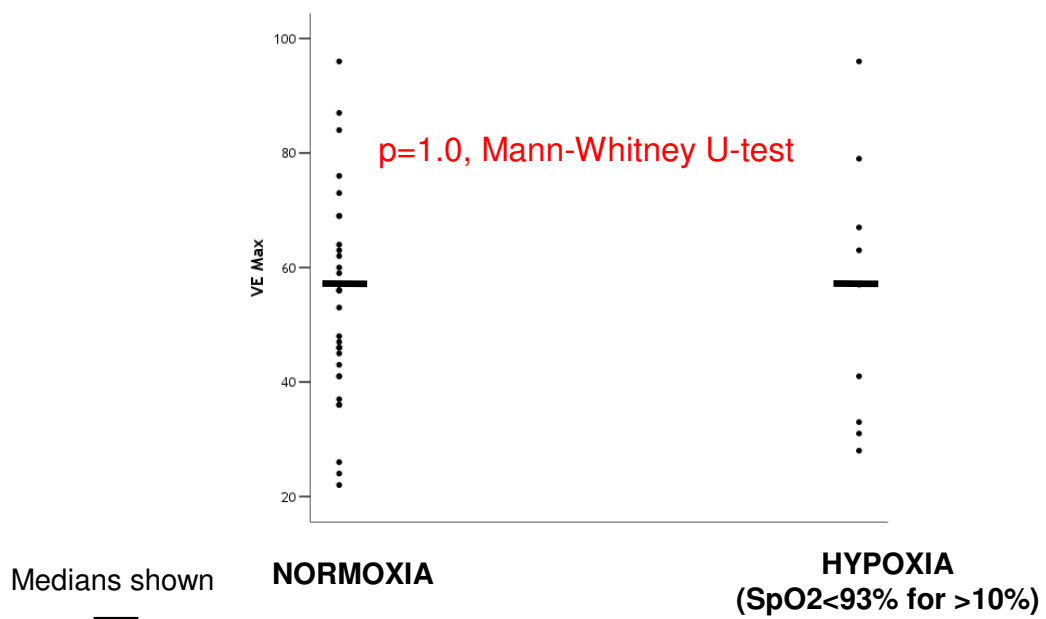
Subjects with nocturnal hypoxia had a trend towards a lower SpO_2 on exercise ($p=0.06$), and also reached a lower peak heart rate ($p=0.02$, Mann-Whitney U test). Potential explanations for this may be that there is respiratory limitation to maximal exercise with cardiac reserve, or that adaptation has occurred in response to nocturnal hypoxia leading to a reduced peak heart rate on exercise. Analogous to athletes who are exposed to repeated periods of hypoxia whilst training, the hypoxic stimulus in our hypoxic CF cohort is repeated every evening whilst asleep. Improved cardiovascular efficiency following a period of mountaineering training has been reported (Purkayastha *et al.* 2000), although more recent work found no difference in heart rate changes between groups of healthy athletes ($n=23$) randomised to hypoxic or sea-level training (Truijens *et al.* 2008).

4.4.6.2.2 Hypoxia and maximal exercise ventilation

No statistically significant differences in minute ventilation (V_E) were seen between hypoxic and normoxic groups (Figure 4.10), despite differences in lung volumes as assessed by spirometry (section 4.4.2).

Figure 4.10

Effects of hypoxia ($\text{SpO}_2 < 93\%$ for $>10\%$ sleep time) on minute ventilation in children with CF

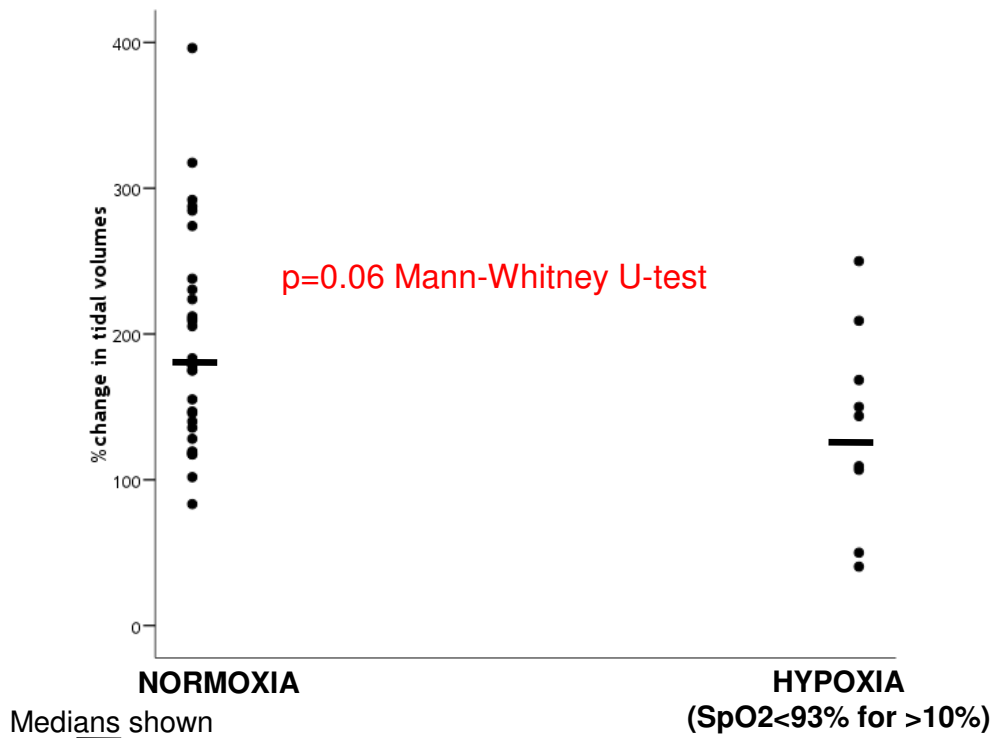


The implication of this finding is that increases in minute ventilation in children with CF and hypoxia are similar to those of their normoxic counterparts. The reasons for this remain unclear, but some possible explanations are discussed below. First, hypoxic subjects have statistically lower vital capacity measured on forced manoeuvres (section 4.4.2), and one might expect greater incremental increases in respiratory rate on exercise in the hypoxic subjects, to account for the similarities observed in minute ventilation. Previous work (Godfrey and Mearns 1971) has shown that increasing disease severity in CF leads to increases in physiological dead space and limitation of tidal volumes that are accompanied by markedly increased respiratory rate on exercise to maintain V_E . However, no differences in respiratory rate at baseline ($p=0.22$), anaerobic threshold ($p=0.5$), or peak exercise ($p=0.11$) were noted (Mann-Whitney U-test).

Secondly, an alternative explanation could be that improved airway clearance occurs on exercise leading to an ability to increase tidal volumes and maintain rate of increase in minute ventilation. Similar median (IQR) tidal volumes were noted between hypoxic and normoxic subjects at peak exercise [0.85(0.6, 1.25) L versus 1.0(0.84, 1.3) L] ($p=0.32$, Mann-Whitney U-test), as well as at AT [0.66(0.52, 0.83) versus 0.6(0.52, 0.78), $p=0.92$; Mann-Whitney U-test]. This suggests that tidal volumes can increase on near-maximal exercise in both hypoxic and normoxic CF subjects. However, the percentage increase in tidal volumes between rest and peak exercise showed a trend ($p=0.06$, Mann-Whitney U-test) towards a smaller median (IQR) % increase in V_T in hypoxic [144(78,189)] subjects compared with normoxic [179(133,232)] controls (Figure 4.11).

Figure 4.11

Percentage change in tidal volumes from rest to peak exercise in hypoxic ($SpO_2 < 93\%$ for $>10\%$) and normoxic children with CF

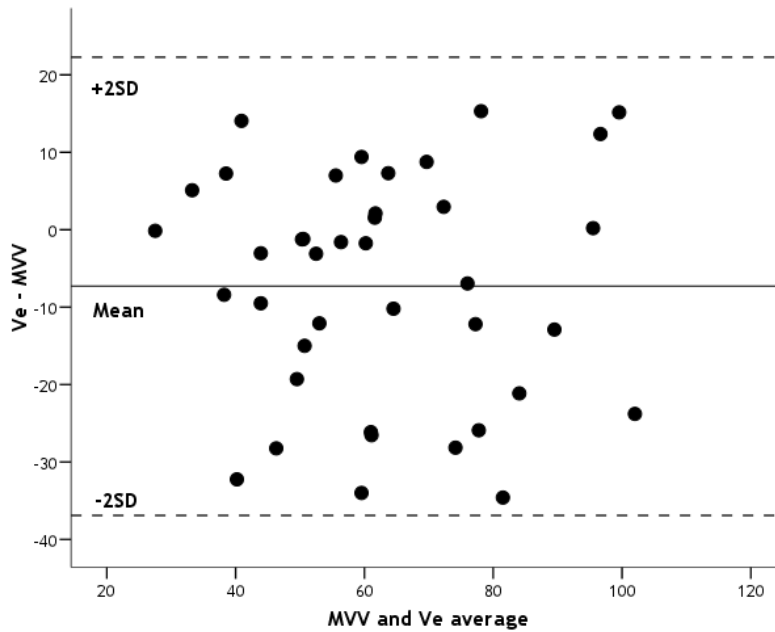


Finally, it could be argued that, as the hypoxic group are taller, this may result in elevated minute ventilation as a result of higher tidal volumes at baseline. However, median (IQR) baseline V_T were 0.40 (0.32, 0.51) L in the hypoxic ($SpO_2 < 93\%$ for $>10\%$) group, compared with 0.36 (0.29, 0.46) L in normoxic controls ($p=0.4$, Mann-Whitney U-test).

Measures of maximal voluntary ventilation (MVV), predicted by $35 \times FEV_1$ (Fulton *et al.* 1995, Sexauer *et al.* 2003) were exceeded by the measured minute ventilation on exercise (maximal V_E) in 14 of our subject group. For the subject group as a whole, the maximal V_E ($V_{E\max}$) achieved on exercise was a mean (sd) of 7.3 (14.8) litres lower than the predicted MVV. This is displayed as a Bland and Altman plot below (Figure 4.12).

Figure 4.12

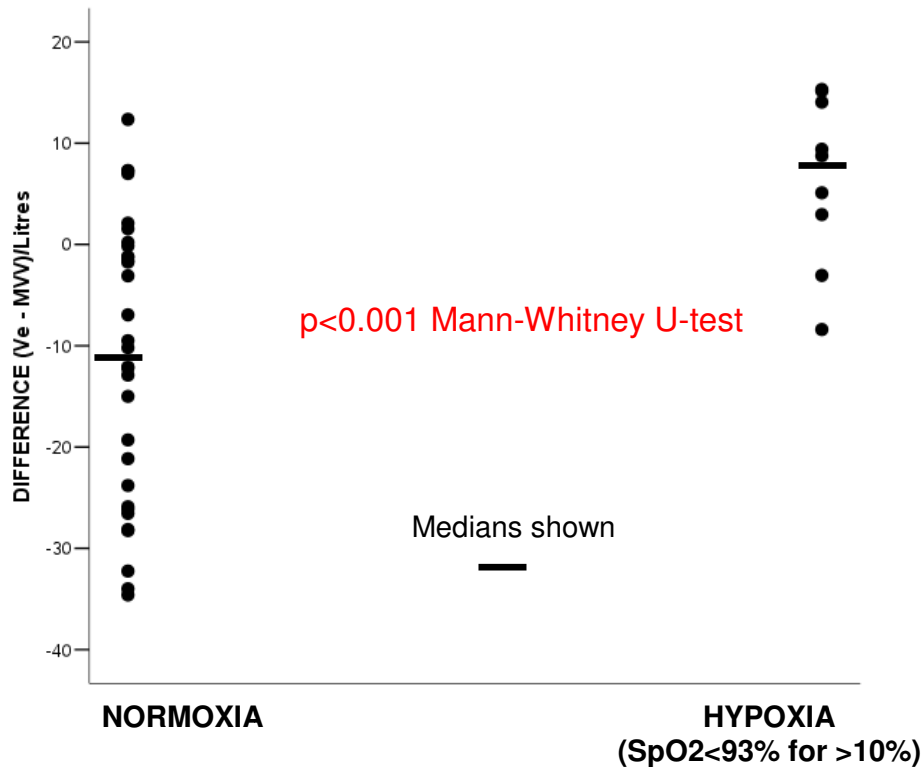
Bland and Altman plot showing the relationship between predicted maximal voluntary ventilation, and minute ventilation measured at maximal exercise



However, if we dichotomise on the basis of hypoxia, it can be seen that the hypoxic group ($SpO_2 < 93\%$ for $>10\%$ sleep time) are, on average, able to exceed their predicted MVV (Figure 4.13).

Figure 4.13

The relationship between predicted MVV and V_E on exercise when comparing normoxic and hypoxic ($SpO_2 < 93\%$ for $>10\%$ sleep time) subjects



In the hypoxic ($\text{SpO}_2 < 93\%$ for $>10\%$ sleep time) group, maximal V_E measured on exercise is a median (IQR) of 8.8 (0, 14.6) litres higher than the predicted ($35 \times \text{FEV}_1$) MVV. In the normoxic group, predicted MVV was 10.2 (-25.9, -0.2) litres higher than measured maximal V_E on exercise. A statistically significant difference is seen between hypoxic and normoxic subjects ($p < 0.001$, Mann-Whitney U test).

This concurs with early work on exercise and CF (Godfrey and Mearns 1971) which showed that exercise ventilation exceeded MVV in all those with more severe disease ($\text{MVV} < 50 \text{L} \cdot \text{min}^{-1}$).

4.4.7 Quality of life measures

The cystic fibrosis questionnaire (CFQ) was successfully completed for 37 of the 41 subjects. In three cases the questionnaire was incorrectly completed, or incomplete making scoring impossible. On one occasion, the questionnaire was not carried out.

4.4.7.1 Demographic data for CFQ

Twenty-three caregiver (6-13 years) CFQ questionnaires were completed, along with 13 self-assessment questionnaires (14+ years), 13 self-assessment questionnaires (12-13 years), and 2 Interviewer format (6-11 year olds) questionnaires.

For purposes of standardisation, either caregiver (children aged 13 or under) or aged 14+ questionnaires (children aged 14 or over) were entered into the study database for analysis. However, for two patients, 12/13 year old self-assessment questionnaires were analysed in the absence of a parental report.

CFQ score (both average and for individual modalities) is expressed as a percentage, whereby a score of 100 represents perfect quality of life and a score of zero, abject misery. The average CFQ score varied between 39.1 and 94.4% with a median (IQR) quality of life score of 71.4% (58.3 to 86). Furthermore, the CFQ is divided into number of individual modalities. Median (IQR) scores and ranges of scores for each modality are shown below (Table 4.11).

Table 4.11

CFQ data demographics for the study population (n=37)

<i>CFQ variable</i>	Number of subjects	Median (IQR)	Range
Age	37	13.1 (10.7, 14.8)	7.98-16.22
Sex	19 Male/ 18 Female	-	-
CFQ Average	37	71.4 (58.3, 86)	39.1-94.4
CFQ Physical	37	83.3 (53.8, 96.3)	25-100
CFQ School	23	88.9 (55.6, 77.8)	11.1-100
CFQ Role	12	75 (66.7, 97.9)	25-100
CFQ Vitality	35	60 (53.3, 80)	33.3-100
CFQ Emotional	37	86.7 (73.3, 93.3)	53.3-100
CFQ Social	14	75 (70.2, 83.3)	61.1-88.9
CFQ Body	37	66.7 (44.4, 94.5)	11.1-100
CFQ Eating	37	100 (50, 100)	0-100
CFQ Treatment	37	55.6 (33.3, 77.8)	11.1-100
CFQ Health	35	77.8 (66.7, 88.9)	33.3-100
CFQ Weight	35	66.7 (33.3, 100)	0-100
CFQ Respiratory	37	72.2 (50, 88.9)	22.2-100
CFQ Digestion	37	88.9 (55.6, 100)	11.1-100

Maximum score for each modality = 100 (CFQ score is a percentage)

Because different scoring components are included in the CFQ 14+ and parent/caregiver (6 to 13 year olds) questionnaire, some modalities (school, role, social) are collated on small numbers of individuals.

4.4.7.2 Hypoxia and quality of life as assessed by CFQ

Data for both sleep studies and CFQ scores were obtained on 37 subjects.

Dichotomisation into hypoxic and normoxic groups gave the following results (Table 4.12).

Table 4.12

Effects of hypoxia ($\text{SpO}_2 < 93\%$ for $>10\%$ sleep time) on CFQ modalities in children with CF

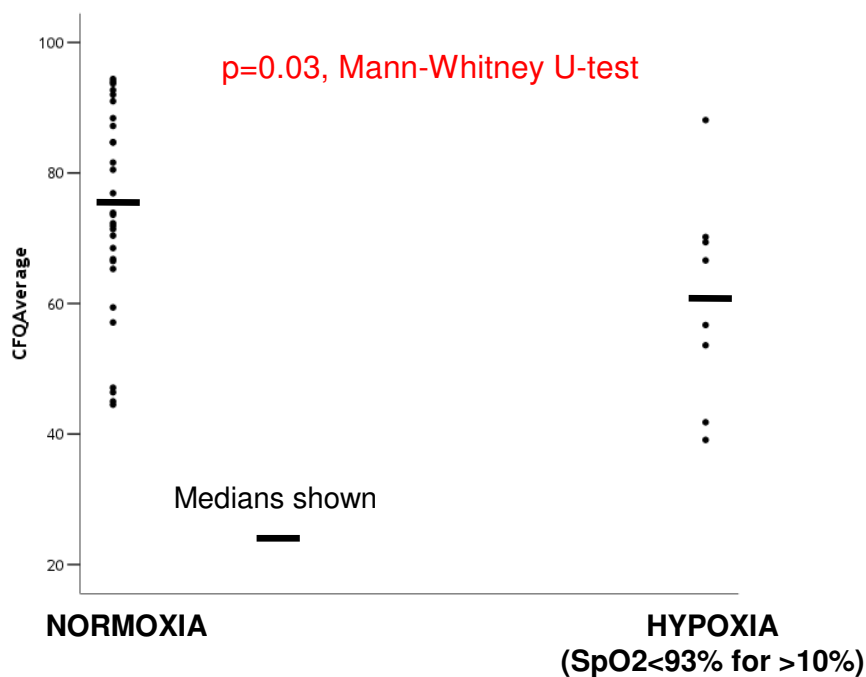
Variable	Study population (n=37)		
	Hypoxic (n=8) ($\text{SpO}_2 < 93\%$ for $>10\%$ time)	Normoxic (n=29)	p-value
CFQ Average	62 (45, 70)	74 (66, 88)	p=0.03*
CFQ Physical	50 (30, 86)	89 (70, 96)	p<0.05*
CFQ Emotional	80 (68, 93)	87 (77, 93)	p=0.46*
CFQ Eating	56 (37, 67)	100 (53, 100)	p=0.06*
CFQ Weight	33 (0, 92)	100 (33, 100)	p=0.17*
CFQ Treatment	44 (14, 78)	56 (44, 83)	p=0.35*
CFQ Respiratory	58 (44, 85)	78 (61, 89)	p=0.19*
CFQ Digestion	94 (67, 100)	89 (56, 100)	p=0.53*

All values are median (IQR) *Mann-Whitney U-test

CF children with sleep hypoxia were noted to have statistically significantly lower average CFQ scores (Figure 4.14) than normoxic individuals.

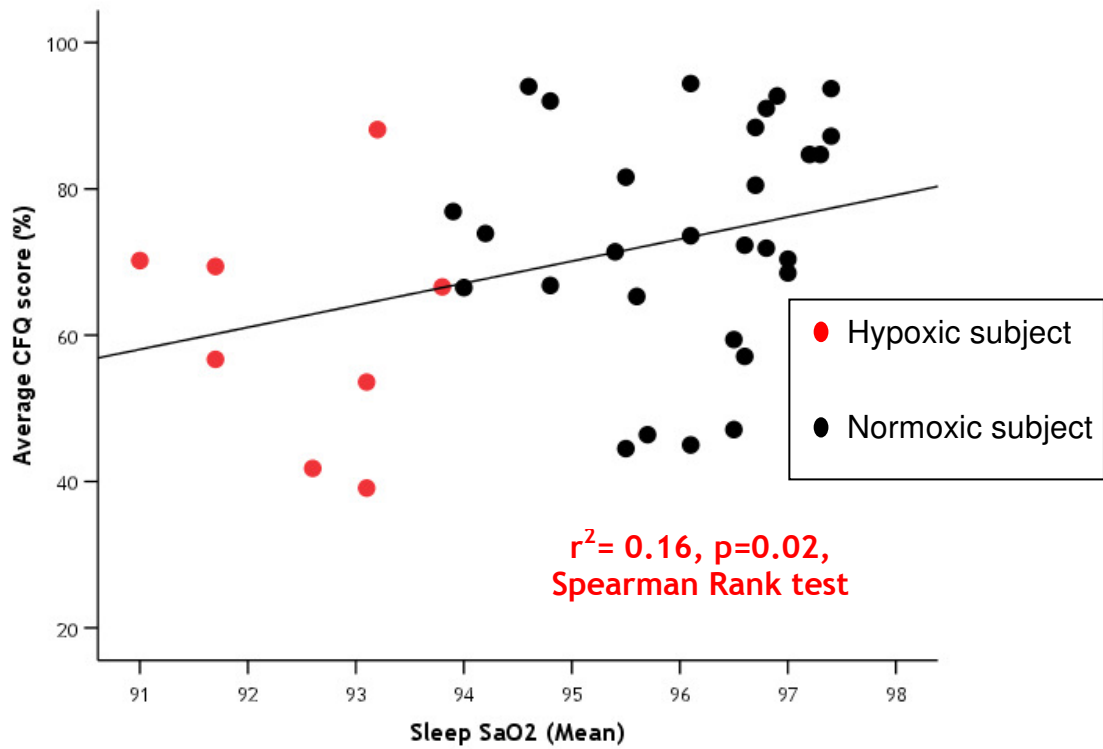
Figure 4.14

Effects of hypoxia ($\text{SpO}_2 < 93\%$ for $>10\%$ sleep time) on quality of life (as assessed by the average CFQ-UK score)



Additionally, a weak relationship between mean sleep SpO_2 and CFQ ($r = 0.4$, $p = 0.02$ Spearman Rank test) was observed (Figure 4.15).

Figure 4.15
Relationship between average CFQ score and mean sleep SpO₂



Significant (but weak) relationships were also noted between mean sleep SpO₂ and the health ($r^2 = 0.24$, $p = 0.003$), body ($r^2 = 0.12$, $p = 0.04$), physical ($r^2 = 0.15$, $p = 0.02$), and respiratory ($r^2 = 0.17$, $p = 0.01$) components of the CFQ score.

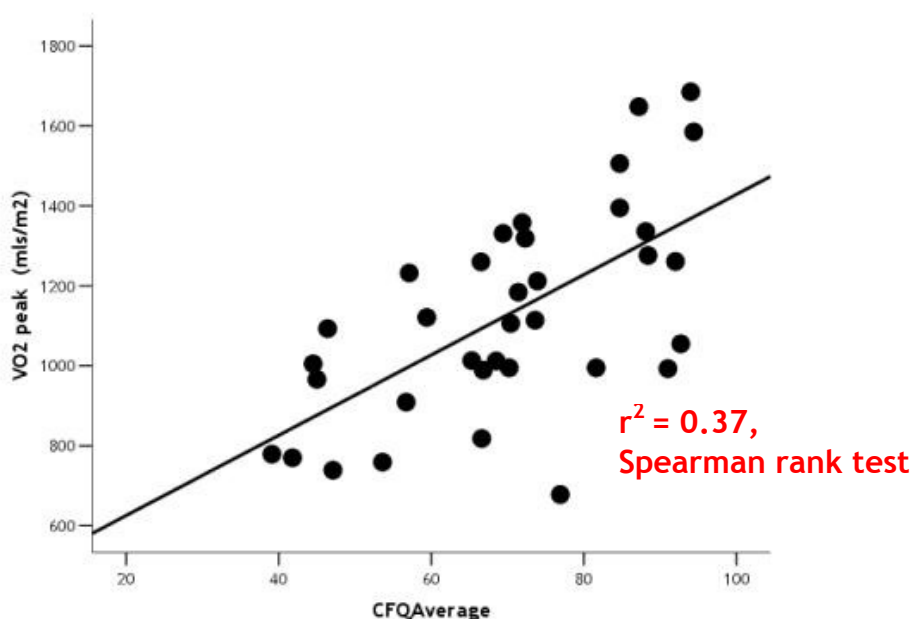
4.4.7.3 Effects of exercise on quality of life as assessed by CFQ

CPET testing and satisfactory completion of the CFQ-UK questionnaire was successfully undertaken in 37 subjects.

Firstly, aerobic fitness as assessed by peak oxygen uptake (VO_2 peak) in $\text{mls.m}^{-2}.\text{min}^{-1}$ shows a significant relationship with average CFQ quality of life score ($r^2 = 0.37$, $p < 0.001$, Spearman rank test), as illustrated below (Figure 4.16).

Figure 4.16

Relationship between average CFQ score and VO_2 peak



Similar associations were seen between VO_2 peak and individual CFQ modalities for physical ($r^2 = 0.42$, $p < 0.001$) and emotional ($r^2 = 0.34$, $p < 0.001$) well-being, as well as health ($r^2 = 0.46$, $p < 0.001$), vitality ($r^2 = 0.37$, $p < 0.001$), respiratory ($r^2 = 0.29$, $p = 0.001$), eating ($r^2 = 0.18$, $p < 0.01$), and body ($r^2 = 0.19$, $p < 0.01$) scores (Spearman rank test).

The associations between VO_2 at AT in $\text{mls.m}^{-2}.\text{min}^{-1}$ and quality of life scores are less strong than those seen at peak exercise. However, statistically significant associations were seen between VO_2 at AT and the average CFQ score ($r^2 = 0.13$, $p = 0.03$), as well as physical ($r^2 = 0.19$, $p < 0.01$) and emotional ($r^2 = 0.16$, $p = 0.01$) well-being modalities, and health ($r^2 = 0.26$, $p < 0.01$), vitality ($r^2 = 0.17$, $p = 0.02$) and respiratory ($r^2 = 0.14$, $p = 0.02$) scores (Spearman rank test).

4.4.8 Echocardiographic measures

Echocardiography was undertaken in 40/41 of the study population.

4.4.8.1 Demographic data for the study population

Displayed below (Table 4.13) are the spread of echocardiographic measures recorded in the study population. It should be noted that 12 subjects had no detectable TR.

Table 4.13

Demography of echocardiogram data for the study population

<i>Variable</i>	<i>Study population (n=40)</i>	
	Range	Median (IQR)
Age	8 to 16.2	12.9 (10.7, 14.7)
Sex	19M:21F	-
LV wall thickness (in diastole)		
Interventricular septum - IVSd (mm)	3.9 – 9	6.4 (5, 7)
Posterior wall - PWd (mm)	4 - 10	6 (5.5, 6.7)
LV dimension #		
LVD (mm)	30 - 56.2	43.4 (40.5, 47)
LV fractional shortening		
LVFS (%)	28 - 46	33 (30, 36)
LV ejection fraction		
LVEF (%)	42 - 78	62 (58, 66)
RV wall thickness (mm)	2 – 6.1	4 (3.2, 5)
Tricuspid Regurgitation		
TRmax (m.s ⁻¹)	0 – 2.8	1.9 (0, 2.2)
Systolic pulmonary artery pressure		
sPAP (mmHg)	5 - 37	19.2 (5, 24.3)

n=27

4.4.8.2 Hypoxia and echocardiographic measures

Dichotomisation using the definition SpO₂<93% for >10% sleep time as denoting hypoxia in CF, was undertaken and the echocardiogram data obtained from our study population was analysed thus (Table 4.14).

Table 4.14Effects of hypoxia (SpO₂<93% for >10% sleep time) on the heart in children with CF

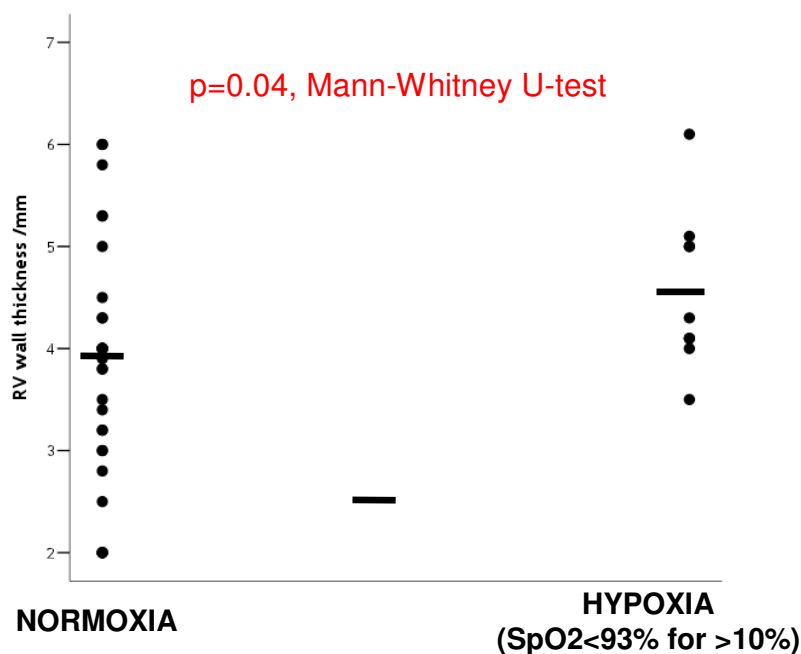
Variable	Study population (n=40)		
	Hypoxic (n=9) (SpO ₂ <93% for >10% time)	Normoxic (n=31)	p-value
LV wall thickness (in diastole)			
IVSd (mm)	6.5 (5.5, 7.1)	6.4 (5, 7)	0.68*
PWd (mm)	6.5 (6.1, 7.6)	6 (5.3, 6.4)	0.04*
LV dimension #			
LVD (mm)	46 (42.3, 46.9)	42.6 (40.2, 47)	0.48*
LVFS (%)	35 (30, 38)	32 (29, 36)	0.44*
LVEF (%)	65 (59, 71)	62 (57, 66)	0.35*
RV wall thickness (mm)	4.3 (4.1, 5.1)	4 (3, 4.4)	0.04*
TRmax m.s ⁻¹	1.8 (0.5, 2.6)	1.9 (0, 2.6)	0.50*
sPAP mmHg	18 (7, 31)	19 (5, 24)	0.55*

All values are median (IQR) *Mann-Whitney U-test

Only 23 normoxic and 4 hypoxic patients had LV dimension measured

Statistically greater right ventricle thickness was seen in hypoxic subjects (Figure 4.17).

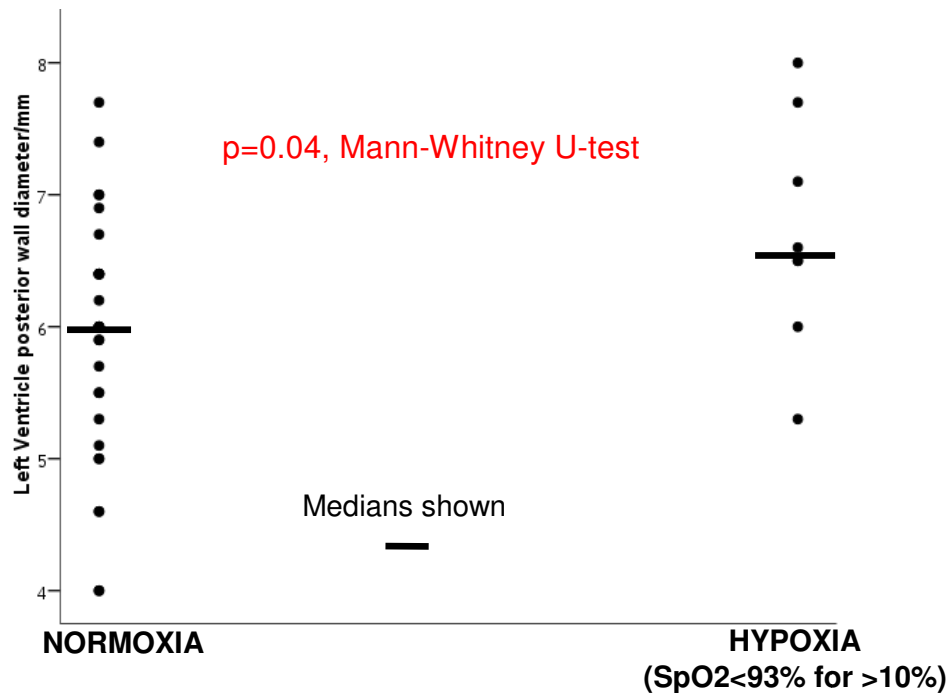
Additionally, increased LV posterior wall diameter was noted (Figure 4.18).

Figure 4.17Effects of hypoxia (SpO₂<93% for >10% sleep time) on RV wall thickness

The finding of increased RV wall thickness may suggest that this measure is a precursor of pulmonary hypertension, at a time when no differences in sPAP were noted between the groups. Indeed no TR whatsoever was recordable in over ¼ of patients studied.

Figure 4.18

Effects of hypoxia (SpO₂<93% for >10% sleep time) on LV posterior wall thickness



The finding of increased LV geometry is not previously reported in CF, but has been reported in obstructive sleep apnoea, another paediatric disorder causing intermittent, repeated hypoxic episodes (Amin *et al.* 2002).

Amin and colleagues (Amin *et al.* 2002) calculated LV mass using a published formula (Devereux *et al.* 1986). This formula allows calculation of LV mass as: $0.8 \{1.04[(LV \text{ dimension} + IVSd + PWd)^3 - LV \text{ dimension}^3]\} + 0.6$ where IVSd and PWd refer to septal and posterior wall thickness in cm. measured in diastole, and LV dimension to the end-diastolic dimension (in cm.) of the LV. Division by (height)^{2.7} is used to construct the LV mass index (de Simone *et al.* 1992). Unfortunately, only 27/41 subjects in our study population had a measure of LV dimension made, including only 4/9 of the hypoxic group. This makes statistical comparison between the two groups impossible. No trends were seen, and the 23 normoxic subjects had a median (IQR) LV mass index of 29.6 (23.9, 32.3) g m^{-2.7} compared with values of 26.2 (18, 30.9) in the 4 hypoxic subjects on whom LV dimension were measured (p=0.58, Mann-Whitney U-test).

4.5 Discussion

4.5.1 Which is more important – sleep hypoxia or exercise hypoxia?

Sleep and exercise hypoxia may represent different pathophysiological defects. At the outset of this work it was unknown which is the more relevant assessment with the greatest clinical impact (section 3.2).

Following the analysis of the above results, clear differences can be seen between children with CF and nocturnal hypoxia and their normoxic counterparts. Subjects with sleep hypoxia ($SpO_2 < 93\%$ for $>10\%$ sleep time) in CF have, when compared to their normoxic counterparts:

- Reduced exercise capacity
- Lower Body Mass Index
- Lower FEV_1
- Lower FVC
- Lower mid-expiratory flows
- Worse gas-trapping as measured by elevation of RV/TLC ratio
- Higher Chrispin-Norman chest radiograph scores
- Greater number of intravenous antibiotic courses
- Worse quality of life as assessed by the CFQ-UK
- Increased right ventricle thickness
- Increased left ventricle (posterior wall) diameter

Such differences were not observed when subjects with exercise-induced arterial hypoxia were compared with controls (data not shown). Previous papers suggest the pathophysiological mechanisms for sleep and exercise hypoxia in CF are different. Airflow limitation related to increase in physiological dead space is reported to be the mechanism by which exercise hypoxia ensues (Godfrey and Mearns 1971), although no evidence for this was found in our study population, with no differences in resting mid-expiratory flows between EIAH and normoxic groups. Flow-volume loops associated with exercise were not analysed, however. Alternatively, dynamic hyperinflation during exercise has been put forward as a potential mechanism for causation of EIAH, but again, no differences in RV/TLC ratios between those with EIAH and the normoxic group were noted.

It appears that hypoxia during sleep, a frequently-repeated (daily) and more prolonged hypoxic insult than EIAH, is associated with greatest clinical impact in CF. The effect of sample size cannot however be discounted, and subject numbers with EIAH were smaller.

4.5.2 Sleep Hypoxia – definitions and outcomes

Previous authors (Bradley *et al.* 1999, Coffey *et al.* 1991) have noted that hypoxia in CF is more likely to occur in sleep than on exercise, because of changes in muscle tone and relative hypoventilation, suggesting that assessment during sleep may be the most relevant assessment of hypoxia in CF.

Various definitions have been used to define hypoxia in CF based on its relationship to inflammation, as outlined in Chapter 3 and led us to propose a new definition of sleep hypoxia (SpO₂<93% for >10% sleep time).

4.5.3 Who needs a sleep study? - Prediction of nocturnal hypoxia in CF

The American Thoracic Society (ATS) recommendations published in 1996 (*Am J Respir Crit Care Med* 1996; **153**: 866-878) suggest that sleep studies should be performed in CF as follows:

1. CF subjects with awake PaO₂ <70mmHg (or SpO₂ <95%) should have an overnight oximetry during a period of disease stability.
2. Patients with polycythaemia or *cor pulmonale*, and those who complain of headache on wakening, daytime sleepiness, or disturbed sleep patterns should have an overnight oximetry.
3. Patients with CF receiving supplemental oxygen may require a full polysomnography (PSG) to rule out OSA in those with snoring and sleep disturbance.
4. PSG may also help assess potential adverse effects of oxygen in CF i.e. promotion of hypercapnia in those with advanced lung disease.

Whilst recommendations 3 and 4 are self-evident, recommendations 1 and 2 require closer scrutiny.

Recommendation 1: Comment

Firstly, daytime SpO₂ are a poor predictor of sleep desaturation in CF. Versteegh and colleagues reported SpO₂<94% as the best independent predictor of nocturnal hypoxaemia (Versteegh *et al.* 1990) in patients with CF. The other reference alluded to in the ATS statement studied daytime blood gas recordings in 14 CF patients aged 9-34 years (Montgomery *et al.* 1989), and compared these to overnight oximetry data. However, blood gases are not routinely done in children with CF.

Frangolias' group studied 70 adults with CF in whom 40% had significant nocturnal hypoxaemia (Frangolias *et al.* 2001). Using resting SpO₂ of 93% as a cut-off for predicting nocturnal hypoxaemia they found that 3/3 patients with a resting SpO₂<93% were hypoxemic at night, but 24/67 with resting SpO₂>93% had nocturnal hypoxaemia also. These data can be used to construct sensitivity (11%) and specificity (100%) values for SpO₂ of <93% as a predictor of nocturnal hypoxaemia in CF. One can construct Likelihood Ratios (LR+ infinite, LR- 0.89), and using a prevalence of nocturnal hypoxaemia of 40%, post-test probabilities can be calculated. One starts with a pre-test probability of nocturnal hypoxaemia of 40% in an adult CF patient group. If resting SpO₂ is below 93%, then the probability of nocturnal hypoxaemia rises to 100%, whereas if resting SpO₂ is above 93%, the probability of nocturnal hypoxaemia falls to 37%. Therefore, resting SpO₂ when less than 93% (and it rarely is) is a useful predictor of nocturnal hypoxaemia, but a sleep study is needed to confidently rule in/rule out hypoxaemia in the remainder.

Similarly, an Australian study reported that in children with significant nocturnal hypoxaemia (average SpO₂ during sleep <90%), 19% had resting SpO₂>94% and therefore 1 in 5 would have been missed without a sleep study (Milross *et al.* 2001).

Recommendation 2: Comment

For subjects with polycythaemia or *cor pulmonale*, and those who complain of headache on wakening, daytime sleepiness, or with disturbed sleep patterns, an overnight oximetry alone is an inappropriate and incomplete investigation. Monitoring of CO₂ is required, be it transcutaneous pCO₂ recording, early-morning arterial or capillary blood gas analysis, or end-tidal measures as part of a full PSG. Clearly full PSG may not be available in all centres, but would provide the gold standard in such scenarios, as sleep disturbance can be directly correlated with stage of sleep.

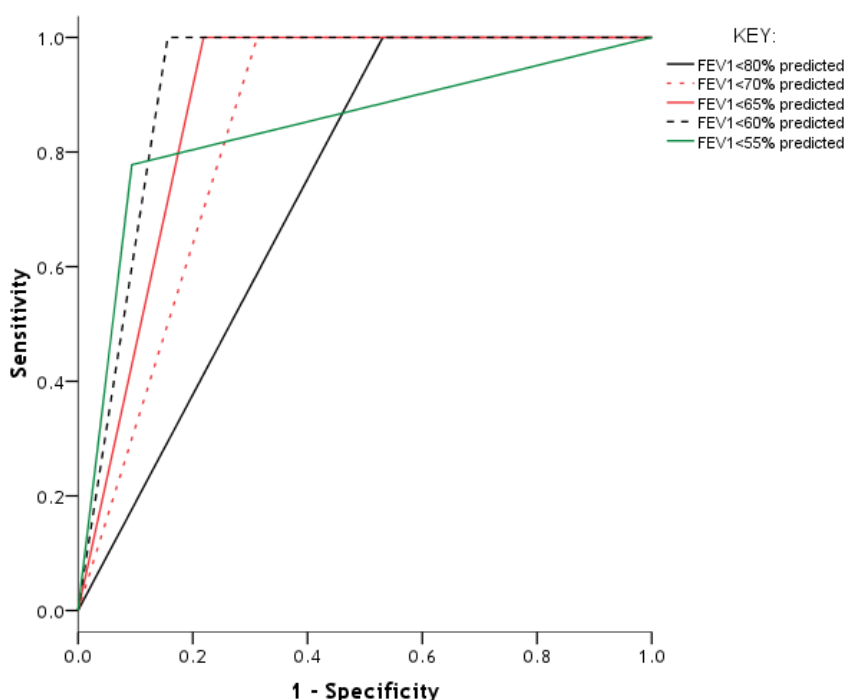
4.5.3.1 Who needs a sleep study? – The role of lung function in the prediction of nocturnal hypoxia in CF

Lung function did not feature in the 1996 ATS recommendations, although an association between sleep SpO₂ and FEV₁ has been reported (Pond and Conway 1995). Our work supports the notion that FEV₁ may be a potentially important predictive index for nocturnal desaturation. Using the *de novo* paediatric definition of sleep hypoxia (SpO₂ <93% for >10% sleep time), the utility value of lung function cut-offs were assessed using ROC statistics (Figure 4.19). Lung function cut-offs [Rosenthal] were considered thus:

- i) FEV₁ <80% predicted
- ii) FEV₁ <70% predicted
- iii) FEV₁ <65% predicted
- iv) FEV₁ <60% predicted
- v) FEV₁ <55% predicted

Figure 4.19

ROC curve of performance of various cut-offs of FEV₁ in the identification of childhood CF subjects with hypoxia (SpO₂<93% for >10% sleep time)



Area under curve:

FEV₁ <80% predicted = 0.73

FEV₁ <70% predicted = 0.84

FEV₁ <65% predicted = 0.89

FEV₁ <60% predicted = 0.92

FEV₁ <55% predicted = 0.84

Previous authors have suggested a cut off of FEV₁ <65% predicted to be the most sensitive and specific predictor of hypoxia (section 1.3.5.2.3.2). Versteegh and colleagues established that an FEV₁ <65% predicted was 88% sensitive and 50% specific (positive predictive value [PPV] 47%) in detecting hypoxia measured as a lowest hourly mean SpO₂ below 90% (Versteegh *et al.* 1990). Frangolias' group report 93% sensitivity and 44% specificity (PPV 51%) for FEV₁ <65% predicted in detecting SpO₂<90% for >5% sleep time (Frangolias *et al.* 2001).

If a cut off of FEV₁ <65% predicted were used to detect hypoxia defined as SpO₂<93% for >10% sleep time in our study population, this would be 100% sensitive and 78% specific in the detection of hypoxia with a positive predictive value of 56%.

The greatest area under the curve is, however, seen for FEV₁ <60% predicted, and this is the predictive measure with the best sensitivity/specificity trade-off (Table 4.15).

Table 4.15

2x2 table on use of FEV₁ <60% predicted in the detection of hypoxia (SpO₂ <93% for >10% sleep time) in children with CF

	Hypoxia: SpO ₂ <93% for >10% sleep time	Normoxia:
FEV ₁ <60% predicted	9	5
FEV ₁ >60% predicted	0	27

FEV₁ <60% predicted

Sensitivity for detection of sleep hypoxia: 100%

Specificity for detection of sleep hypoxia: 84%

Positive predictive value for detection of sleep hypoxia: 64%

It therefore appears that lung function assessment may be useful in the detection of hypoxia in CF. For example, if we were to study all CF children with FEV₁ <60% predicted, two hypoxic (SpO₂<93% for >10% sleep time) children would be detected for every 3 sleep studies performed.

4.5.4 Limitations of study

As discussed in section 3.8.2.3, the use of oximetry alone as our sleep study method has some disadvantages namely:

a) False negative results

Oximetry-only studies may be associated with false negative results, i.e. subclinical obstructive events or arousals may be missed. However, hypoxia is either present or absent on the study, and oximetry appears both sensitive and specific in the detection of hypoxia.

b) Lack of information on sleep state

Sleep state is unknown, to the point that subjects could have been awake all night, although verbal reports from parents confirmed all were sleeping. Baseline SpO₂ were low, with little heart rate variability or SpO₂ dips seen on analyses of oximetry studies of each of the hypoxic individuals; suggesting hypoventilation is occurring throughout sleep (both REM and non-REM). Without EEG monitoring the sleep phases are unknown, however.

c) Cause of hypoxia on study remains speculative

Furthermore, an assumption is made that desaturations seen in our study population were not due to obstruction. This appears a reasonable assumption to make, given the rationale of point b) above, and non-obstructive hypoventilation seems most likely to explain the study findings. A limitation of the study is that data on snoring were not collected.

The strengths of oximetry were the performance of the study at home, and at a time of clinical stability. Each case was standardised so that oximetry preceded the measurement of clinical and inflammatory outcomes. A number of the previously published studies alluded to in this thesis used oximetry alone to assess hypoxia during sleep (Coffey *et al.* 1991, Versteegh *et al.* 1990, Frangolias *et al.* 2001) whilst others (Milross *et al.* 2001) performed full PSG. Furthermore, oximetry alone is the recommended first-line investigation of hypoxaemia in CF in the ATS recommendations for cardiopulmonary sleep studies in children as discussed in section 4.5.3.

A further study limitation was in the small number of subjects studied.

d) Study numbers

Further limitations lie in the study numbers and the inability to perform multiple regression analyses to assess for potential confounding factors that might have an effect on outcome measures. For example, the relationship between hypoxia, lung function and *Pseudomonas aeruginosa* (PA) may be important. However, the fact that only 4/41 subjects had never grown PA, and these were all in the normoxic group made such a relationship impossible to ascertain. Larger numbers would have allowed identification of, and correction for, other factors/confounders.

4.5.5 Adaptation to hypoxia in CF

Our data concur with previous work (Stein *et al.* 2003), that use of $35 \times \text{FEV}_1$ as a predictor of MVV (Fulton *et al.* 1995; Sexauer *et al.* 2003) will underestimate the MVV in CF. Furthermore, earlier work (Godfrey and Mearns 1971) showed that V_E max on exercise was increasingly likely to exceed MVV in CF subjects as lung disease severity worsened.

Fourteen of our subject group had V_E max on exercise which exceeded their predicted MVV (Figure 4.13). The degree of underestimation has been reported to be proportional to the degree of airflow limitation (Stein *et al.* 2003). Our data do not directly support these findings, with no significant associations seen between V_E max and z scores for FEV_1 , FVC or mid-expiratory flows. However, the hypoxic group had a V_E max on exercise that was, on average higher than predicted MVV, as well as statistically significantly lower lung volumes and airflows (Table 4.5).

Hypoxic and airflow-limited CF patients have an innate capacity to increase V_E , which is rate-dependent in the absence of ability to further increase V_T . Mechanisms for this may include changes in chemoreceptor sensitivity, central effects on respiratory drive, as well as the effects of preservation of respiratory muscle strength (Aldrich *et al.* 1982), a phenomenon seen in the hypoxic subjects studied. A study of healthy adult volunteers showed that increases in V_E on exercise under hypoxic conditions exceeded the V_E dynamics of exercise in air (Fukoaka *et al.* 2003). Altered carotid body chemoreceptor sensitivity, as well as the effect of muscle chemoreceptors on the respiratory centre were considered most likely explanations for this phenomenon.

Genetic influences such as the known effect of the ACE genotype on hypoxic ventilatory responses (HVR) also are worthy of consideration. Previous work has shown that increases in V_E on exercise under hypoxic condition were significantly greater in those with an II genotype (Patel *et al.* 2003). The effects of ACE polymorphism on V_E in our study population will be considered in Chapter 5. There are more questions than answers, and there is scope for future studies to further our knowledge of HVR in the childhood CF population.

4.6 Conclusions

CF subjects with sleep hypoxia were noted to have, when compared to normoxic controls:

- Reduced exercise capacity
- Lower Body Mass Index
- Lower FEV₁
- Lower FVC
- Lower mid-expiratory flows
- Worse gas-trapping as measured by elevation of RV/TLC ratio
- Higher Chrispin-Norman chest X-ray scores
- Greater number of intravenous antibiotic courses
- Worse quality of life as assessed by the CFQ-UK
- Increased left and right ventricle thickness

Such findings serve to verify that an association between hypoxia and adverse clinical status exists.

The question of hypoxia being cause or effect in these associations remains unanswered. Clearly hypoxia may arise as an end result of severe CF lung disease, but the evidence reviewed in the introductory chapter suggest also that hypoxia could be an effector mechanism for perpetual neutrophilic inflammation, leading to a pro-inflammatory state and deleterious clinical sequelae. Chapter 6 describes a set of *in vitro* experiments designed to assess the effects of hypoxia on IL-8 mediated inflammation in CF bronchial epithelial cells, in an attempt to delineate whether hypoxia can be a direct cause of inflammation in the CF airway.

If it is true that hypoxia can be both cause and effect of lung inflammation in children with CF, then trials of mechanisms of restoration of normoxia (oxygen therapy and/or non-invasive ventilation) and their effects on amelioration of inflammation and clinical status appear worthy of consideration.

The role of exercise as an anti-inflammatory therapy in CF is also an area of interest. Long-term oxygen therapy during exercise in hypoxic CF subjects has potential cumulative benefits (Elphick and Mallory Jr. 2009).

Exercise capacity was chosen as a primary outcome of our study because of the purported relationship that exercise and hypoxia may exert on lung disease in CF (Figure 1.5). It is known that exercise improves airway clearance, and this may be beneficial in minimising bacterial load and prevention of worsening lung inflammation. In addition, an immunological mechanism for exercise exerting anti-inflammatory benefit has been reported (Petersen and Pedersen 2006) and the CF patient group would potentially benefit from this.

Hypoxic subjects achieved lower work rates and have diminished exercise capacity at both peak exercise capacity and AT. Quality of life scores are also lower in the hypoxic group (Table 4.12, Figures 4.14 and 4.15), and our data also show that quality of life is related to exercise capacity (Figure 4.16), with a $VO_2 - CFQ$ association ($r=0.61$, $p<0.001$, Spearman) seen. Therefore, exercise may help to bring about beneficial psychological as well as physiological effects. These data suggest exercise prescription may be a beneficial therapeutic intervention in hypoxic CF subjects.

CHAPTER 5:

Relationship between hypoxia and surrogate measures of inflammation in children with cystic fibrosis

5.1 Introduction

In Chapter 4, the suggested definition of hypoxia ($\text{SpO}_2 < 93\%$ for 10% sleep time) was used to dichotomise the study population and assess the association of hypoxia with clinical, radiological, physiological and psychological outcome measures in children with CF. Chapter 5 seeks to apply this definition of hypoxia to surrogate measures of inflammation in CF.

5.2 Aims

The aim of this chapter is to investigate the relationship between measures of hypoxia and inflammatory parameters, namely:

- ACE genotype
- Exhaled breath condensate inflammatory markers
- Skeletal muscle (Quadriceps and hand-grip) strength measures
- Bone densitometry data

The rationale for measuring each of the above as a surrogate marker of lung inflammation is outlined below:

5.2.1 ACE genotype

The ACE I/D polymorphism described previously codes for an insertion/deletion polymorphism within the ACE gene. Those with DD genotype have increased serum and tissue ACE activity (Danser *et al.* 1995) and are thought to be at increased risk of lung inflammation.

5.2.2 Exhaled breath condensate inflammatory markers

A full raft of pro- and anti-inflammatory cytokines were measured including IL-8 which for reasons above may be central to the process of ongoing airway inflammation in CF.

5.2.3 Skeletal muscle (Quadriceps and hand-grip) strength measures

AND

5.2.4 Bone densitometry data

These measures may be indirect markers of the consequences of inflammation in CF. Putative deleterious effects that may arise from being in a pro-inflammatory state in CF include muscle-wasting (Gan *et al.* 2003), and CF bone disease (Haworth *et al.* 2004). The mechanism by which these effects occur is thought to be due to up-regulation of catabolic cytokines including TNF- α and IL-6.

5.3 Methods

Measures of inflammation (inflammatory markers in exhaled breath), the predisposition to inflammation (ACE genotype) and the end results of inflammation (wasting of muscle and bone) were made. These are each described below.

5.3.1 ACE genotype

Briefly, ACE genotype for the I/D polymorphism was established using a three-primer PCR set up with MADGE gel electrophoresis used to visualise the DNA amplification products (Section 2.2.2.6.7). This work was carried out in conjunction with Dr Christina Hubbart, Rayne Institute, University College London.

5.3.2 Exhaled breath condensate (EBC)

Samples were collected as per the methods of section 2.2.2.5.1. Oral inhalation-exhalation for 20 minutes of tidal breathing was undertaken, using a commercially available system (ECoScreen, Jaeger, Hoechberg, Germany). A nose-clip was worn in accordance with the ATS/ERS recommendations (Horvath *et al.* 2005).

EBC was immediately frozen at -80°C and used for later cytokine analyses. A human 10-plex kit (BioSource International Inc., Camarillo, California) was used to assay EBC levels of IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IFN- γ , GM-CSF and TNF- α , using Luminex technology (section 2.2.2.5.2.2). The methodology is identical to that used for the analysis of serum by this method. Furthermore, an ultrasensitive ELISA assay for quantification of interleukin-8 (IL-8) levels in EBC (section 2.2.2.5.2.1).

5.3.3 Skeletal muscle strength measurement

Measures of proximal (quadriceps isometric voluntary contraction), and distal (handgrip strength dynamometry) musculature were undertaken:

5.3.3.1 Quadriceps isometric voluntary contraction

Maximal voluntary isometric contractions were measured using a custom-built isometric dynamometer (University College [Royal Free Campus] Medical School Physiology Department), as per the methodology of section 2.2.2.9.1.

Briefly, with the subject sat upright with knee flexed to 90⁰, a clamp placed above the ankle transmitted knee extensor force via an inextensible chain to a strain gauge bar. Subjects maintained maximal contraction for 2-3 seconds and then relaxed. Forces from the strain gauge bar were amplified and displayed on a chart recorder. Maximal forces achieved by voluntary quadriceps contraction in our study subjects were converted to standard deviation scores (SDS) using validated reference data (Backman *et al.* 1989).

5.3.3.2 Handgrip strength dynamometry

Distal skeletal muscle strength was assessed by peak force measurements made using a Jamar hydraulic hand dynamometer (Sammons Preston, Bolingbrook, IL, USA), in accordance with the methods of section 2.2.2.9.2.

Briefly, alternate dominant and non-dominant hand grip strength measures were made one minute apart (3 measures per side). The maximal force generated was recorded. Maximal handgrip strength values from our study subjects were converted to standard deviation scores (SDS) using this validated reference data (Mathiowetz *et al.* 1986).

5.3.4 Bone densitometry

Briefly, children underwent dual energy X-ray absorptiometry (DXA) scans (Lunar Prodigy, GE Medical Systems). Bone mineral density (BMD) measures were calculated from measures of bone mineral content and bone area and converted to z scores. However, it is known that BMD is underestimated in small subjects, and overestimated in tall subjects (Fewtrell *et al.* 2003). Therefore, a volumetric assessment – the bone mineral apparent density (BMAD) was undertaken to adjust for calculated bone volume rather than bone area, and again values were converted to BMAD z scores.

5.4 Results

First, anthropometric and demographic data for the study population has already been displayed (section 4.4.1), as well as a breakdown for groups when dichotomised for sleep and exercise hypoxia.

5.4.1 ACE genotype

ACE genotyping was successfully undertaken in forty of the 41 subjects.

5.4.1.1 Distribution of ACE genotype across study population

Subjects ranged from 7.98 to 16.22 years of age, with a mean (sd) age of 12.6 (2.4) years, and a median age of 12.9 years. Twenty-one subjects analysed were female, and 19 male. The reason that no ACE genotype was established in 1 subject was due to insufficient blood being obtained. The distribution of ACE genotype in our study population was as follows (Table 5.1). The observed gene frequency in our study population - D allele frequency 56%, I allele 44% is similar to that reported in the literature for a UK-based sample population - 49% D allele and 51% I allele (Nagi *et al.* 1995).

Table 5.1

Gene frequencies for ACE polymorphism

<i>ACE genotype</i>	<i>Study population (n=40)</i>	
	OBSERVED	EXPECTED
ID	19	19.7
DD	8	7.6
II	13	12.7

$\chi^2=0.048$, $p>0.50$

The ACE polymorphism obeys Hardy-Weinberg equilibrium in our study population. The full Hardy-Weinberg equilibrium calculation is provided in Appendix 5.

5.4.1.2 Hypoxia and ACE genotype

Similar distribution for ACE genotype was seen for both hypoxic and normoxic groups when existing definitions of sleep hypoxia were applied (Table 5.2), with Hardy-Weinberg equilibrium preserved.

Table 5.2ACE genotype and hypoxia defined as SpO₂ <93% for 10% sleep time

	<i>Study population (n=40)</i>		
	ID	II	DD
Hypoxia (n=9)	4	1	4
Normoxia (n=31)	15	12	4

5.4.2 Exhaled breath condensate (EBC) cytokine analyses**5.4.2.1 EBC cytokine levels by Luminex analyses**

The human 10-plex cytokine assay utilised (BioSource International Inc., Camarillo, California) uses Luminex technology to assay levels of 10 different cytokines – IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, Interferon gamma (IFN- γ), granulocyte and macrophage colony stimulating factor (GM-CSF) and TNF- α . Using this kit to assay raw EBC samples (neither concentrated or diluted), no cytokines were detected. For interleukin-8, the range in which IL-8 can be detected is from 16.5 to 4000 pg.mL⁻¹.

5.4.2.2 EBC interleukin-8 [IL-8] levels

An ultrasensitive IL-8 assay was also used to detect lower concentrations of IL-8 (Biosource ultrasensitive IL-8 [KHC0084] ELISA assay). This assay will detect IL-8 levels in the range of 0.39pg.mL⁻¹ to 25pg.mL⁻¹.

In our hands the KHC0084 ELISA kit detected only 1 positive EBC sample at a concentration of 0.4pg.mL⁻¹. The kit did however correctly pick up a sample spiked with a known concentration of IL-8 and variability between this sample and standard of similar concentration was <10%. The subject identified was markedly hypoxic with 78.9% of the night spent with SpO₂ <93%, and a lowest exercise saturation of 85%.

5.4.3 Skeletal muscle strength

Measures of proximal muscle strength (quadriceps maximal isometric voluntary contraction) and distal muscle strength (handgrip) are presented.

5.4.3.1 Quadriceps maximal isometric voluntary contraction (MIVC)

Quadriceps force was successfully measured in all forty-one subjects (Table 5.3).

Table 5.3

Quadriceps MIVC for the study population as a whole

<i>Variable</i>	<i>Study population (n=41)</i>	
	Range	Median (IQR)
Dominant Leg		
Quadriceps MIVC (N)	82 to 498	172 (136, 239)
Quadriceps MIVC (N.m ⁻²)	63 to 295	144 (112, 173)
Quadriceps MVC SDS	-3.6 to 4.4	-0.7 (-1.5, 0.1)
Non-dominant Leg		
Quadriceps MIVC (N)	70 to 447	154 (126, 216)
Quadriceps MIVC (N.m ⁻²)	61 to 274	128 (103, 165)
Quadriceps MVC SDS	-3.7 to 4.1	-1.3 (-2, -0.5)

As a group, our study population had median quadriceps strength values that are approximately 0.7 to 1.3 standard deviations below that of the reference population. However, there were children within our study population with stronger than muscles than healthy subjects.

5.4.3.1.1 Hypoxia and quadriceps MIVC

No differences in quadriceps strength were observed when either existing or *de novo* definitions of nocturnal hypoxia were used to dichotomise the study population (Table 5.4).

Table 5.4

Quadriceps MIVC and hypoxia defined as SpO₂<93% for >10% sleep time

<i>Variable</i>	<i>Study population (n=41)</i>		
	Hypoxic (n=9)	Normoxic (n=32)	p-value
Dominant Leg			
Quadriceps MIVC (N)	185 (129, 280)	166 (138, 232)	0.59*
Quadriceps MIVC (N.m ⁻²)	146 (109, 170)	143 (110, 178)	0.89*
Quadriceps MIVC SDS	-0.6 (-2, -0.1)	-0.8 (-1.6, 0.5)	0.99*
Non-dominant Leg			
Quadriceps MIVC (N)	175 (119, 232)	151 (126, 208)	0.61*
Quadriceps MIVC (N.m ⁻²)	122 (98, 162)	129 (103, 166)	0.63*
Quadriceps MIVC SDS	-1.3 (-1.9, -0.9)	-1.3 (-2, 0)	0.63*

Figures given are median (IQR) * Mann-Whitney U-test

5.4.3.2 Handgrip strength dynamometry

Handgrip strength of the dominant hand was successfully measured in all forty-one subjects (Table 5.5), but that of the non-dominant hand was only measured in 40 subjects as one child had a fracture of the non-dominant wrist.

Table 5.5

Handgrip strength for the study population as a whole

<i>Variable</i>	<i>Study population (n=41)</i>	
	Range	Median (IQR)
Dominant Hand		
Handgrip force (N)	8 to 41	17 (14, 23)
Handgrip force SDS	-5.1 to 0.7	-1.2 (-1.9, 0.5)
Non-dominant Hand		
Handgrip force (N)	7 to 37	17 (12, 21)
Handgrip force SDS	-4.9 to 0.4	-1.0 (-1.7, -0.5)

5.4.3.2.1 Hypoxia and handgrip strength

No differences in handgrip strength were observed when the *de novo* definition of nocturnal hypoxia was used to dichotomise the study population (Table 5.6).

Table 5.6

Handgrip strength and hypoxia defined as SpO₂<93% for >10% sleep time

<i>Variable</i>	<i>Study population (n=40)</i>		
	Hypoxic (n=9)	Normoxic (n=31)	p-value
Dominant Hand			
Handgrip force (N)	20 (17, 27)	16 (14, 22)	0.15*
Handgrip force SDS	-1.1 (-1.3, -0.4)	-1.2 (-2.2, -0.5)	0.17*
Non-dominant Hand			
Handgrip force (N)	18 (16, 23)	14 (12, 19)	0.18*
Handgrip force SDS	-0.9 (-1.8, -0.6)	-0.9 (-1.2, -0.3)	0.26*

Figures given are median (IQR) * Mann-Whitney U-test

5.4.4 Bone densitometry

Thirty subjects out of the total study population underwent bone densitometry within a year of the date of their exercise test, either for clinical reasons or as part of a separate research study.

5.4.4.1 Bone densitometry data for study population

The baseline characteristics of the 30 subjects who underwent bone densitometry are displayed below (Table 5.7). The baseline characteristics are similar to that for the whole study population (Table 4.1). Bone densitometry data, in the form of raw (BMD) and adjusted (BMAD) bone mineral density z scores are displayed also.

Table 5.7

Demographic data for the 30 subjects that underwent bone densitometry

<i>Variable</i>	<i>Study population (n=30)</i>	
	Range	Median (IQR)
Age	8 to 15.7	12.4 (10.7, 13.9)
Sex	15M:15F	-
Height SDS	-2.1 to +1.8	-0.4 (-1.1, +0.2)
Weight SDS	-2 to 1.7	-0.6 (-1.2, +0.4)
BMI SDS	-1.9 to +2	-0.3 (-0.8, +0.3)
CFTR genotype		
• ΔF508/ΔF508	18	-
• ΔF508/Other	10	-
• Other/Other	2	-
Lung function		
FEV ₁ SDS	-5 to 1.2	-1.7 (-3, -0.9)
FVC SDS	-4 to 1.3	-0.7 (-2, 0)
<i>Pseudomonas aeruginosa</i> infection (Ever versus never)	Ever (n=26) versus Never (n=4)	- -
<i>Staphylococcus aureus</i> infection (Ever versus never)	Ever (n=24) versus Never (n=6)	- -
BMD z score	-4.2 to +0.7	-0.8 (-1.4, -0.3)
BMAD z score	-2.8 to +2.2	-0.4 (-1.2, +0.3)

5.4.4.1.1 Bone densitometry data and hypoxia

Hypoxic subjects ($\text{SpO}_2 < 93\%$ for $>10\%$ sleep time) were noted to have statistically lower size corrected bone density (BMAD), when compared with normoxic controls (Table 5.8, Figure 5.1).

Table 5.8

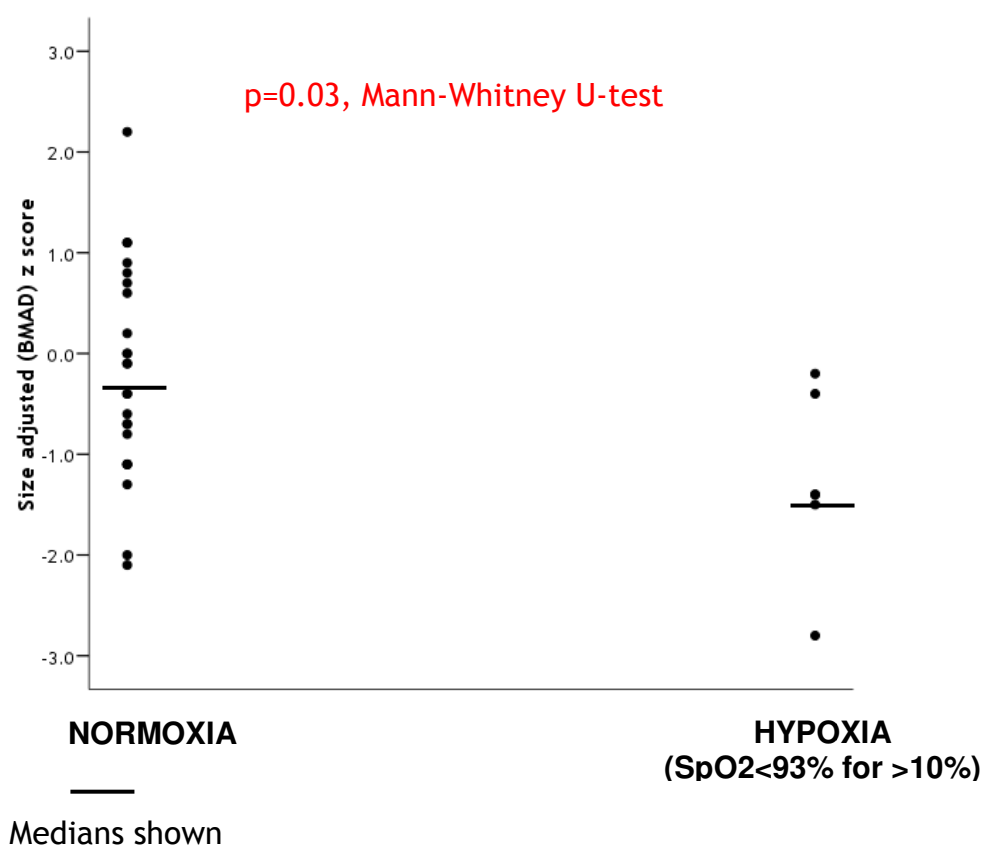
Bone mineral density and hypoxia defined as $\text{SpO}_2 < 93\%$ for $>10\%$ sleep time

Variable	Study population (n=33)		
	Hypoxic (n=6) ($\text{SpO}_2 < 93\%$ for $>10\%$ sleep time)	Normoxic (n=24)	p-value
BMD z score	-1.1 (-2.3, -0.7)	-0.6 (-1.4, -0.1))	0.16*
BMAD z score	-1.4 (-1.8, -0.4)	-0.3 (-1, +0.7)	0.03*

Figures given are median (IQR) * Mann-Whitney U-test

Figure 5.1

Distribution of BMAD z scores in normoxic CF patients and those with hypoxia defined as $\text{SpO}_2 < 93\%$ for $>10\%$ sleep time



5.5 Discussion

The results presented suggest that hypoxia in children with CF is associated with reduced bone density, a potential end result of increased inflammation. No differences in skeletal muscle strength were noted between hypoxic and normoxic groups; although when compared with historical controls, muscle strength was lower in children with CF.

The results would also question the potential usefulness of exhaled breath condensate as a clinical tool.

5.5.1 ACE genotype

The numbers in our study were too small to assess the effect of genotypic differences in ACE genotype and effects on clinical parameters. It is known from published data that subjects possessing II genotype have improved hypoxic ventilatory responses (Patel *et al.* 2003), with larger potential increases in minute ventilation (V_E). A phenomenon of higher V_E to MVV ratio was seen in the subjects with sleep hypoxia (Figure 4.13), and indeed despite differences in lung volumes as assessed by spirometry, no statistically significant differences in minute ventilation (V_E) were seen between hypoxic and normoxic groups (Figure 4.11). It is possible that modification of hypoxic ventilatory responses may be a potential explanation for this phenomenon. From our small numbers however, it is impossible to assess whether this associated with any effects of the effects of ACE genotype.

Genetic modifier studies in CF are hampered by small numbers and inadequate power to demonstrate outcome differences, as well as differences in phenotypic classification of disease severity (Accurso and Sontag 2003, Davies 2004). However, inclusion of a modifier gene as a study outcome can be justified, for if large differences between groups exist then these might be detected despite relatively small study numbers. An example of this lies in the association reported between reduced lung function and mannose binding lectin (MBL) polymorphism in 11 subjects with MBL polymorphism, matched with 11 controls from a study total of 164 subjects (Gabolde *et al.* 1999).

A note of caution is that a positive association between a polymorphism and clinical phenotype does not prove that the gene studied is the cause for phenotypic differences. For example other genes may travel with the candidate gene and account for such differences (Accurso and Sontag 2003).

5.5.2 Exhaled breath condensate

It was not possible to detect markers of inflammation in EBC in our study population, apart from in a single child who was severely hypoxic. Reasons for this could be that there is no inflammation present, or that the levels of inflammatory markers in EBC are below the limits of detection for the assays used. It is implausible that inflammation is absent in EBC, for CF lung disease is characterised by persistent neutrophilic inflammation. Large amounts of circulating interleukin-8 (IL-8) are present in the inflamed CF lung, leading to neutrophil chemoattraction, release of neutrophil enzymes such as elastase, and resultant parenchymal lung damage. Bronchoalveolar lavage (BAL) fluid taken from CF subjects contains elevated levels of IL-8, IL-6, IL-1 β and TNF- α (Bonfield et al. 1995). BAL is an invasive procedure, and a bedside test such as EBC, would therefore be beneficial in evaluating lung inflammation.

The ATS/ERS task force reported that IL-8 and IL-6 have been detected in EBC (Horvath *et al.* 2005), although a note of caution was added that unconcentrated EBC samples may have cytokine levels below the lower limits of detection for conventional ELISA kits. IL-8 was detectable in only 33% of EBC samples (Cunningham *et al.* 2000) in a group of children with CF. However, more recent work (Bodini *et al.* 2005) has suggested that IL-8 can be detected in 90% EBC samples in CF. A wide discrepancy in EBC IL-8 levels detected in CF was noted between these studies. Cunningham *et al.* detected IL-8 in seven of 21 CF patients studied with median (range) concentration of 49 (8 to 90) pg.mL⁻¹. Bodini's group studied thirty CF patients and 10 controls. IL-8 was undetectable in only 4 patients due to insufficient EBC volume. The mean (range) IL-8 level detected in CF patients with *Pseudomonas aeruginosa* infection was 0.73 (0.58-0.87) pg.mL⁻¹ compared to 0.34 (0.29-0.43) pg.mL⁻¹ in healthy controls.

There are methodological differences between the two. Firstly, the mechanism of collection for Cunningham and colleagues was to collect EBC using Teflon tubing immersed in ice, whereas Bodini's group used a condensing device made of 2 glass chambers. Secondly, the assays were different with Cunningham using an in-house IL-8 ELISA that had a lower limit of detection of 0.5pg.mL⁻¹, and Bodini using the same ultrasensitive IL-8 ELISA kit as ourselves (Biosource ultrasensitive IL-8 [KHC0084] ELISA assay), but presumably with an extra standard dilution to achieve a standard curve running from 0.18-25 pg.mL⁻¹.

In our hands, the Biosource ultrasensitive IL-8 [KHC0084] ELISA assay yielded a solitary positive EBC sample at a concentration of 0.4pg.mL^{-1} . Reasons for failure to detect IL-8 in our EBC samples may include the following:

- a) Collection and storage problems
- b) Inhibition of cytokine detection due to salivary proteases
- c) True IL-8 concentration lay below detection limits of the assay

a) Collection and storage

The use of a commercially available device for collection of EBC (ECoScreen, Jaeger, Hoechberg, Germany) clearly differs from the two published papers on EBC in CF noted above. The method is, however, a validated method for EBC collection. In addition, the rapid freezing of samples (section 2.2.2.5.1) is in accordance with the handling recommendations for EBC of the ATS and ERS (Horvath *et al.* 2005), and is unlikely to explain the negative findings in our study population.

b) Cytokine inhibition due to salivary proteases

This appears unlikely, as the kit correctly detected a sample spiked with a known concentration of IL-8. The variability between this sample and standard of similar concentration was less than 10%.

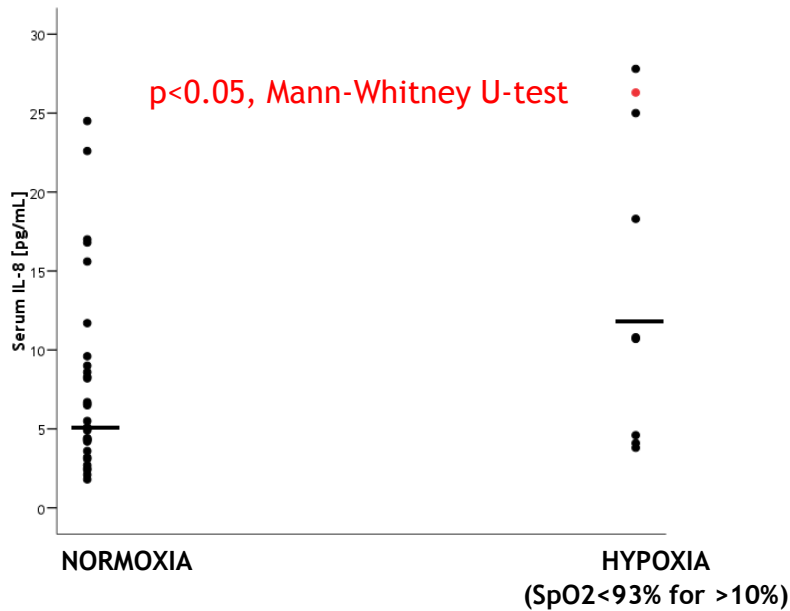
c) True IL-8 concentration lying below the detection limits of the assay

To summarise, Cunningham's group detected IL-8 in EBC in 33% of the CF patients that they studied with median concentrations of 49pg.mL^{-1} , whereas Bodini detected IL-8 in 90% CF patients and controls but with concentrations that were all below 1pg.mL^{-1} . The EBC IL-8 concentrations detected by Bodini were 50-fold lower than those reported by Cunningham. The assay appears to function correctly, for a spiked sample was correctly identified. The only positive patient sample occurred in one of our most hypoxic patients, and one might surmise that he was in a pro-inflammatory state as a result of hypoxia, with up-regulated IL-8, the detection of which, at 0.4pg.mL^{-1} was only just possible with the ultrasensitive IL-8 assay. The remainder of the study population may have varied levels of IL-8 expression, albeit below the lower limit (0.39pg.mL^{-1}) of detection of the assay.

It is noted that the sole subject with elevated IL-8 in EBC, also had an elevated serum IL-8 (●) – Figure 5.2.

Figure 5.2

Serum IL-8 levels and hypoxia defined as $SpO_2 < 93\%$ for $>10\%$ sleep time



Medians shown

- Subject with elevated EBC IL-8,
- Remaining subjects

IL-8 concentrations lying below the detection limits of the assay seems a plausible explanation for the lack of detection of IL-8 in our EBC samples. As a clinical tool, therefore, measuring IL-8 in EBC appears fraught with difficulty and of limited usefulness. Techniques such as lyophilisation to concentrate the EBC sample may have improved the yield of cytokine detection (Wells *et al.* 2005).

5.5.3 Skeletal muscle

Median quadriceps strength and also handgrip strength are each approximately 1 standard deviation below the strength values of the reference population. The CF lung and its' pro-inflammatory state and consequent cytokine up-regulation may provide an explanation for the observed reduction in skeletal muscle strength in CF. Alternative explanations could include the use of corticosteroids, although the pattern of wasting associated with steroid use would tend to be a disproportionate wasting of proximal muscle (quadriceps) compared with distal (handgrip), and this was not observed in our study population.

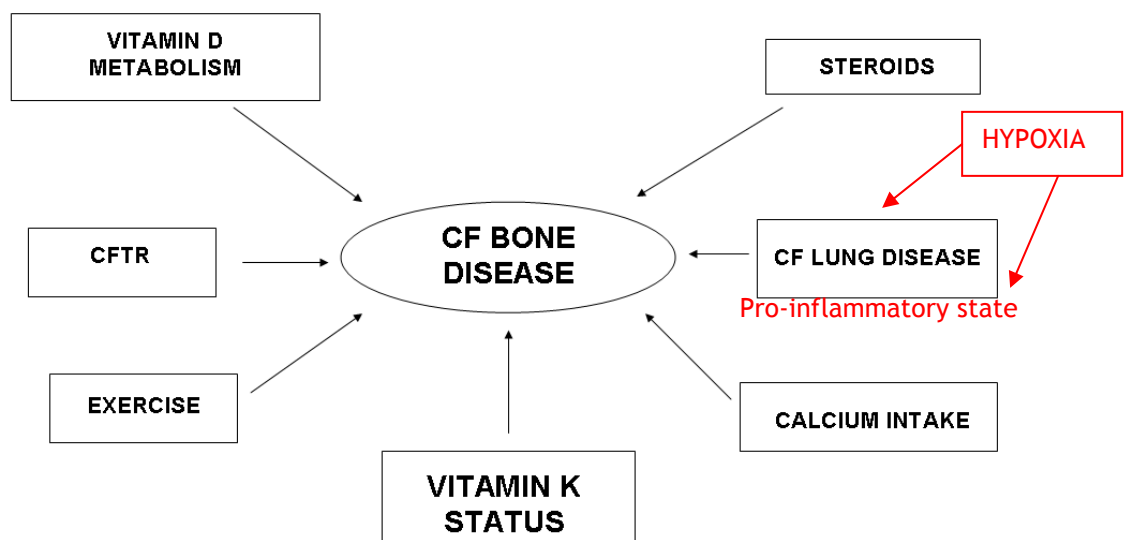
However, it should also be noted that within our study population, there were individuals with stronger quadriceps than reference population (one child had a quadriceps strength that was 4 standard deviations above the mean). A potential explanation for this may be that exercise promotion in CF has led these children to undertake more exercise than their peers, with published work suggesting that children with mild CF undertake greater exercise than healthy age-matched controls (Selvadurai *et al.* 2004).

5.5.4 Bone mineral density

Subjects with nocturnal hypoxia were noted to have statistically lower size corrected bone density (BMAD), than normoxic controls (Figure 5.1). CF bone disease is multifactorial (Figure 5.3), and the various potential causes of CF bone disease are considered.

Figure 5.3

Aetiological factors for CF bone disease (adapted from Urquhart *et al.* 2007)



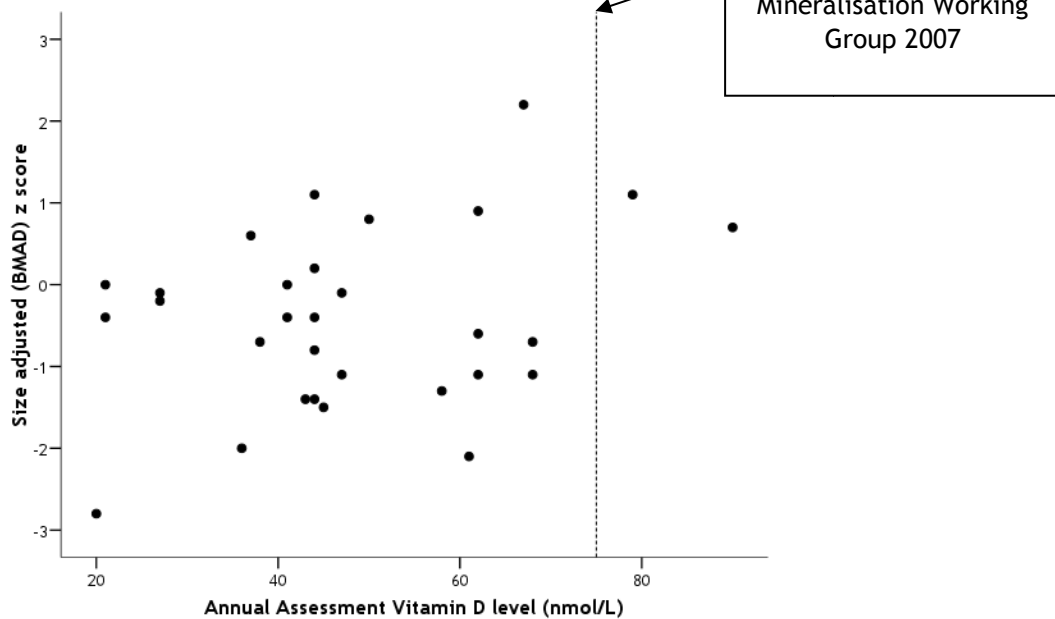
Vitamin D status:

The reduction in BMAD observed in hypoxic subjects appears to be independent of vitamin D status. No differences in median (IQR) vitamin D levels recorded at annual assessment were observed between normoxic [47 (37, 62) nmol.L⁻¹] and hypoxic [44 (30, 52)] subjects (p=0.35, Mann-Whitney U test).

No correlation was seen between BMAD and vitamin D levels (Figure 5.4). Also of note is that only 2/40 patients in whom Vitamin D levels were measured had levels above those recommended by the UK CF Trust guidelines (UK CF Trust 2007).

Figure 5.4

Vitamin D levels and BMAD z scores



CFTR:

No differences in BMD ($p=0.66$) or BMAD ($p=0.23$) between CFTR genotypes (considered as $\Delta F508/\Delta F508$, $\Delta F508/Other$ and $Other/Other$) were observed (Kruskal-Wallis test).

Vitamin K status, Calcium intake, Steroid therapy:

No data were collected.

Exercise:

In our study population, BMD was statistically significantly lower (Table 5.9) in subjects with reduced exercise capacity denoted by $VO_{2peak} < 32 \text{ mls.kg}^{-1}.\text{min}^{-1}$ as exercise capacity below this level has been shown to relate to mortality in children with CF (Pianosi *et al.* 2005), echoing a previously reported association between VO_{2max} and BMD (Frangolias *et al.* 2003). Statistical significance was however lost when size correction (BMAD) was carried out. It is known that subjects with nocturnal hypoxia had lower exercise capacity than normoxic controls (see section 4.4.6).

Table 5.9

Exercise capacity and BMD

	VO₂peak <32 mls.kg⁻¹.min⁻¹	VO₂peak ≥32 mls.kg⁻¹.min⁻¹	p-value
BMD Z scores	-1.2 (-1.5, -0.8)	-0.5 (-1.3, -0.1)	<0.05*
BMAD z scores	-0.8 (-1.5, -0.3)	-0.1 (-1.1, 0.1)	0.14*

Figures given are median (IQR) * Mann-Whitney U-test

CF lung disease:

It therefore appears plausible that the pro-inflammatory lung status associated with hypoxia, and the consequent up-regulation of cytokines that promote wasting of bone may provide an explanation for the finding of reduced bone density in this hypoxic CF patient group. However, no differences in BMD or BMAD were observed when the groups was dichotomised on the basis of FEV₁, FVC or MEF₅₀ <-2 z scores; and no correlation between IL-8 levels and BMAD z scores ($r^2=0.02$, $p=0.46$, Spearman) were observed.

Further work to evaluate the cause of reduced bone density in hypoxic CF subjects is required, and may include collection of longitudinal bone density data as well as more detailed profiling of pro-inflammatory cytokines.

5.6 Conclusions

The results described in Chapter 5 suggest a possible association between hypoxia and bone mineral density. Deleterious effects are reported to arise from being in a pro-inflammatory state in CF including muscle-wasting (Gan *et al.* 2003), and progression of CF bone disease (Haworth *et al.* 2004).

Additionally, the data presented on exhaled breath condensate would suggest that this is not a useful bedside or clinic technique for assessing fluctuations in the levels of inflammatory markers in children with CF.

Similar to the associations described in chapter 4, it is unclear whether the observed relationship between hypoxia and bone density has occurred because hypoxia is simply a mere endpoint of severe CF lung disease and thus a state of increased inflammation, or whether hypoxia may be a contributor to a pro-inflammatory state and progression of bone disease. Chapter 6 describes a set of *in vitro* experiments designed to assess the effects of hypoxia on IL-8 mediated inflammation in CF bronchial epithelial cells, in an attempt to delineate whether hypoxia can be a direct cause of inflammation in the CF airway.

Following on from the conclusions of chapter 4, trials of mechanisms of restoration of normoxia (oxygen therapy and/or non-invasive ventilation) and their effects on amelioration of inflammation and clinical status appear worthy of consideration. Furthermore the effects of exercise as a therapy also may be beneficial due to the promotion of new bone formation, as well as the preservation of skeletal muscle strength.

CHAPTER 6:

Effects of hypoxia on inflammation in CF airway epithelial cells

6.1 Introduction

The results of Chapters 4 and 5 suggest an association between hypoxia and adverse clinical status in hypoxic CF subjects. The effects of hypoxia on adverse clinical status in CF may be causal, but it is also possible that hypoxia is consequent to disease severity.

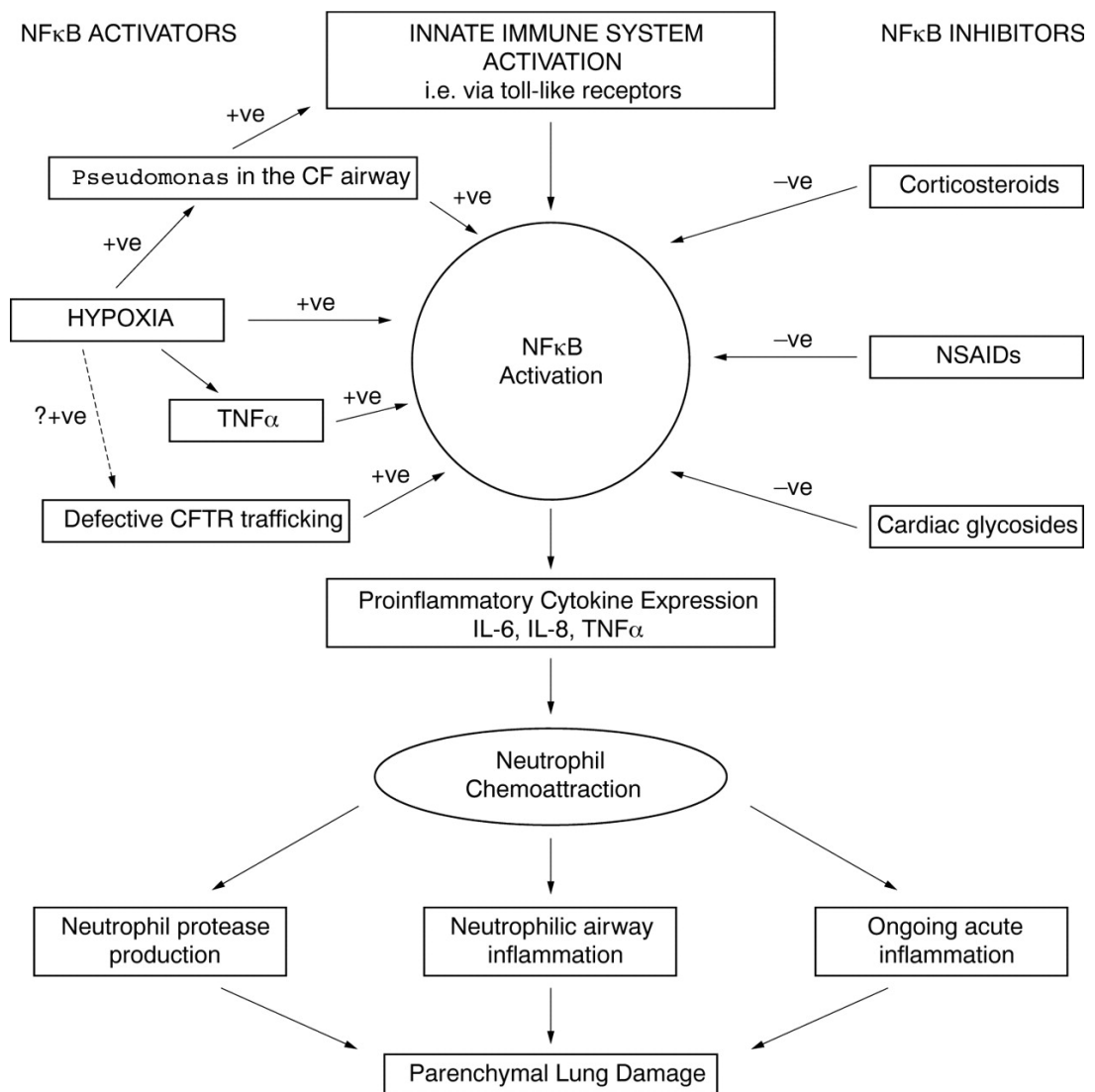
There is *in vitro* evidence that hypoxia may exert a direct pro-inflammatory effect (Koong *et al.* 1994, Leeper-Woodford and Detmer 1999, Taylor and Cummins 2009) in non-CF disease states, and in this chapter a set of experiments were undertaken to directly investigate the effects of tissue culture in an hypoxic environment on inflammation, as assessed by interleukin-8 (IL-8) secretion in CF airway epithelial cells.

6.1.1 Inflammation and the CF lung

Briefly (as this area is covered in detail in Chapter 1), perpetual neutrophilic inflammation is found in the CF airway which may contribute to parenchymal lung damage. Neutrophils are attracted to the airway by IL-8, a chemokine released as part of the innate immune response to infection. IL-8 levels are regulated by transcription factors of the NF κ B family.

The NF κ B signalling pathway may be key to inflammation in the CF airway, and is activated by a number of factors including bacterial lipopolysaccharide from organisms such as *Pseudomonas aeruginosa* (Hajjar *et al.* 2002), cytokines, endoplasmic reticulum (ER) stress (Hershenson 2004) and possibly directly by CFTR itself (Schroeder *et al.* 2002). The potential effects of hypoxia as an activator of this cascade are of interest also. The activation of NF κ B results in increases in pro-inflammatory cytokines including IL-6 and IL-8, with resultant neutrophil chemo-attraction, as well as the initiation and propagation of chronic airway inflammation (Figure 6.1).

Figure 6.1
Simplified mechanisms for NFκB signalling and the CF airway
(from Urquhart *et al.* 2005)



6.1.2 Hypoxia and CFTR

Evidence exists that CF patients with the $\Delta F508$ mutation (85% UK CF patients) have ongoing NF κ B-driven inflammation, due to cell stress caused by overload of $\Delta F508$ CFTR in the endoplasmic reticulum (ER) (Bauerle and Baltimore 1995), implying that neutrophilic inflammation in the CF airway may be associated with CFTR dysfunction as well as infection - 38/41 (93%) of our study population carry the $\Delta F508$ mutation. *In vitro* work has shown increased NF κ B activation in $\Delta F508$ CF cell lines (Weber *et al.* 2001); whilst *in vivo* studies report increased IL-8 levels in BAL fluid of young children (Balough *et al.* 1995, Noah *et al.* 1997) and infants (Khan *et al.* 1995) with CF in the absence of infection. Thus, if CFTR protein function could be improved (by improving trafficking to the cell surface), inflammation might be reduced.

In vitro work suggested that cell surface CFTR protein expression in a canine kidney cell line is impaired by hypoxia (Bebok *et al.* 2001), and hypoxia is also reported to reduce CFTR mRNA, protein expression and function in human cell lines (Guimbellot *et al.* 2008). This is backed by animal work that has shown reduced CFTR mRNA expression in the airways, gut and liver of mice subjected to hypoxia when compared with normoxic control mice (Guimbellot *et al.* 2008).

Furthermore, *in vivo* human data exist to suggest that hypoxia may inhibit CFTR function. In mountaineers with high altitude pulmonary oedema (HAPE), CFTR mRNA levels fell by 60% at altitude, (Mairbaurl *et al.* 2003). It is possible that a trafficking deficit could account for such CFTR dysfunction due to hypoxia. Additionally, levels of CFTR mRNA expression were observed to be lower in the lung epithelia of hypoxaemic lung transplant recipients at the time of transplantation, when compared to of non-hypoxaemic donors (Guimbellot *et al.* 2008).

Thus *in vitro*, rodent and *in vitro* work support a role for hypoxia having deleterious action on CFTR function, and a novel therapeutic role for oxygen in CF is suggested: CF disease severity correlates with the amount of functional CFTR protein expression on airway epithelial cells, and research into molecular chaperoning has studied the ability of a number of agents in facilitating CFTR trafficking – guiding $\Delta F508$ CFTR protein from the endoplasmic reticulum to the cell surface. As yet, the trafficking effects of oxygen in a $\Delta F508$ CF cell line remain undocumented.

6.1.3 Hypoxia and NFκB

As previously discussed above, *in vitro* evidence suggests that hypoxia increases NFκB activation (Koong *et al.* 1994, Leeper-Woodford and Detmer 1999) leading to proinflammatory cytokine expression, neutrophil recruitment and consequent CF airway inflammation. The role of supplemental oxygen in modulating CF inflammation would be both of interest and also of great potential therapeutic benefit.

Although addressing the interaction of hypoxia and NFκB, the above studies focused on acute hypoxia, over periods of 2 (Koong *et al.* 1994) to 48 hours (Leeper-Woodford and Detmer 1999). The work discussed in 6.1.1 regarding the potential inhibitory effects of hypoxia on CFTR trafficking, and the potential for mutant CFTR to activate NFκB, provide another potential mechanism by which hypoxia may propagate inflammation in the CF lung.

As a counterbalance to the above evidence, there is also *in vitro* work reporting that hyperoxia too is an activator of NFκB (Horowitz 1999), along with *in vivo* work noting an increase in NFκB-mediated inflammatory markers in exhaled breath condensate (EBC) of chronic obstructive pulmonary disease (COPD) patients treated with oxygen (Carpagno *et al.* 2004). Of note however, are the difficulties and inconsistencies reported when measuring inflammatory cytokines in EBC (section 5.5.2).

6.2 Aims

This aims of this chapter were to assess the effects of variable degrees of hypoxia upon NFκB-mediated inflammation (as assessed by cellular IL-8 production) in both CF and non-CF cell lines. Furthermore, the effects of hypoxia on inflammation in CF and non-CF cell lines was compared and contrasted to the effects of a known stimulus of the NFκB cascade, namely bacterial lipopolysaccharide (LPS) from *Pseudomonas aeruginosa*, and in addition variable lengths of incubation under the given environmental conditions were studied up to a maximum of 96 hours. The study hypothesis that hypoxia exerts an *in vitro* effect on NFκB-mediated inflammation in the CF airway was thus tested.

6.3 Methods

The methodology is discussed in detail in section 2.3. A précis is given below.

6.3.1 Tissue Culture

Tissue culture was undertaken using immortalised CF (CFBE41o⁻) and non-CF (1HAEo⁻) cell lines (courtesy of Dieter Gruenert, University of Vermont, USA). The cells were grown in a standard tissue culture incubator (Galaxy-R, Wolf Laboratories Limited, Pocklington, UK) maintained at constant temperature (37⁰C), CO₂ (5%) and humidity levels. Hypoxia was controlled by a Pro-Ox controller (Biospherix Limited, Redfield, NY13437, USA), regulating oxygen concentration within a two-shelf tissue culture chamber (Biospherix Limited), housed in the incubator as illustrated previously (Figure 2.15b). Carbon dioxide within the cell culture chamber was maintained at 5% using a Pro-CO₂ controller (Biospherix Limited, Redfield, NY13437, USA).

In order to maintain a control environment, cells were incubated simultaneously in normoxic conditions (incubator) alongside hypoxic conditions (tissue culture chamber) – see Figure 2.16.

Three sets of experiments were carried out. Firstly, CF and non-CF cells were simultaneously incubated in air and variable degrees of hypoxia (0.1%, 1%, 5%, 10%) over a 48 hour period. Counting of cells and sampling of cell supernatant was carried out at time zero, and then at 8, 24 and 48 hours thereafter, according to the methodology of sections 2.3.3 and 2.3.4.

In order to compare hypoxia to LPS as a stimulus of inflammation, and to assess the potential augmentation of inflammation by LPS in the presence of hypoxia, the above experiments were repeated in the presence of high (50µg.mL⁻¹ media) or low (10µg.mL⁻¹) levels of LPS derived from *Pseudomonas aeruginosa* (L9143 Sigma-Aldrich, St Louis, MO, USA), and in the absence of LPS, for both cell lines and with simultaneous incubation in both air and at variable (0.1%,1% and 10%) levels of hypoxia.

To assess the impact of time, a further set of experiments were carried out comparing extreme hypoxia (0.1%) with normoxia over a 4-day period. In this experiment, cells were incubated with either high-dose LPS (50 µg.mL⁻¹ media) added or no LPS at all, enabling comparison of cell lines with and without LPS under extreme hypoxic and normoxic conditions at each timepoint.

At each timepoint in each of the experiments, cell supernatant was sampled from the T25 flask, and stored in cryovials (Nalge-Nunc International, Roskilde, Denmark) at -80°C for later cytokine analyses.

6.3.2 Interleukin-8 (IL-8) enzyme-linked immunosorbent assay (ELISA)

Given the hypothesis that hypoxia will upregulate NF κ B, and that NF κ B activation in turn leads to IL-8 production, then direct measurement of IL-8 appears an appropriate surrogate measure by which to test this hypothesis. A human IL-8 ELISA kit (ELH-IL8-001, RayBiotech Inc., Norcross, GA, USA) was used to measure IL-8 concentration in tissue culture cell supernatant for both human airway epithelial cells and CF bronchial epithelial cells. The kit detects IL-8 levels in the range of 2.74 to 2000 pg.mL^{-1} .

Prior to ELISA, cell supernatant was centrifuged (Rotina 46-R, Wolf Laboratories Limited, Pocklington, UK) to allow debris to sink to the bottom of the tube, then diluted to 1 in 100 using assay diluent. The protocol was based on manufacturer's instructions. A 96-well plate pre-coated with capture antibody was used with standards generated by 1:3 serial dilutions from 2000 pg.mL^{-1} down to 2.74 pg.mL^{-1} . The assay is summarised in Figure 2.17.

Optical densities of each well were read using an ELISA plate reader (Revelation 4.02, Dynex Technology, Chantilly, VA, USA) at 450nm, and cytokine concentrations in the unknown samples were read from the standard curve.

6.4 Results

Results for each set of experiments are summarised below. The three groups of experiments undertaken were as follows:

1. Effects of variable degrees of hypoxia on inflammation in CF and non-CF cell lines (Section 6.4.1).
2. Effects of variable degrees of hypoxia with or without exposure to lipopolysaccharide on inflammation in CF and non-CF cell lines (Section 6.4.2).
3. Effects of prolonged extreme hypoxia and exposure to lipopolysaccharide on inflammation in CF and non-CF cell lines (Section 6.4.3).

6.4.1 Effects of hypoxia on inflammation in CF and non-CF cell lines

The effects of varying degrees of hypoxia (10%, 5%, 1% and 0.1%) on cell growth and IL-8 production were considered.

6.4.1.1 Cell Counts at experimental time-points

These are displayed below in Table 6.1.

It should be noted that only live cells were counted, using the Trypan blue dye exclusion method described in section 2.3.4.

Growth patterns appeared broadly similar for each cell line at varying degrees of hypoxia. This is demonstrated in Figure 6.2 (a and b). There is a suggestion of a reduction in CFBE cell growth at extreme (0.1%) hypoxia (Figure 6.2 b).

However, when repeatability measures were applied to cell growth across the entire set of experiments, no significant differences in growth patterns within cell-lines and between cell-lines were seen (Section 6.4.4).

Table 6.1
Cell counts ($\times 10^6 \text{ mL}^{-1}$ of media) at times of sampling in variable hypoxia experiments using CF and non-CF cell lines

	Normoxic Control		Normoxia		Hypoxia 10%		Hypoxia 5%		Hypoxia 1%		Hypoxia 0.1%	
	HAE	CFBE	HAE	CFBE	HAE	CFBE	HAE	CFBE	HAE	CFBE	HAE	CFBE
ZERO	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
8 hours	0.1	0.12	0.12	0.12	0.16	0.13	0.08	0.12	0.12	0.14	0.08	0.06
24 hours	0.25	0.26	0.25	0.24	0.19	0.27	0.18	0.2	0.22	0.18	0.17	0.13
48 hours	0.26	0.28	0.28	0.23	0.25	0.38	0.25	0.31	0.23	0.36	0.26	0.18

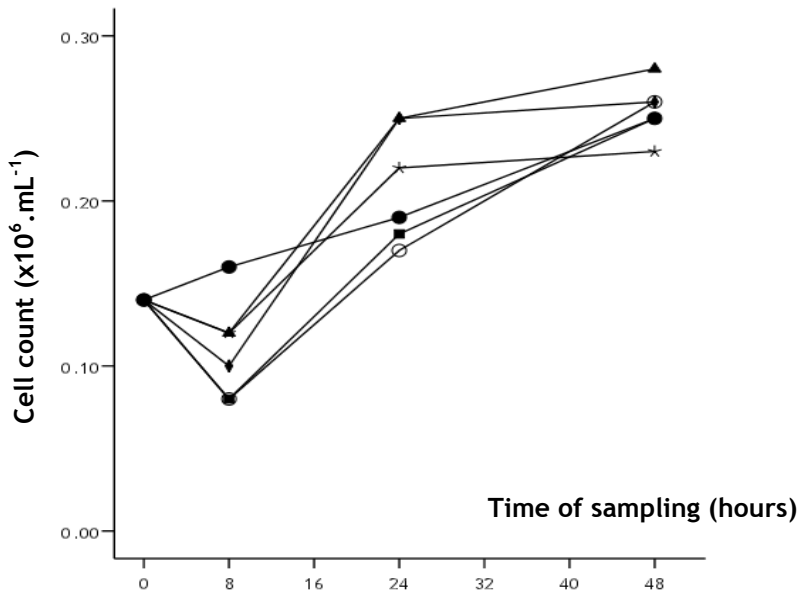
KEY:

HAE = Human airway epithelial cells (1HAEo⁺) i.e. a non-CF cell line

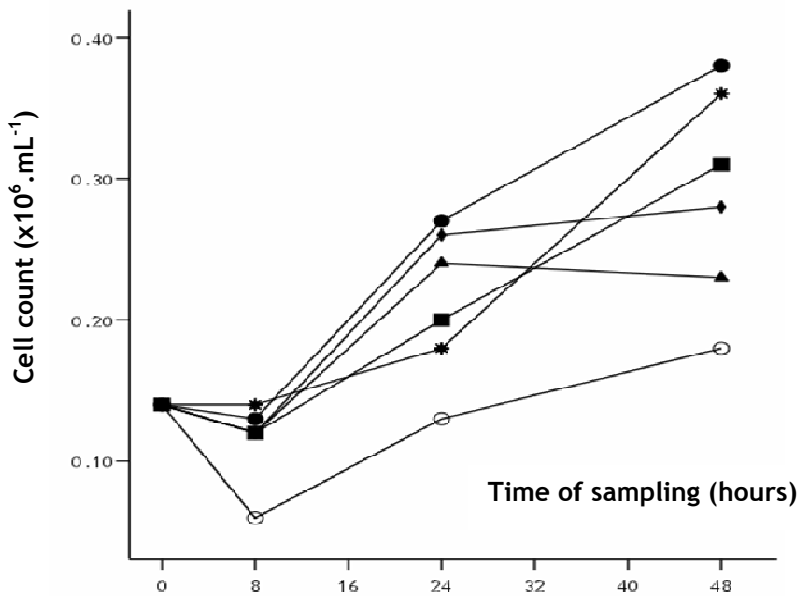
CFBE = Cystic Fibrosis Bronchial Epithelial cells (CFBE41o⁺) i.e. a CF cell line

Figure 6.2
Growth of cells at variable levels of hypoxia

a) HAE cells



b) CFBE cells



◆	Normoxic Controls
▲	Normoxia
●	Hypoxia 10%
■	Hypoxia 5%
☆	Hypoxia 1%
○	Hypoxia 0.1%

6.4.1.2 IL-8 levels at experimental time-points

Having established survival of cells despite extreme hypoxia, it was decided not to assay each sample for IL-8 levels, as the same experiments were in effect to be repeated as part of the next stage of work – namely, incubation in the presence or absence of lipopolysaccharide (LPS) at varying degrees of hypoxia. An ELISA was however carried out to establish the optimum dilution to be used for subsequent assays. Figure 6.3 shows the standard curve, and the optimum dilution is seen to be that which gives an optical density within the limits of detection of the assay. Table 6.2 lists the optical densities for each dilution.

Figure 6.3

Standard curve for IL-8 ELISA

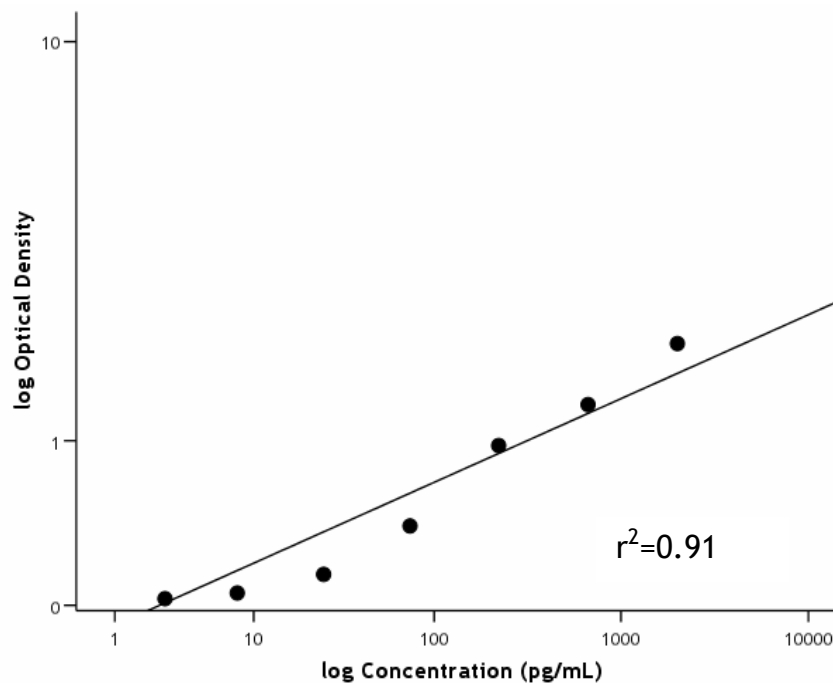


Table 6.2
Establishing optimum dilution

[Optical densities]

	NORMOXIA						1% HYPOXIA					
	HAE			CFBE			HAE			CFBE		
	1:10	1:50	1:100	1:10	1:50	1:100	1:10	1:50	1:100	1:10	1:50	1:100
0	0	0	0	0	0	0	0	0	0	0	0	0
8	1	0.3	0.2	0.6	0.1	0.1	0.6	0.2	0.1	1.3	0.4	0.2
24	1.2	0.3	0.1	1.6	0.4	0.2	1.4	0.3	0.1	1.4	0.5	0.3
48	1.4	0.5	0.2	1.4	0.5	0.3	1.8	0.5	0.4	1.8	0.9	0.6

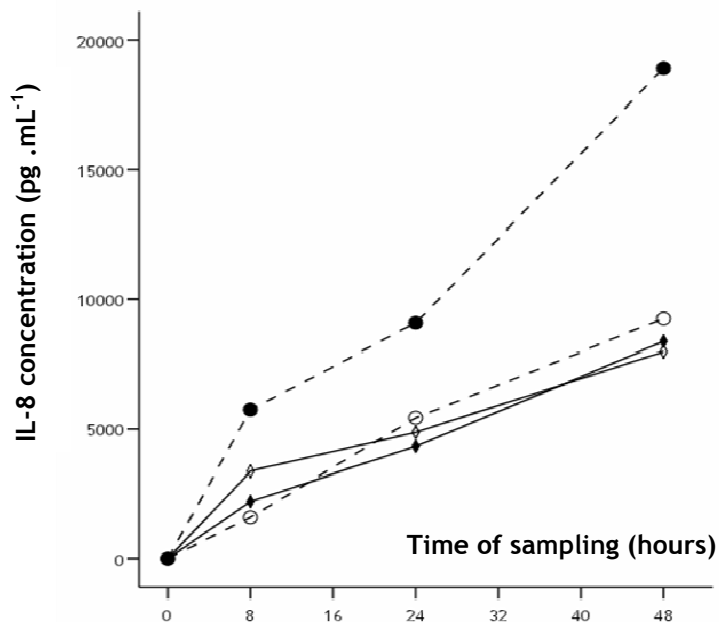
Extrapolation of these optical densities to the standard curve suggests that an optical density below 1 might be desirable, so a dilution of 1:50 or 1:100 appears most likely to provide optical densities within this range (at least when unstimulated by LPS).

Using the data available from the 1 in 50 dilutions, IL-8 levels for each time-point and for each condition could be plotted (Figure 6.4). Absolute concentrations in pg.mL^{-1} (a) as well as concentrations corrected for cell counts in $\text{ng.10}^{-6} \text{ cells.mL}^{-1}$ (b), are shown.

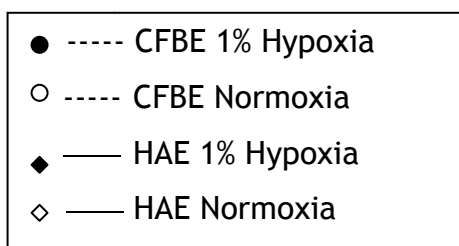
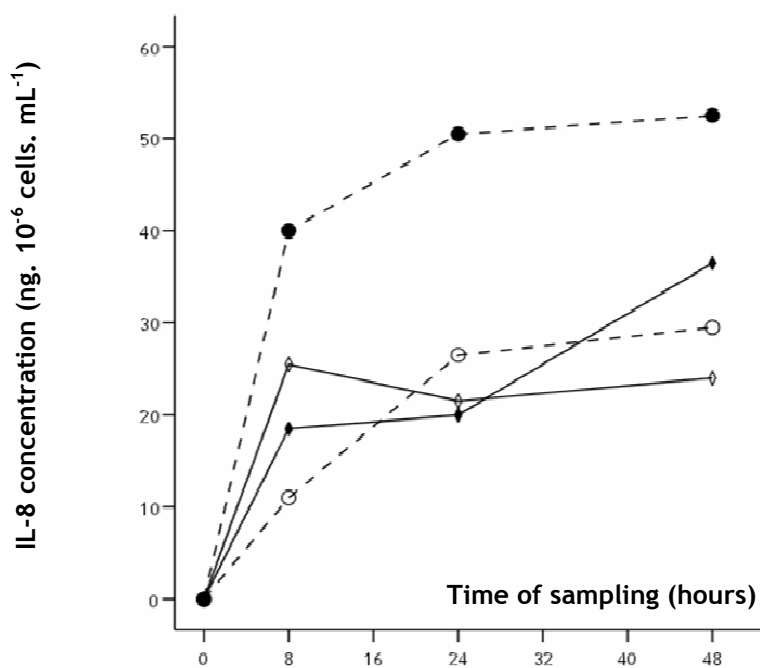
Figure 6.4

IL-8 production by CF and non-CF airway epithelial cells under normoxic and 1% hypoxic conditions

a) IL-8 concentrations in pg.mL^{-1}



b) IL-8 concentrations in $\text{ng.10}^{-6} \text{ cells.mL}^{-1}$



This experiment was only undertaken on one occasion, but suggests that IL-8 production increases with time in both normoxic and hypoxic environments, and in both cell lines. Hypoxia may augment such an inflammatory process, and this is particularly noted for the CFBE cell studies. It would be necessary to repeat this experiment on a number of occasions to assess the reproducibility of these results, and clearly this is a limitation in our study findings.

Aspects of this initial experiment were, therefore, incorporated into the next stage of experiments, which was designed to compare variable degrees of hypoxia to a known stimulus of NF κ B activation (and hence IL-8 production), namely exposure to LPS derived from *Pseudomonas aeruginosa*. The LPS preparation used was the L9143 preparation from Sigma-Aldrich (St Louis, MO, USA) which is phenol-extracted *Pseudomonas aeruginosa* serotype 10.

6.4.2 Effects of variable degrees of hypoxia with or without exposure to lipopolysaccharide on inflammation in CF and non-CF cell lines

6.4.2.1 Cell Counts at experimental time-points

These are displayed below in Table 6.3.

For this set of experiments, LPS was added to the media in high (50 $\mu\text{g}\cdot\text{mL}^{-1}$) or low (10 $\mu\text{g}\cdot\text{mL}^{-1}$) concentrations, or standard media (no LPS added) was used. Sampling was carried out at time zero, 24 and 48 hours.

Growth patterns appeared broadly similar for each cell line at varying degrees of hypoxia, with and with each concentration of LPS. This is demonstrated in Figure 6.5 (a and b). Of significance is that the best growing CFBE cell conditions were normoxia in the absence of LPS, and similar to Figure 6.2 (b), CFBE cells at 1% hypoxia appear to grow more slowly than do their normoxic counterparts.

Again, however, when repeatability measures were applied to cell growth across the entire set of experiments, no significant differences in growth patterns within cell-lines and between cell-lines were seen (Section 6.4.4).

Table 6.3

Cell counts ($\times 10^6 \cdot \text{mL}^{-1}$ of media) at times of sampling in variable hypoxia experiments using CF and non-CF cell lines in the presence or absence of lipopolysaccharide (LPS)

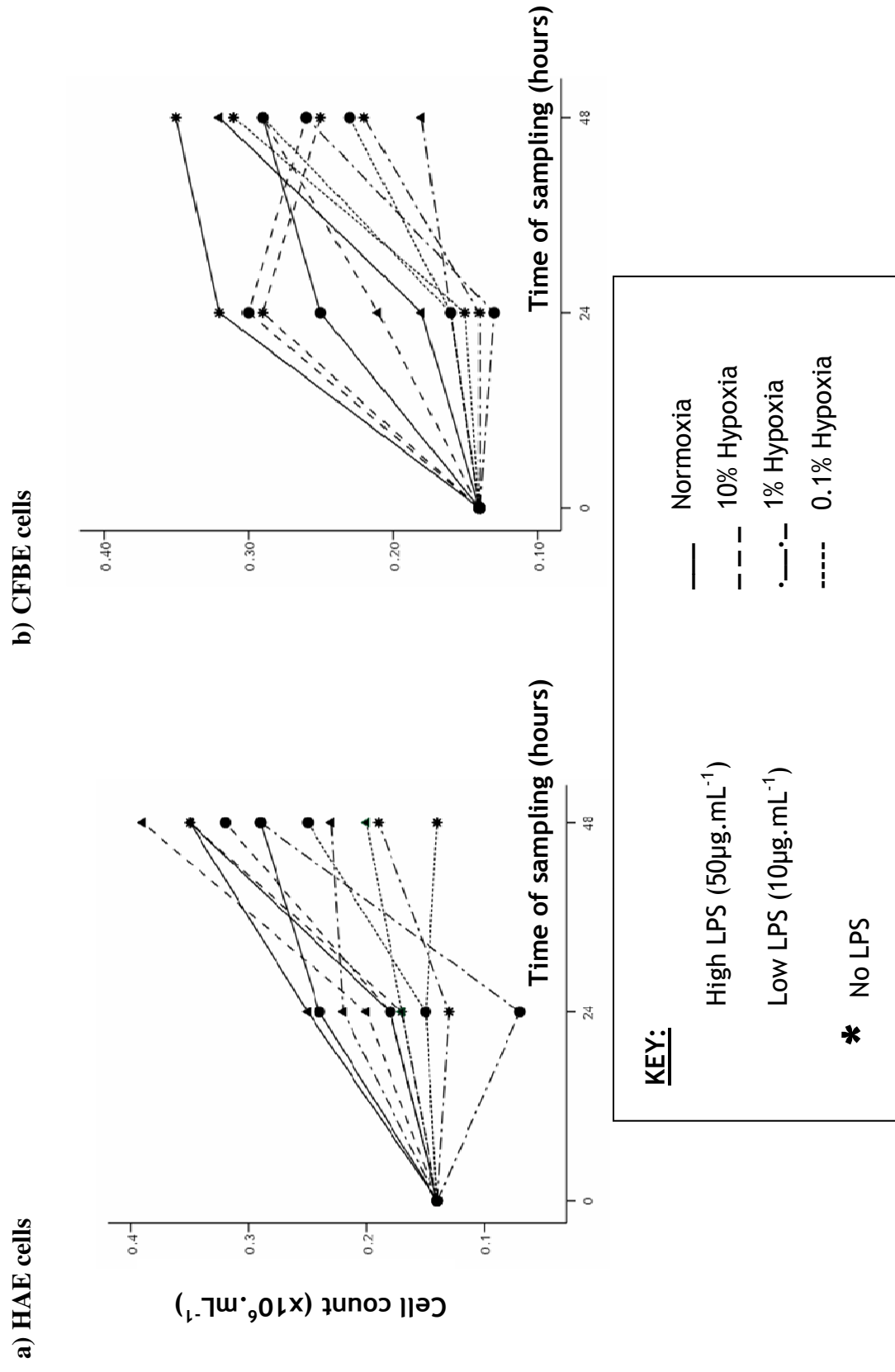
	NORMOXIA						HYPOXIA 10%						HYPOXIA 1%						HYPOXIA 0.1%						
	HAE		CFBE		HAE		CFBE		HAE		CFBE		HAE		CFBE		HAE		CFBE		HAE		CFBE		
	High	Low	No	High	Low	No	High	Low	No	High	Low	No	High	Low	No	High	Low	No	High	Low	No	High	Low	No	
Zero	LPS	0.14	0.14	0.14	LPS	0.14	0.14	0.14	LPS	0.14	0.14	0.14	LPS	0.14	0.14	0.14	LPS	0.14	0.14	0.14	LPS	0.14	0.14	0.14	
24	LPS	0.18	0.25	0.25	0.18	0.32	0.18	0.17	0.2	0.3	0.21	0.29	0.07	0.13	*	0.13	0.16	0.14	0.15	0.15	0.17	0.16	0.16	0.15	
hours																									
48	LPS	0.35	0.35	0.29	0.32	0.35	0.32	0.35	0.39	0.26	0.29	0.25	0.29	0.19	*	0.26	0.18	0.22	0.25	0.14	0.2	0.23	0.29	0.31	
hours																									

* Sample missing due to error in seeding

KEY:

- HAE = Human airway epithelial cells (1HAEo) i.e. a non-CF cell line
- CFBE = Cystic Fibrosis Bronchial Epithelial cells (CFBE41o) i.e. a CF cell line
- High LPS = LPS concentration of $50 \mu\text{g}\cdot\text{mL}^{-1}$ in cell culture media
- Low LPS = LPS concentration of $50 \mu\text{g}\cdot\text{mL}^{-1}$ in cell culture media
- No LPS = No LPS added to cell culture media

Figure 6.5
Growth of cells under variable degrees of hypoxia when incubated in the presence or absence of LPS



6.4.2.2 IL-8 levels at experimental time-points

The first stage of this second round of experiments was to compare the effect of a known stimulus for IL-8 production (LPS) with the effect produced by hypoxia (1% hypoxia arbitrarily chosen), and to assess for any synergy between the two – section 6.4.2.2.1.

Secondly, the effects of variable degrees of hypoxia on this process were analysed – section 6.4.2.2.2.

6.4.2.2.1 Comparison of IL-8 production in the presence or absence of LPS under normoxic and hypoxic (1%) conditions

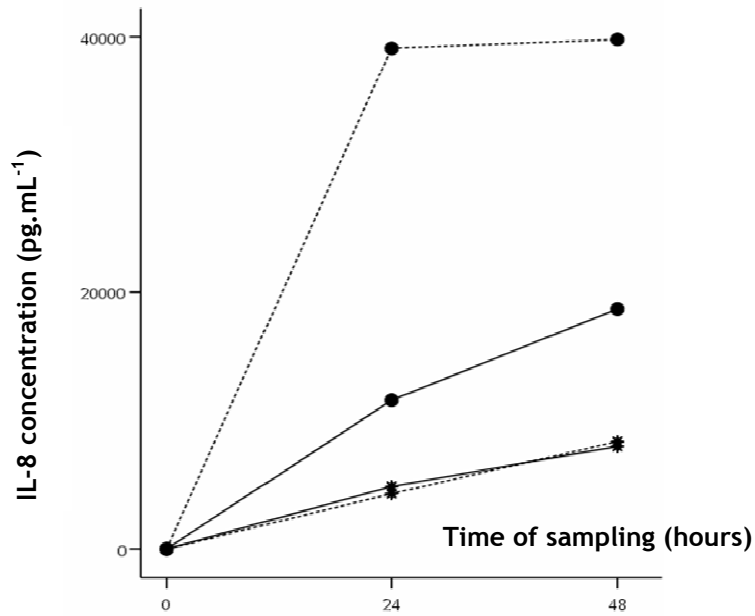
This work was undertaken at the same time as the ELISA study described in section 6.4.1.2. The standard curve for this assay is shown in Figure 6.4. The conditions studied were the effects of high LPS concentrations ($50 \mu\text{g.mL}^{-1}$) versus no LPS, in HAE and CFBE cells under both normoxic and hypoxic (1%) conditions. Both absolute concentrations in pg.mL^{-1} (a) and concentrations corrected for cell counts in ng.10^{-6} cells.mL⁻¹ (b) are shown (Figure 6.6). A dilution factor of 1:100 was needed for LPS stimulated samples to achieve optical densities within the desired range.

Figure 6.6

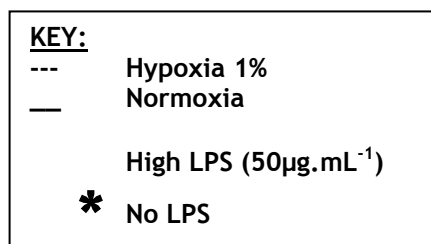
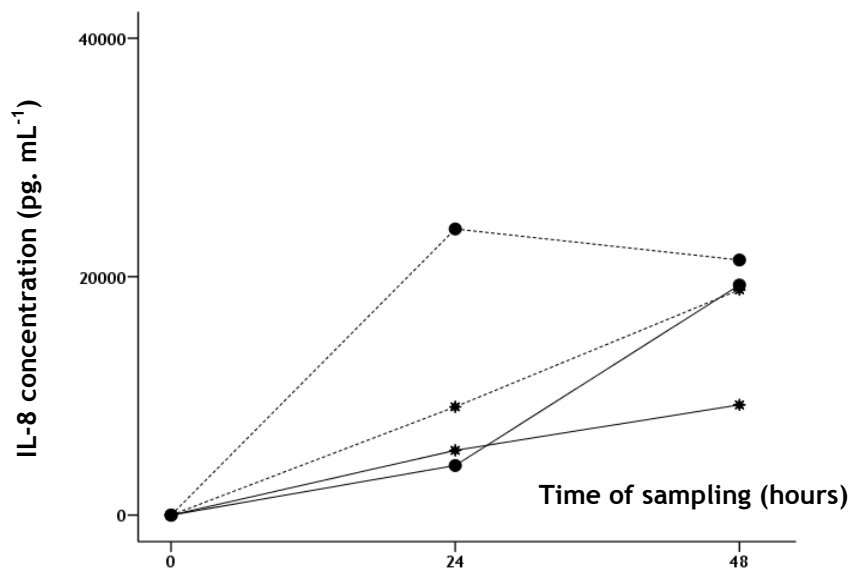
IL-8 production by CF and non-CF airway epithelial cells under normoxic and 1% hypoxic conditions, and in the presence or absence of LPS ($50\mu\text{g.mL}^{-1}$)

a) IL-8 concentrations in pg.mL^{-1}

i) HAE cells

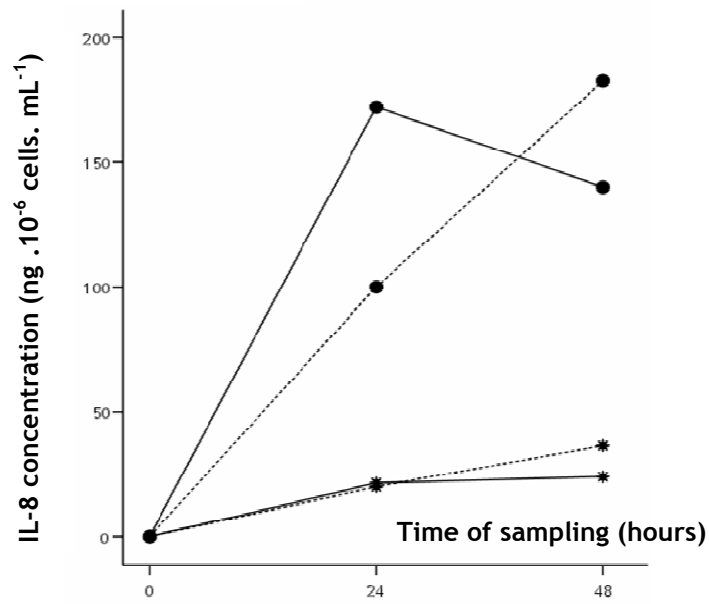


ii) CFBE cells

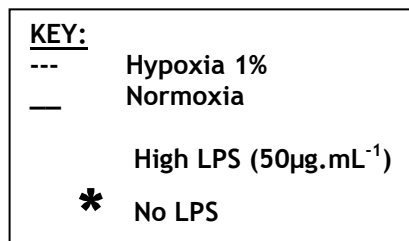
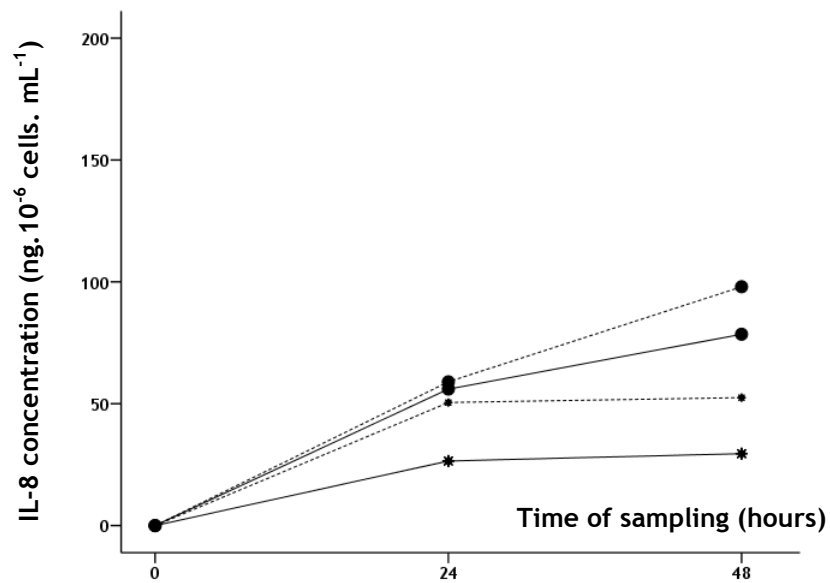


b) IL-8 concentrations in $\text{ng} \cdot 10^{-6} \text{ cells} \cdot \text{mL}^{-1}$

i) HAE cells



ii) CFBE cells



These data would appear to bear out the findings of experiment 1 (Figure 6.5), namely that IL-8 production is increased under hypoxic conditions when compared to normoxia. Furthermore, the effect of hypoxia on IL-8 production appears to be considerably lower than that of stimulation with LPS. Finally a synergistic effect on IL-8 production is suggested by hypoxia and LPS in combination.

It should however also be noted that the IL-8 concentration seen in HAE cells when stimulated with LPS is greater than that observed in the CFBE cell population. Although there is evidence that baseline IL-8 secretion may be lower in CF cell lines when compared with wild-type (Massengale *et al.* 1999), reduced IL-8 secretion when stimulated with LPS is an unexpected finding, for it is known that the innate immune response to *Pseudomonas* LPS within the CF airway is exaggerated and prolonged compared with normal individuals.

It is reported that *Pseudomonas aeruginosa* changes its configuration within the CF airway from a penta-acylated to a hexa-acylated LPS form (Hajjar *et al.* 2002). Human TLR4 recognises this change in configuration, and activation of NFκB is 100 times greater with the hexa-acylated form of LPS than with the penta-acylated form (Hajjar *et al.* 2002). It is postulated that it is this differential recognition of LPS in the CF airway which leads to massive activation of innate immune response. The above experiment was performed with penta-acylated *Pseudomonas* LPS (L9143 Sigma-Aldrich, St Louis, MO, USA), which may explain why levels of IL-8 production were similar for the two cell lines. This differs from the *in vivo* situation, where a change in LPS configuration would take place in the CF airway leading to differential cytokine activation.

Having shown that 1% hypoxia in combination with LPS exerts an effect on inflammation as measured by IL-8 production, the effects of varying degrees of hypoxia on this inflammatory process (in the presence and absence of LPS) were then studied.

6.4.2.2.2 Effect of variable hypoxia (10% v 1% v 0.1%) on IL-8 production in the presence or absence of LPS

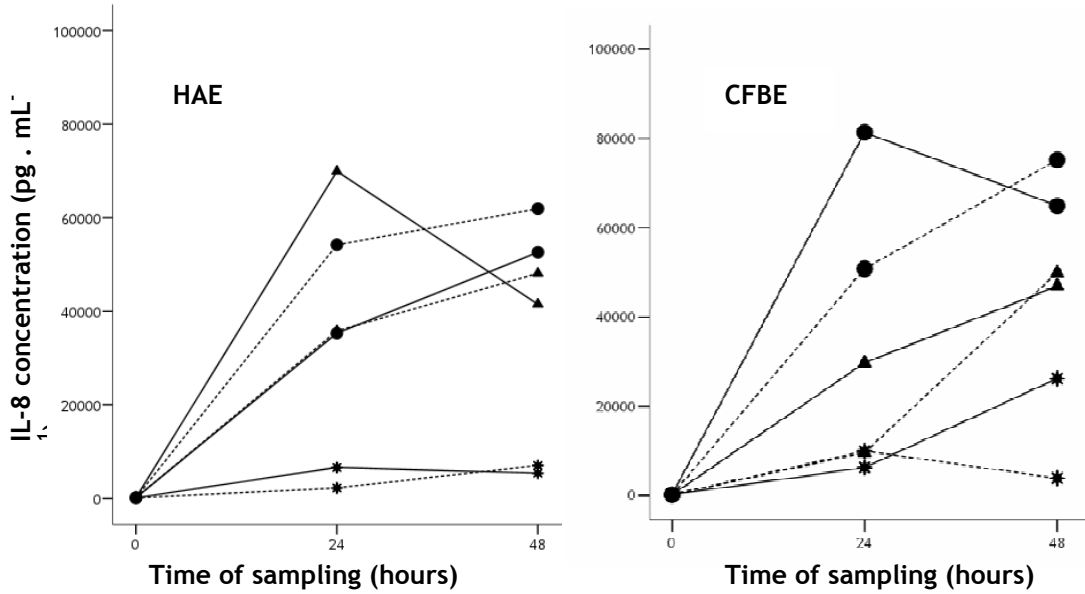
The next stage of analysis was to compare the effects of varying degrees of hypoxia on IL-8 production in the presence or absence of LPS using ELISA. The effects of 10%, 1% and 0.1% hypoxia were compared to time-matched normoxic control cells over a 48-hour culture period. The findings are illustrated below in Figure 6.7.

Figure 6.7

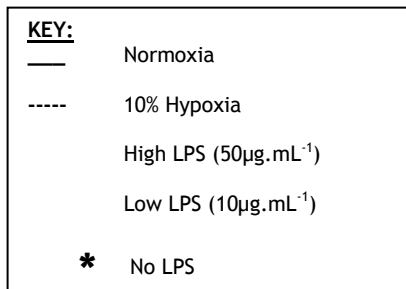
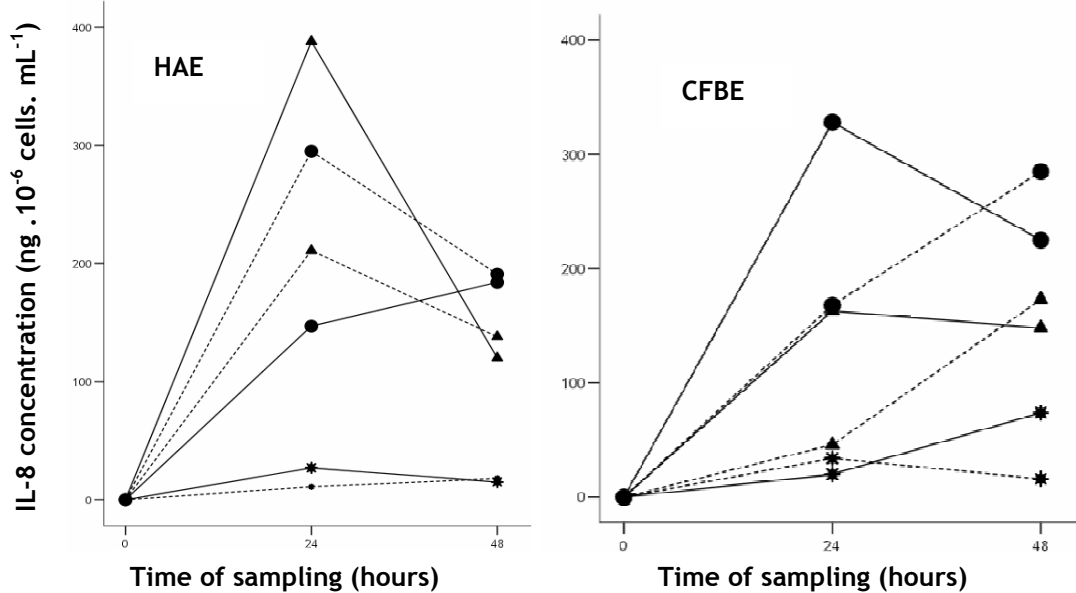
IL-8 production by CF and non-CF cells under variable degrees of hypoxia when incubated in the presence or absence of LPS

a) 10% Hypoxia

i) Absolute concentrations ($\text{pg} \cdot \text{mL}^{-1}$)

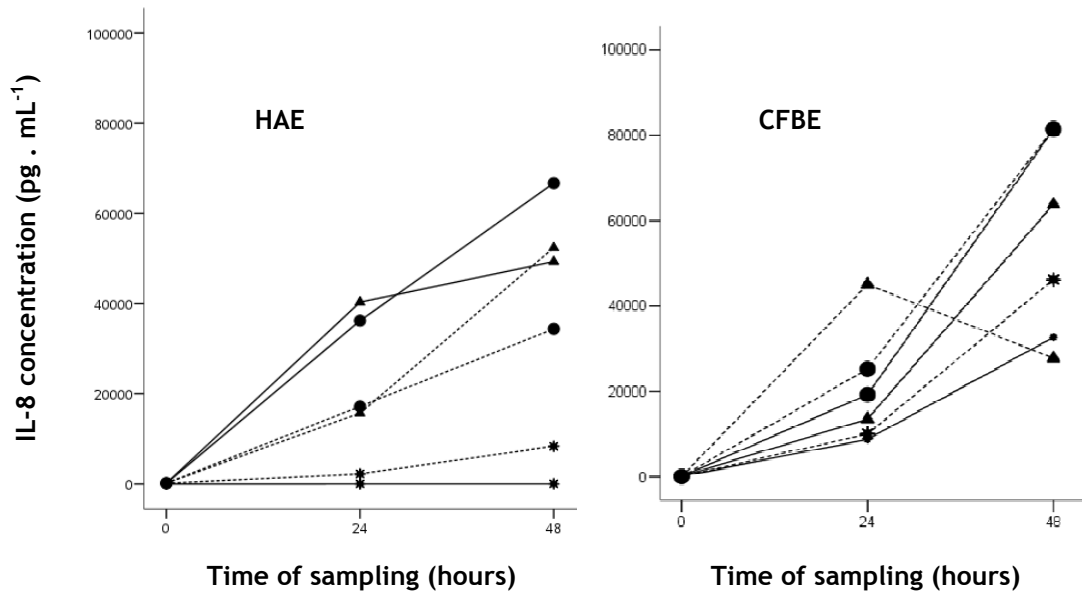


ii) Concentrations corrected for cell count ($\text{ng} \cdot 10^{-6} \text{ cells} \cdot \text{mL}^{-1}$)

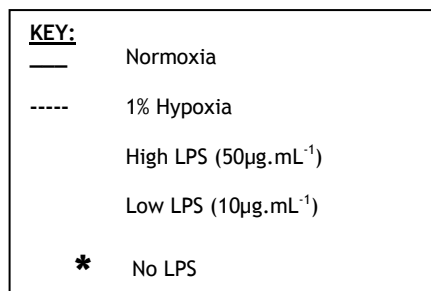
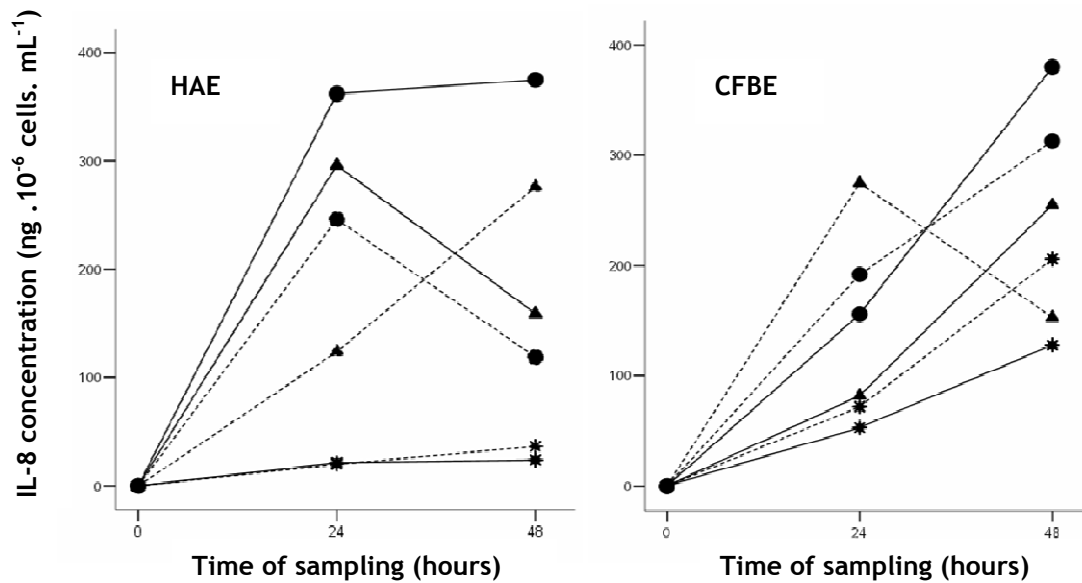


b) 1% Hypoxia

i) Absolute concentrations (pg.mL⁻¹)

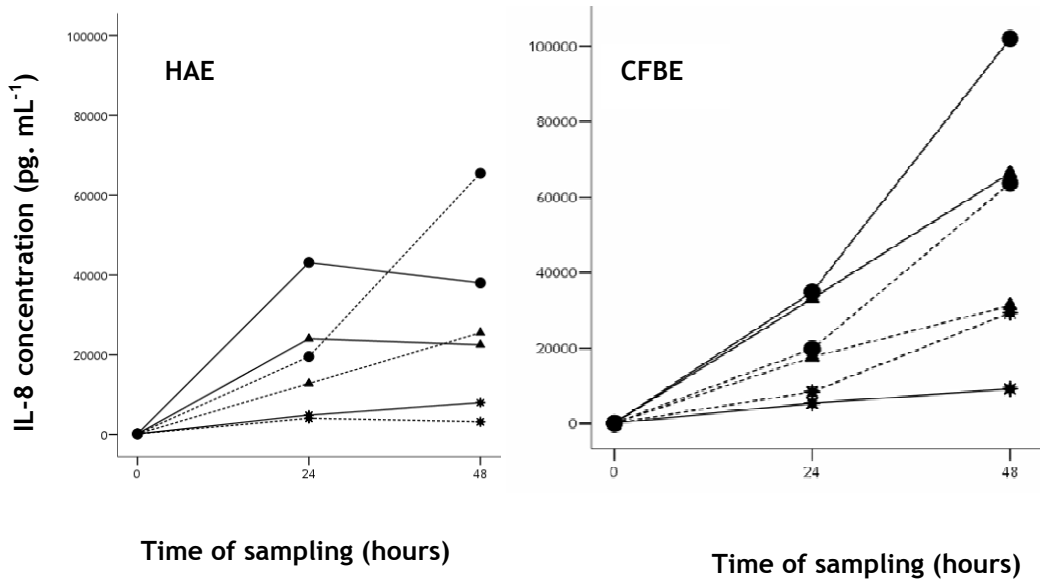


ii) Concentrations corrected for cell count (ng.10⁻⁶ cells.mL⁻¹)

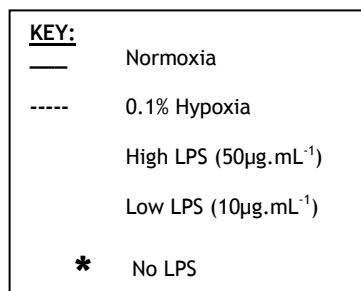
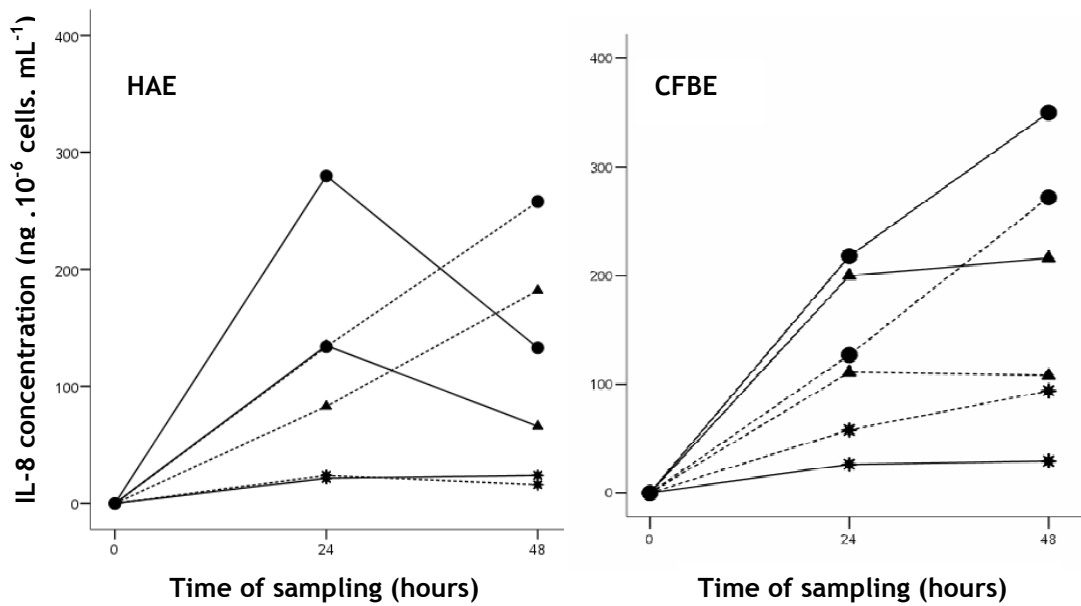


c) 0.1% Hypoxia

i) Absolute concentrations (pg.mL⁻¹)



ii) Concentrations corrected for cell count (ng.10⁻⁶ cells.mL⁻¹)



Although the data are less clean than the results of the previous set of experiments, the following trends are seen:

- Lipopolysaccharide (LPS) appears to act as a greater stimulus of IL-8 production than does hypoxia with a dose gradient for LPS concentration being observed
- IL-8 production appears to still be rising at 48 hours i.e. a peak may not have yet been reached
- Some evidence to support the findings from the first set of experiments that hypoxia acts a stimulus for IL-8 production, and may exert a synergistic action with LPS.

Bearing these findings in mind, the third set of experiments was devised.

6.4.3 Effects of prolonged extreme hypoxia and exposure to lipopolysaccharide on inflammation in CF and non-CF cell lines

The final experiments were designed to compare the effects of extreme hypoxia (0.1% hypoxia) on IL-8 production in the presence or absence of LPS over a prolonged (96-hour) time course. In this set of experiments only one concentration of LPS (50 $\mu\text{g.mL}^{-1}$) was used.

6.4.3.1 Cell Counts at experimental time-points

These are displayed below in Table 6.4.

Table 6.4

Cell counts ($\times 10^6$.mL⁻¹ of media) at times of sampling in extended timeframe hypoxia experiments using CF and non-CF cell lines incubated in the presence and absence of lipopolysaccharide

	Normoxic Control			Normoxia With LPS			Hypoxia 0.1%			Hypoxia 0.1% With LPS		
	HAE	CFBE	HAE	CFBE	HAE	CFBE	HAE	CFBE	HAE	CFBE	HAE	CFBE
ZERO	0.07	0.07	0.07	0.07	0.07	0.07	0.08	0.08	0.08	0.07	0.07	0.07
24 hours	0.09	0.11	0.09	0.08	0.09	0.08	0.09	0.09	0.09	0.06	0.06	0.09
48 hours	0.18	0.16	0.18	0.15	0.18	0.15	0.21	0.21	0.16	0.2	0.2	0.11
72 hours	0.22	0.17	0.15	0.28	0.15	0.28	0.26	0.26	0.24	0.19	0.19	0.13
96 hours	0.51	0.28	0.36	0.37	0.36	0.37	0.18	0.18	0.38	0.16	0.16	0.35

KEY:

HAE = Human airway epithelial cells (1HAEo⁺) i.e. a non-CF cell line

CFBE = Cystic Fibrosis Bronchial Epithelial cells (CFBE41o⁺) i.e. a CF cell line

LPS = Lipopolysaccharide

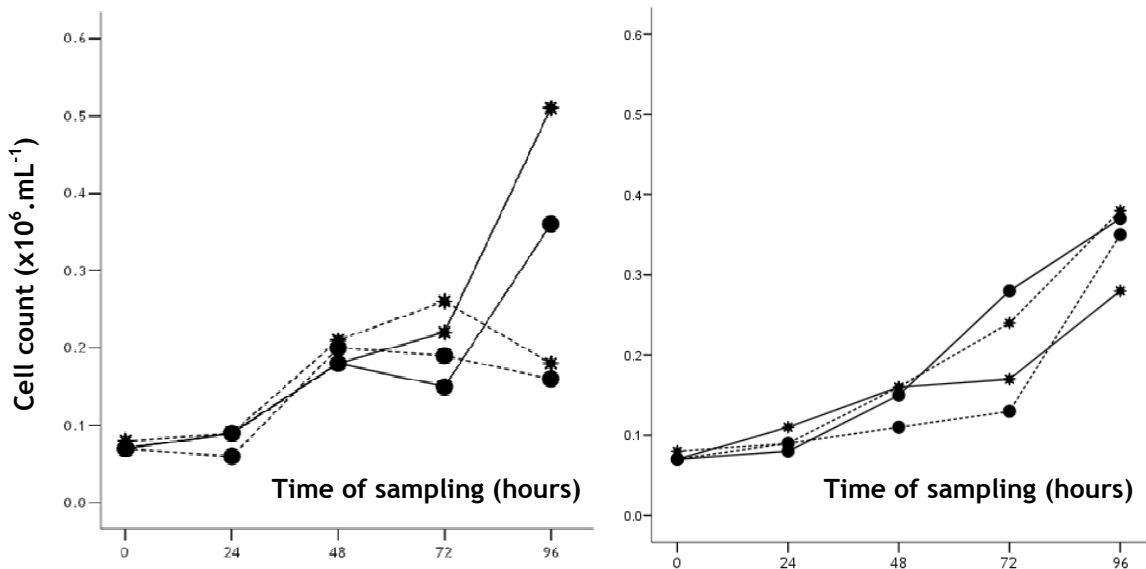
There is an observed reduction in growth of HAE cells under hypoxic conditions, which only becomes apparent at 96 hours (0.1%), as demonstrated in Figure 6.8 (a), although for the CFBE cells growth patterns appear unaffected by hypoxia - Figure 6.8 (b).

Figure 6.9

Growth of cells over 96-hour exposure to extreme hypoxia

a) HAE cells

b) CFBE cells



KEY:

— Normoxia

- - - 0.1% Hypoxia

● LPS (50µg.mL⁻¹)

* No LPS

Both CFBE41o⁻ and 1HAEo⁻ cells appear to adapt to prolonged periods of extreme hypoxia and survive. Previous work has shown that airway cells (human epithelial-like cell line A549) can survive extreme hypoxia, and even anoxia (0%) for up to 24 hours (Ahmad *et al.* 2003); whilst exposure of the CF cell line used in the above experiment (CFBE41o⁻) to hypoxia (1% O₂) over a 48 hour time-course experiment (Guillembellot *et al.* 2004) resulted in a reduction in CFTR mRNA levels when compared with normoxic CFBE41o⁻ cells although no report on growth kinetics was however, made.

6.4.3.2 IL-8 levels at experimental time-points

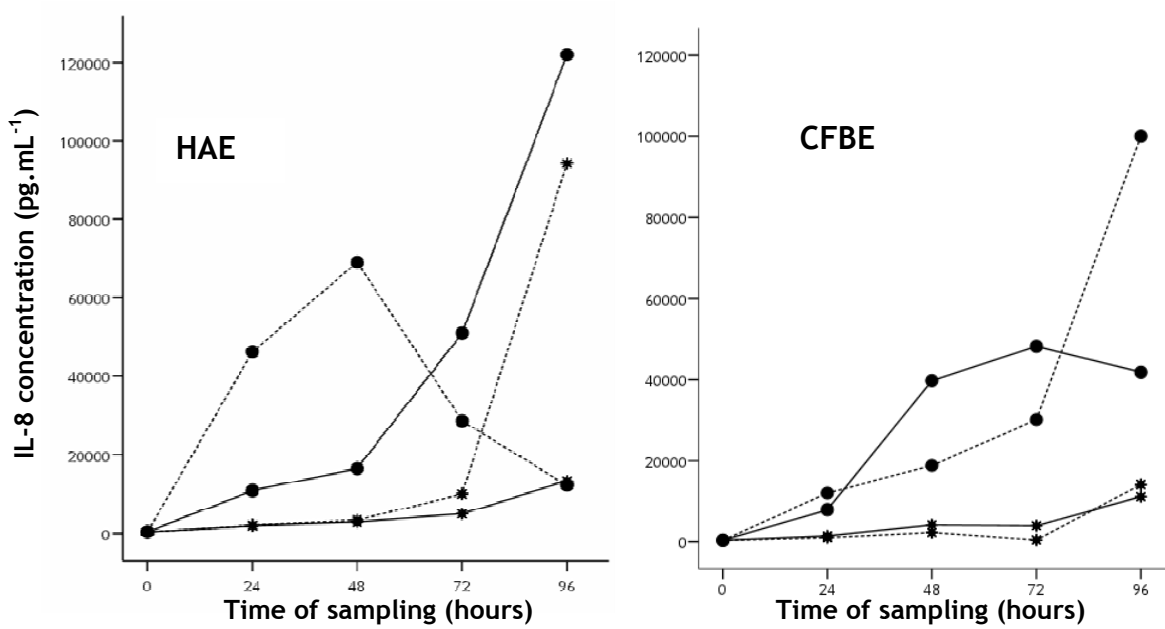
The next stage of analysis was to study the effects of a prolonged period of extreme hypoxia on IL-8 production in the presence or absence of LPS using ELISA.

The effects of 0.1% hypoxia with and without LPS were compared to time-matched normoxic control cells over a 96-hour culture period. The findings are illustrated overleaf in Figure 6.9.

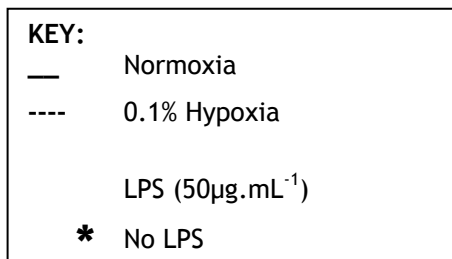
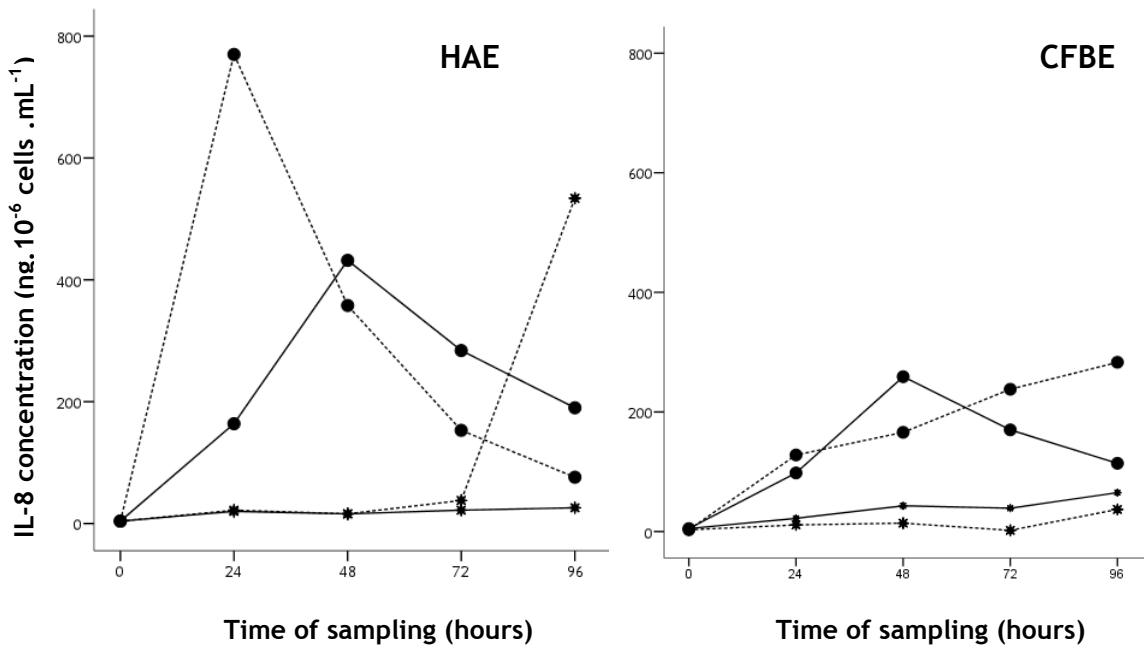
Figure 6.9

IL-8 production by CF and non-CF cells at extreme hypoxia (0.1%) when incubated in the presence or absence of LPS

i) Absolute concentrations (pg.mL^{-1})



ii) Concentrations corrected for cell count ($\text{ng.10}^{-6} \text{ cells.mL}^{-1}$)



To summarise the effects of prolonged hypoxic cell culture of CF and non-CF airway epithelial cells, the following trends were noted:

- The largest stimulus for IL-8 production in both HAE and CFBE cells was incubation in the presence of both hypoxia and also LPS.
- The stimulus from LPS alone is much higher than that seen with hypoxia alone.

A potential erroneous IL-8 level was noted at 96 hours for HAE cells incubated at 0.1% hypoxia in the absence of LPS, as this appears much higher than would be expected from the value obtained at 72 hours, and from previous experiments. The sample was run in duplicate and co-efficient of variation was 3.4% only. Adjacent wells were from 96 hour samples exposed to LPS under normoxic conditions which had very similar IL-8 concentrations making contamination in the form of overspill from adjacent wells a possibility.

6.4.4 Experimental repeatability

The sample size was small and therefore limited these analyses, with experiments under the same conditions being replicated on two to four occasions.

6.4.4.1 Cell counts

The repeatability of cell counts was assessed using a repeated measures ANOVA test. As expected, cell counts significantly increased with time over the 48 hour incubation period (Wilks' Lambda, $p < 0.001$). No evidence of difference in cell counts were seen within cell lines exposed to the same environmental conditions on repeated occasions (Wilks' Lambda, $p = 0.10$). However, the sample size was small and these data could lack the power to detect a difference.

After adjustment for repeated experiments, no statistical differences between cell lines and/or environmental conditions over time were observed ($p = 0.17$). The small sample size precluded meaningful *post hoc* analysis (Bonferroni) to detect differences between the groups, although on visual assessment of these data, CFBE cells exposed to extreme (0.1%) hypoxia and coincubated with LPS had lower cell counts.

6.4.4.2 IL-8 measures

A significant increase in IL-8 levels with time over the 48 hour incubation period was noted (Wilk's Lambda, $p < 0.001$), as well as a trend towards a difference in IL-8 levels (Wilk's Lambda, $p = 0.06$) within the same environmental condition (cell line, FiO_2 , LPS concentration) on repeated occasions. The effects of successive passages of cells on IL-8 production (i.e. cells with higher passage numbers may be more stressed) appears to be the most likely explanation for these findings. Although affecting the ability to compare IL-8 levels from separate experiments to one another, in each experiment the test conditions (variable hypoxia +/- LPS co-incubation) were matched with a contemporaneous normoxic control of the same passage number allowing direct comparison of each test condition with a normoxic control.

After adjustment for repeated experiments on the same cell line, there was a difference observed for IL-8 production between environmental combinations - cell line, FiO_2 , LPS concentration ($p = 0.05$). However, once again the small sample size precluded meaningful *post hoc* analysis (Bonferroni) to detect differences between the groups.

When IL-8 levels were adjusted for cell count, differences were again noted over time (Wilk's Lambda, $p < 0.001$), and similar within-group trends were noted for tests under the same environmental conditions (Wilk's Lambda, $p = 0.05$). After adjustment for repeated experiments on the same cell line, there was also a difference in observed IL-8 production per cell ($p = 0.007$). *Post hoc* analyses suggested that the greatest differences were between extremely hypoxic (0.1%) HAE cells exposed to LPS which produced greater amounts of IL-8 per cell than normoxic HAE controls ($p = 0.02$).

Whilst such trends are interesting, and may suggest that hypoxia (especially in conjunction with LPS) can exert an effect on inflammation, the work would need to be repeated on multiple occasions, ideally at the same time (same cell passage, atmospheric pressure, etc.) to accurately assess the biological variability and repeatability of the experiments.

6.5 Discussion

To summarise the overall results for this series of experiments, the following trends were apparent.

1. Cell growth appears largely preserved, even on exposure to extreme hypoxic conditions.
2. Hypoxia alone may exert an effect on IL-8 production in both CF and non-CF airway epithelial cells. This finding was however, not consistent across the range of experiments undertaken.
3. Lipopolysaccharide (LPS) stimulation of airway epithelial cells exerts a several-fold effect on IL-8 production in both CF and non-CF airway epithelial cells when compared to baseline levels of IL-8 production in the absence of LPS.
4. The highest levels of IL-8 production are noted in the presence of both hypoxic incubation conditions and LPS stimulation suggesting possible synergy between the two.

These results would support the notion that hypoxia may exert a pro-inflammatory effect in CF, supporting the *in vivo* findings of Chapter 5, where elevated WBC, neutrophil counts, and IL-8 levels were seen in hypoxic CF subjects, along with a reduction in bone density that may be related to such a pro-inflammatory (and therefore catabolic) state.

This experimental work has some limitations, and a number of potential sources of experimental error need to be acknowledged.

6.5.1 Methodology and data acquisition

Firstly, pipetting error may have led to differences in seeding densities between each of the tissue culture flasks. Although attempting to seed at a given density (0.7 or 0.3×10^6 cells per flask) based on cells counted after a new passage from stock cell culture flasks, errors in seeding densities are perhaps less important, as cell counts were undertaken at each timepoint, enabling IL-8 levels to be expressed in ng per million cells per mL of culture media.

Second, the use of a haemocytometer to undertake cell counts may be a source of error at two points in the experiment. Cell counts at the seeding stage may be inaccurate, leading to inaccurate seeding densities. More importantly, however is the potential source of inaccuracy at the counting stage at each experimental time point. Because the numbers of cells being counted within the haemocytometer field were small, then a clump of cells within the counting field could lead to an overestimation of cell numbers, and an underestimation of IL-8 production ($\text{ng} \cdot 10^{-6} \text{ cells} \cdot \text{mL}^{-1}$). There was no readily available alternative to count cells however, and data have been expressed throughout as absolute IL-8 concentrations as well as those corrected for cell numbers.

In experiments 1 and 2, an *a priori* assumption was made that time zero readings for IL-8 production would be zero. This hypothesis was tested along with the ELISA that was run for experiment 3. Time zero readings for IL-8 in blank media were of the order of 150 pg mL^{-1} , and for HAE and CFBE cells placed in media, then removed and stored, the time zero readings were between 250 and $350 \text{ pg} \cdot \text{mL}^{-1}$. The 24-hour readings for unstimulated (normoxia, no LPS) HAE and CFBE were 1926 and $1415 \text{ pg} \cdot \text{mL}^{-1}$ respectively.

A further question to address may have been to study the implications of exposing a sample of blank media to both hypoxic and normoxic conditions for the 24/48/72/96 hour time period, and carry out analysis of IL-8 production. This could be undertaken to assess whether cellular contamination of the media may be exerting a role in IL-8 production, and may be useful to be performed in future work.

The effects of successive passages of cells on IL-8 production (i.e. cells with higher passage numbers may be more stressed) were hopefully eradicated by each experiment being done with a simultaneous normoxic control.

Additionally, a new batch of LPS was used in the third set of experiments, but running alongside cells simultaneously incubated in the absence of LPS should have ensured that any confounding effect of this new reagent was eradicated.

Therefore, although the work has limitations - in particular, statistical analyses were hampered by the lack of repeatability for each experimental stage, the work was undertaken as a feasibility study for setting up a hypoxic tissue culture environment which was successful. Furthermore, the work shows some supporting evidence for the proof of the principle that direct activation of CF airway inflammation may occur in the presence of hypoxia. The *in vitro* work was not the main aim of the thesis, but nonetheless has proved an interesting aside, placing the *in vivo* findings in context, and generating a number of future research directions.

6.5.2 Interpretation of results

The combination of both hypoxia and LPS was chosen because of the *in vivo* effects of *Pseudomonas aeruginosa* (PA) on the lungs of a child with CF, whereby bacterial LPS from PA coupled with hypoxia may cause damage to the lung of a CF child.

The potential synergy of LPS and hypoxia is therefore of huge potential importance to the care of patients with CF. This may lead to the development of future research directions to examine this interaction.

These should include:

- a) Repeated experiments to examine the effects of extreme hypoxia in the presence or absence of LPS on production of IL-8 in CF and non-CF airway epithelial cells. This is essential to assess for potential biological variability in the experiments described above, and also to verify their findings.

It should, however, be noted that cells may differ in their morphology and behaviour with time and number of passages (divisions), as may environmental conditions.

Therefore, whilst some repeatability data is presented, across the series of experiments; if designing the experiments again, it would be desirable to run each set in duplicate such that repeatability could be assessed without the potential confounding effects of time, environmental pressure and cell passage number.

- b) In an experimental model similar to that described in a) to analyse not just the effects on IL-8 production, but also on other aspects of the NF κ B cascade. This may include work to study downstream signalling regulators within the NF κ B family, in a bid to tease out the signalling mechanism that is activated by hypoxia in the CF airway.
- c) To examine the effects in primary cell lines from CF subjects rather than immortalised cell lines, and undertake translational research on the effects of hypoxia and LPS stimulation in animal models such as CF and non-CF mice.
- d) To study the effects of extreme hypoxia on CFTR trafficking by estimation of CFTR protein reaching the cell surface. This may need to be undertaken in primary cells in order that the apical cell surface can be identified. This could hopefully be achieved by selecting an optimal antibody to stain for CFTR protein (Mendes *et al.* 2004, Farinha *et al.* 2004) in conjunction with confocal microscopy to evaluate trafficking to the cell surface using similar techniques to those described previously by Dormer and colleagues (Dormer *et al.* 2005). This series of experiments in a CF cell line may help to delineate whether up-regulation of inflammation in CF airway cells under hypoxic conditions is mediated by CFTR dysfunction.
- e) Finally, the effects of oxygen therapy with and without aggressive anti-pseudomonal treatment, and their effects on lung inflammation appear worthy of evaluation in animal models, and perhaps later in human subjects.

Although preliminary, these research findings are novel, and not without clinical application. Further research will hopefully confirm or refute their importance.

6.6 Conclusions

The results discussed in Chapter 4 suggest that CF subjects with sleep hypoxia have a reduction in clinical well-being with reductions in exercise capacity, anthropometric measures (BMI), lung function (lower FEV₁ and FVC), and quality of life, as well as increased gas trapping (elevated RV/TLC ratio), chest radiograph (Chrispin-Norman) scores, number of intravenous antibiotic courses and measures of ventricular wall (LV and RV) thickness. Chapter 5 proposes an association between hypoxia and reduced BMAD z scores.

The paradigm exists of whether hypoxia is simply an effect of, or whether also a possible contributor to CF lung inflammation remains contentious. However, the results of chapter 6 would suggest that hypoxia may indeed exert a direct effect on inflammation (as assessed by IL-8 production) in HAE and CFBE cells. Furthermore, the greatest levels of IL-8 production were seen when hypoxia was combined with a known stimulus of IL-8 production in the CF airway, namely bacterial LPS from *Pseudomonas aeruginosa*.

Therefore, this thesis provides some supporting evidence that hypoxia may directly contribute to a pro-inflammatory state in the CF airway. Further research should, however, require to be undertaken to assess the repeatability of these findings.

If hypoxia can be both a contributor to, and a consequence of, decline in clinical status and pro-inflammatory state in CF, then strategies to ameliorate hypoxia and its sequelae should be evaluated. Chapter 7 (Discussion) will consider this in detail.

Chapter 7: Hypoxia in children with cystic fibrosis – Conclusions and future directions

7.1 Definition of hypoxia in CF

At the beginning of this thesis, no unifying definition of hypoxia in CF was available, and one of the principal study aims was to select a definition of hypoxia in CF that best correlates with measures of inflammation.

The most sensitive and specific definition for detecting measures of inflammation in children with CF was that of nocturnal SpO₂ <93% for >10% sleep time. This is proposed as the optimal definition of hypoxia in children with CF.

7.1.1 Predicting hypoxia in CF - Recommendations

The work presented in this thesis concurs with the findings of previous authors (Coffey *et al.* 1991, Bradley *et al.* 1999), in suggesting that hypoxia during sleep occurs more commonly than exercise hypoxia. It is proposed that all subjects with exercise-associated desaturation should undergo overnight oximetry.

A further recommendation from this thesis is that all CF subjects with FEV₁ <60% predicted should undergo an overnight oximetry at a time of clinical stability (100% sensitive, 84% specific and positive predictive value of 64%) in the detection of nocturnal hypoxia using the above definition.

7.2 Future research directions for hypoxia and childhood CF

Future research hypotheses can be generated in areas allied to both *in vivo* and *in vitro* aspects of the research presented in this thesis. A call for randomised, controlled trials of restoration of normoxia is made.

7.2.1 Mechanisms of hypoxia - Human work

Follow-up of the 41 subjects in the thesis cohort at intervals throughout later childhood and into adulthood would be of immense value. In particular, delineating the time of onset of measurable pulmonary hypertension, and its correlation with hypoxia would be important, as it would confirm whether the increased right ventricle wall thickness seen in our hypoxic subjects was indeed a true precursor of pulmonary hypertension. Evaluation of the longer term effects of hypoxia on life expectancy, rate of decline in lung function, exercise capacity, bone density, and quality of life would also be both interesting and informative.

Replication of the study findings in a second population would be vital to future research into both the causal mechanisms of, adaptation to, and potential treatment of hypoxia in CF.

7.2.2 Mechanisms of hypoxia - *In vitro* work

The preliminary data in this thesis suggest that the initial hypothesis that hypoxia is associated with inflammatory changes was correct. The data were limited by the small numbers of experiments but demonstrated ‘proof-of-principle’. Some strategies for future *in vitro* research are outlined in section 6.5.2.

7.2.3 Therapeutic trials of restoration of normoxia in hypoxic CF children

If, as suggested, clinically important nocturnal hypoxia is more prevalent than is estimated from the numbers currently receiving oxygen, this may act as a call for research into the effects of restoration of normoxia. The adverse effects observed in hypoxic CF subjects in this study, and the putative deleterious effects that may arise from existing in a pro-inflammatory state in CF (muscle-wasting, CF bone disease, ongoing neutrophilic lung inflammation, etc.) support research into strategies to restore normoxia.

The effects of restoration of normoxia on inflammation are important to measure not only because of the associations seen between hypoxic CF subjects and markers of inflammation in this study, but because in other childhood disease models of nocturnal hypoxia (namely OSA), restoration of normoxia is reported to be associated with a reduction in inflammation (Gozal *et al.* 2008). If hypoxia can be both contributor to, and consequence of decline in clinical status and pro-inflammatory state in CF, then strategies to ameliorate hypoxia and its sequelae should be evaluated.

7.2.3.1 Restoration of normoxia using oxygen therapy

A randomised, controlled trial of oxygen therapy in CF with entry criteria including a definition of hypoxia as SpO₂ <93% for >10% sleep time may be a reasonable starting point. It is essential that night-time oxygen is titrated to provide the amount required to restore normoxia at night – a major limitation of the study design of the randomised trial of oxygen in CF undertaken by Zinman and colleagues was that nocturnal oxygen therapy flows were decided by the flow required to normalise daytime PaO₂. Since daytime measures are poorly sensitive indicators of sleep hypoxia (section 4.5.3), it is possible that patients in the oxygen arm of the study may have remained hypoxic and any treatment effect may have been underestimated (Zinman *et al.* 1989).

Outcome measures should include exercise capacity, lung function and quality of life data, as well as markers of inflammation including neutrophil counts, CRP, measures of NFκB-mediated inflammation (IL-8, and possibly direct assay of NFκB). Monitoring for side-effects is important, and should include measurement of transcutaneous CO₂ in view of the theoretical effects on blunting of respiratory drive.

7.2.3.2 Restoration of normoxia with non-invasive ventilation (NIV)

An alternative strategy to restore normoxia would be to use non-invasive ventilatory pressure support driven with air. When compared with either oxygen or placebo, NIV alone (without supplemental oxygen) had beneficial effects on nocturnal hypoventilation and daytime functioning including exercise capacity in 8 CF subjects with both hypoxia and hypercapnia (Young *et al.* 2008). Additionally, NIV alone led to a change in mean (95% CI) SpO₂ of +3% (+0.7, +6.7) from baseline. This was similar to the order of increase in SpO₂ seen in the oxygen arm of this cross-over trial (Young *et al.* 2008). Although encouraging, these data act as a call for further research into the effects of NIV in CF, echoing the need for adequately powered randomised trials of NIV in CF highlighted in the 2007 Cochrane review (Moran *et al.* 2007). Larger studies with adequate power may also be useful to compare the effects of NIV with oxygen therapy utilising similar measures of clinical and inflammatory status to that described above (section 7.4.3.1).

7.2.3.3 Restoration of normoxia using Heliox21™

A further approach to consider in the future is the use of Heliox™ - a mixture of helium and air. Use of heliox in CF is confined to two single case reports (Stucki *et al.* 2002, Henchey 2003). Heliox has a density that is one third of that of air, and the lower density of heliox, when compared with air enhances flow in obstructed airways by decreasing the pressure gradient across these airways, resulting in reductions in both dyspnoea and work of breathing (Levine 2008). Furthermore, heliox reduces turbulence in the airways, restores laminar flow and enhances the deposition of aerosolised agents to the distal lung spaces (Piva *et al.* 2002), which may be of benefit to those with CF who receive mucolytic, antibiotic, bronchodilator, and corticosteroid therapy via a nebulised or inhaled route.

Various helium/oxygen mixtures are available including Heliox 21™, which is a mixture of 79% helium and 21% oxygen that provides the same FiO₂ as air, which may offer the restoration of normoxia without hyperoxia.

7.3 Summary: The proposed circular relationship of hypoxia, inflammation and lung disease in CF

An association between hypoxia in CF and clinical, radiological, psychological and immunological outcome measures is demonstrated. The adverse clinical outcomes associated with the hypoxia could be argued to reflect increased disease severity for lung function, radiological evidence of lung disease, number of antibiotic courses, exercise capacity, and measures of inflammation are all worse in the hypoxic subjects.

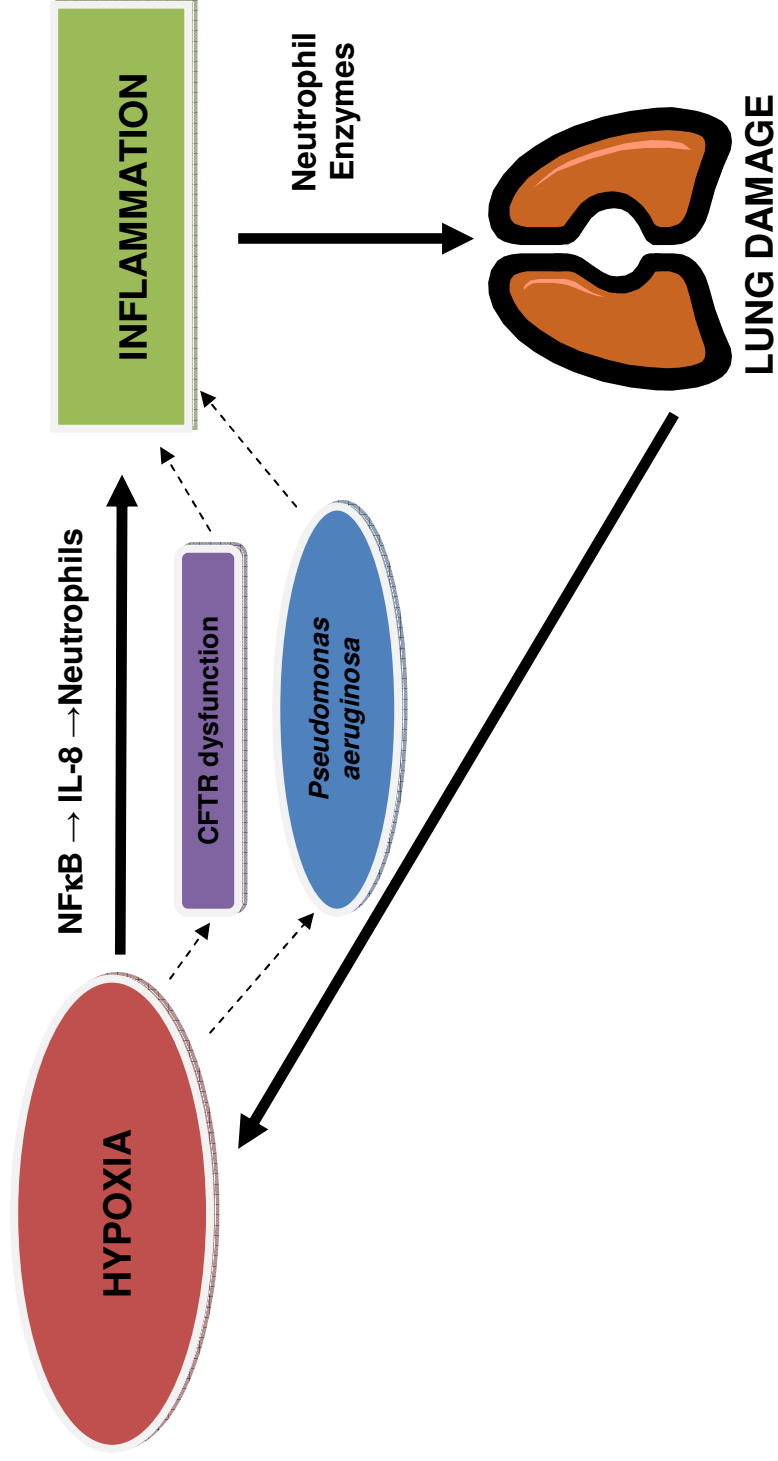
Hypoxia may, however, be both ‘cause’ and ‘effect’ in this sequence. As discussed in section 1.5, there are *in vitro*, rodent and *in vivo* data which support a causative role for hypoxia as an effector mechanism of perpetual neutrophilic inflammation, leading to a pro-inflammatory state and adverse clinical sequelae. The case for hypoxia acting directly on lung inflammation in CF is strengthened by the *in vitro* work of Chapter 6 of this thesis, whereby hypoxia is a potential stimulus for NFκB mediated inflammation and IL-8 production in a CF airway epithelial cell model.

Quality of life was lower in children with sleep hypoxia, which too may simply be because children felt bad as a result of severe CF lung disease. However, published evidence suggests that hypoxia may have an independent, negative impact on psychological well-being in CF, as, in the only long-term study of oxygen therapy in CF, it is reported that 83% of those in oxygen maintained school/work attendance at 12 months compared with only 20% of the air group ($p < 0.01$) (Zinman *et al.* 1989).

Therefore, whilst advancing lung disease undeniably promotes hypoxia, it appears also to be the case that hypoxia exerts an effect on lung disease (via upregulation of inflammation), thus perpetuating a downward pathophysiological spiral. Further research is needed to tease out the effector signalling mechanisms (including effects on the NFκB signalling cascade and CFTR trafficking) by which hypoxia may mediate CF lung inflammation, and also the extent to which restoration of normoxia may impact on inflammation in the CF lung. The proposed circular relationship is illustrated in Figure 7.1.

A uniform approach to defining hypoxia needs to be adopted by paediatric specialist CF centres, along with guidelines for utilising oxygen therapy in children with CF. Any change in practice must be evidence-based and the need for adequately-powered randomised, controlled trials of oxygen therapy and/or NIV in CF is apparent.

Figure 7.1
The proposed circular relationship between hypoxia, inflammation and lung disease in CF



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APPENDIX ONE

RESEARCH ETHICS COMMITTEE APPROVAL

Institute of Child Health/Great Ormond Street Hospital Research Ethics Committee

The Institute of Child Health
30 Guilford Street
London
WC1N 1EH

15 March 2005

Dr Donald S Urquhart
Cystic Fibrosis Research Fellow
Great Ormond Street Hospital for Children
Cystic Fibrosis Office, Level 8 Nurses Home,
Great Ormond Street Hospital,
London
WC1N 3JH

Dear Dr Urquhart

Full title of study: ***Definition of hypoxic phenotype in childhood cystic fibrosis***
REC reference number: ***05/Q0508/19***
Protocol number: ***Research proposal, not dated***

Thank you for your folder of extra information in support of the above project, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information was considered at the meeting of the Sub-Committee of the REC held on 15 March 2005. A list of the members who were present at the meeting is attached.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Conditions of approval

The favourable opinion is given provided that you comply with the conditions set out in the attached document. You are advised to study the conditions carefully.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type:	Version:	Dated:	Date Received:
Application		12/01/2005	12/01/2005
Investigator CV	Dr Urquhart's CV		13/01/2005
Protocol	Research Proposal - MD Research Project 2004 - 5		12/01/2005
Participant Information Sheet	Parent Information Sheet		12/01/2005
Participant Information Sheet	Patient Information Sheet, 14+		12/01/2005
Participant Information Sheet	Patient Information Sheet, 8-14		12/01/2005
Response to Request for Further Information	Answers to LREC questions		25/02/2005
Response to Request for Further Information	Information about peer review		14/01/2005
Other	Minutes from a Statistics meeting		02/03/2005
Other	Further information about application	24/01/2005	24/01/2005

Management approval

The study should not commence at any NHS site until the local Principal Investigator has obtained final management approval from the R&D Department for the relevant NHS care organisation.

Membership of the Committee

The members of the Ethics Committee who were present at the meeting were: Dr V Larcher (Consultant Paediatrician) and Dr R Gilbert (Senior Lecturer, Paediatric Epidemiology and Biostatistics).

Notification of other bodies

The Committee Administrator will notify the research sponsor that the study has a favourable ethical opinion.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

05/Q0508/19

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project,

Yours sincerely,

Taki Austin (Mrs)
Research Ethics Coordinator
E-mail: t.austin@ich.ucl.ac.uk

Enclosures *Standard approval conditions*

Site approval form (SF1)

APPENDIX TWO

MODIFIED CHRISPIN-NORMAN X-RAY SCORING SYSTEM

Patient's name	Review date	/	/
Hospital Number	Scoring date	/	/
D.O.B.	Scored by		

Chrispin-Norman X-ray score

Feature	Not present	Present but not marked	Marked
Overinflation			
Diaphragmatic depression	0	1	2
Chest wall shape	0	1	2
Lung fields	0	1	2
Bronchial Line Shadows			
Right upper zone	0	1	2
Left upper zone	0	1	2
Right lower zone	0	1	2
Left lower zone	0	1	2
Ring Shadows			
Right upper zone	0	1	2
Left upper zone	0	1	2
Right lower zone	0	1	2
Left lower zone	0	1	2
Mottled Shadowing			
Right upper zone	0	1	2
Left upper zone	0	1	2
Right lower zone	0	1	2
Left lower zone	0	1	2
Large Soft Shadows			
Right upper zone	0	1	2
Left upper zone	0	1	2
Right lower zone	0	1	2
Left lower zone	0	1	2
			TOTAL

Instructions

The frontal and lateral x-rays are inspected for the typical abnormalities seen in CF. These include increases in lung volume – **hyperinflation**, which result in forward bowing of the sternum and spinal kyphosis (lateral film) and the degree of diaphragm depression (frontal and lateral films). Each individual item is given a score of: 0 - not present; 1 – present but not marked; 2 – marked; depending on the degree of change. The lung fields are then **divided into four zones on the PA film**; right upper, left upper, right lower, left lower. Each field is then reviewed for parenchymal lung changes which are a consequence of bronchial mucus plugging and infection. These are seen as bronchial wall thickening, ring shadows, mottled shadowing and areas of confluent consolidation – large soft shadows. A score of 0, 1 or 2 is given according to severity for each zone in relation to these changes. The increased **bronchial line shadowing** indicates thickening of the walls of the airways; these are usually seen as longitudinal shadows with a straight line branching pattern and also as end-on bronchi. **Ring shadows** are formed by a central area of increased radiolucency circumscribed by a discrete shadow of lesser radiancy. These shadows are about 5mm in diameter and are predominantly seen in peripheral areas of the lung; they represent bronchiectasis at the lobular level. **Mottled shadows** indicate sputum collection at the microlobular level and show as small rounded opacities with ill-defined edges which are seen as confluent areas of increased radiolucency. **Large “soft” shadows** are areas of more generalised lung collapse and consolidation affecting a lobe or segment within a lobe. A score of 0, 1 or 2 is given for the presence and severity of each of these signs in the four quadrants. The radiograph is best reviewed by two observers simultaneously in order to reduce inter-individual error. In clinical practice a score of 20 or more indicates advanced disease.

Ref. Chrispin AR, Norman AP. The systematic evaluation of the chest radiograph in cystic fibrosis. *Pediatr Radiol* 1974; **2**: 101-5.

APPENDIX THREE

CYSTIC FIBROSIS QUESTIONNAIRES [CFQ-UK]

	<u>Pages</u>
- Adolescents and adults [CFQ 14+]	279-282
- Parents and caregivers [CFQ-child P]	283-286
- Self-completion format for children aged 12-13 years	287-290

NB: Interviewer format for children aged 6-11 yrs also available
(not used in data analyses for this study)

Understanding the impact of your illness and treatments on your everyday life can help your healthcare team keep track of your health and adjust your treatments. For this reason, this questionnaire was specifically developed for people who have cystic fibrosis. Thank you for your willingness to complete this form.

Instructions: The following questions are about the current state of your health, as you perceive it. This information will allow us to better understand how you feel in your everyday life.

Please answer all the questions. There are **no** right or wrong answers! If you are not sure how to answer, choose the response that seems closest to your situation.

Section I. Demographics

Please fill-in the information or tick the box indicating your answer.

- A.** What is your date of birth?
 Date

Day	Month					Year	
- B.** What is your gender?
 Male Female
- C.** During the **past two weeks**, have you been on holiday or out of school or work for reasons **NOT** related to your health?
 Yes No
- D.** What is your current marital status?
 Single/never married
 Married
 Widowed
 Divorced
 Separated
 Remarried
 With a partner
- E.** Which of the following best describes your racial background?
 White - UK
 White - other
 Indian/ Pakistani
 Chinese/ Asian
 African
 Caribbean
 Other [not represented above or people whose predominant origin cannot be determined/ mixed race]
 Prefer not to answer this question
- F.** What is the highest level of education you have completed?
 Some secondary school or less
 GCSEs/ O-levels
 A/AS-levels
 Other higher education
 University degree
 Professional qualification or post-graduate study
- G.** Which of the following best describes your current work or school status?
 Attending school outside the home
 Taking educational courses at home
 Seeking work
 Working full or part time (either outside the home or at a home-based business)
 Full time homemaker
 Not attending school or working due to my health
 Not working for other reasons

Section II. Quality of Life

Please tick the box indicating your answer.

<i>During the past two weeks, to what extent have you had difficulty:</i>	A lot of difficulty	Some difficulty	A little difficulty	No difficulty
1. Performing vigorous activities such as running or playing sports.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
2. Walking as fast as others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
3. Carrying or lifting heavy things such as books, shopping, or school bags.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
4. Climbing one flight of stairs.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
5. Climbing stairs as fast as others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

<i>During the past two weeks, indicate how often:</i>	Always	Often	Sometime s	Never
6. You felt well.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
7. You felt worried	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
8. You felt useless.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
9. You felt tired	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
10. You felt full of energy	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
11. You felt exhausted.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
12. You felt sad	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Please circle the number indicating your answer. Please choose only one answer for each question.

Thinking about the state of your health over the last two weeks:

13. To what extent do you have difficulty walking?
 1. You can walk a long time without getting tired
 2. You can walk a long time but you get tired
 3. You cannot walk a long time because you get tired quickly
 4. You avoid walking whenever possible because it's too tiring for you

14. How do you feel about eating?
 1. Just thinking about food makes you feel sick
 2. You never enjoy eating
 3. You are sometimes able to enjoy eating
 4. You are always able to enjoy eating

15. To what extent do your treatments make your daily life more difficult?
 1. Not at all
 2. A little
 3. Moderately
 4. A lot



Adolescents and Adults (Patients 14 Years Old and Older)

CYSTIC FIBROSIS QUESTIONNAIRE - REVISED

- 16. How much time do you currently spend each day on your treatments?
 - 1. A lot
 - 2. Some
 - 3. A little
 - 4. Not very much
- 17. How difficult is it for you to do your treatments (including medications) each day?
 - 1. Not at all
 - 2. A little
 - 3. Moderately
 - 4. Very
- 18. How do you think your health is now?
 - 1. Excellent
 - 2. Good
 - 3. Fair
 - 4. Poor

Please select a box indicating your answer.

Thinking about your health during the past two weeks, indicate the extent to which each sentence is true or false for you.

	Very true	Somewhat true	Somewhat false	Very false
19. I have trouble recovering after physical effort	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
20. I have to limit vigorous activities such as running or playing sports.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
21. I have to force myself to eat	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
22. I have to stay at home more than I want to.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
23. I feel comfortable discussing my illness with others.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
24. I think I am too thin.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
25. I think I look different from others my age	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
26. I feel bad about my physical appearance.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
27. People are afraid that I may be contagious.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
28. I get together with my friends a lot	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
29. I think my coughing bothers others.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
30. I feel comfortable going out at night	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
31. I often feel lonely	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
32. I feel healthy.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
33. It is difficult to make plans for the future (for example, going to college, getting married, getting promoted at work, etc.).....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
34. I lead a normal life	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>



Section III. School, Work, or Daily Activities

Questions 35 to 38 are about school, work, or other daily tasks.

35. To what extent did you have trouble keeping up with your schoolwork, professional work, or other daily activities during the past two weeks?

1. You have had no trouble keeping up
2. You have managed to keep up but it's been difficult
3. You have been behind
4. You have not been able to do these activities at all

36. How often were you absent from school, work, or unable to complete daily activities during the last two weeks because of your illness or treatments?

- Always Often Sometimes Never

37. How often does CF get in the way of meeting your school, work, or personal goals?

- Always Often Sometimes Never

38. How often does CF interfere with getting out of the house to run errands such as shopping or going to the bank?

- Always Often Sometimes Never

Section IV. Symptom Difficulties

Please select a box indicating your answer.

Indicate how you have been feeling during the past two weeks.

	A great deal	Somewhat	A little	Not at all
39. Have you had trouble gaining weight?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
40. Have you been congested?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
41. Have you been coughing during the day?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
42. Have you had to cough up mucus?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

**Go to
Question
44**

43. Has your mucus been mostly: Clear Clear to yellow Yellowish-green Green with traces of blood Don't know

How often during the past two weeks:

	Always	Often	Sometimes	Never
44. Have you been wheezing?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
45. Have you had trouble breathing?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
46. Have you woken up during the night because you were coughing?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
47. Have you had problems with wind?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
48. Have you had diarrhoea?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
49. Have you had abdominal pain?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
50. Have you had eating problems?.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Please make sure you have answered all the questions.

THANK YOU FOR YOUR COOPERATION!



Understanding the impact of your child’s illness and treatments on his or her everyday life can help your healthcare team keep track of your child’s health and adjust his or her treatments. For this reason, we have developed a quality of life questionnaire specifically for parents of children with cystic fibrosis. We thank you for your willingness to complete this questionnaire.

Instructions: The following questions are about the current state of your child’s health, as he or she perceives it. This information will allow us to better understand how he or she feels in everyday life.

Please answer all the questions. There are **no** right or wrong answers! If you are not sure how to answer, choose the response that seems closest to your child’s situation.

Section I. Demographics

Please fill-in the information or tick the box indicating your answer.

A. What is your child’s date of birth?

Date

--	--	--	--	--	--

Day Month Year

E. What is **your** date of birth?

Date

--	--	--	--	--	--

Day Month Year

B. What is your relationship to the child?

- Mother
- Father
- Grandmother
- Grandfather
- Other relative
- Foster mother
- Foster father
- Other (please describe)

F. What is your current marital status?

- Single/never married
- Married
- Widowed
- Divorced
- Separated
- Remarried
- With a partner

C. Which of the following best describes your child's racial background?

- White - UK
- White - other
- Indian/ Pakistani
- Chinese/ Asian
- African
- Caribbean
- Other [not represented above or people whose predominant origin cannot be determined/ mixed race]
- Prefer not to answer this question

G. What is the highest level of education **you** have completed?

- Some secondary school or less
- GCSEs/O-levels
- A/AS-levels
- Other higher education
- University degree
- Professional qualification or post-graduate study

D. During the **past two weeks**, has your child been on holiday or out of school for reasons **NOT** related to his or her health?

- Yes No

H. Which of the following best describes your current work status?

- Seeking work
- Working full or part time (either outside the home or at a home-based business)
- Full time homemaker
- Not working due to my health
- Not working for other reasons

Section II. Quality of Life

Please indicate how your child has been feeling during the past two weeks by ticking the box matching your response.

<i>To what extent has your child had difficulty:</i>	A lot of difficulty	Some difficulty	A little difficulty	No difficulty
1. Performing vigorous activities such as running or playing sports.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
2. Walking as fast as others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
3. Climbing stairs as fast as others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
4. Carrying or lifting heavy objects such as books, a school bag, or rucksack	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
5. Climbing several flights of stairs.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Please tick the box matching your response.

<i>During the past two weeks, indicate how often your child:</i>	Always	Often	Sometimes	Never
6. Seemed happy	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
7. Seemed worried.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
8. Seemed tired.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
9. Seemed short-tempered	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
10. Seemed well	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
11. Seemed grouchy	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
12. Seemed full of energy	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
13. Was absent or late for school or other activities because of his/her illness or treatments	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Please circle the number indicating your answer. Please choose only one answer for each question.

Thinking about the state of your child's health over the past two weeks, indicate:

- 14. The extent to which your child participated in sports and other physical activities, such as P.E. (physical education)
 - 1. Has not participated in physical activities
 - 2. Has participated less than usual in sports
 - 3. Has participated as much as usual but with some difficulty
 - 4. Has been able to participate in physical activities without any difficulty

- 15. The extent to which your child has difficulty walking
 - 1. He or she can walk a long time without getting tired
 - 2. He or she can walk a long time but gets tired
 - 3. He or she cannot walk a long time, because he or she gets tired quickly
 - 4. He or she avoids walking whenever possible, because it's too tiring for him or her



Please tick the box that matches your response to these questions.

*Thinking about your child's state of health during the past **two weeks**, indicate the extent to which each sentence is true or false for your child:*

	Very true	Somewhat true	Somewhat false	Very false
16. My child has trouble recovering after physical effort	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
17. Mealtimes are a struggle	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
18. My child's treatments get in the way of his/her activities.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
19. My child feels small compared to other kids the same age.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
20. My child feels physically different from other kids the same age.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
21. My child thinks that he/she is too thin	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
22. My child feels healthy.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
23. My child tends to be withdrawn.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
24. My child leads a normal life.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
25. My child has less fun than usual	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
26. My child has trouble getting along with others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
27. My child has trouble concentrating	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
28. My child is able to keep up with his/her school work or holiday activities	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
29. My child is not doing as well as usual in school or holiday activities.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
30. My child spends a lot of time on his/her treatments everyday	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Please circle the number indicating your answer. Please choose only one answer for each question.

31. How difficult is it for your child to do his/her treatments (including medications) each day?

1. Not at all
2. A little
3. Moderately
4. Very

32. How do you think your child's health is now?

1. Excellent
2. Good
3. Fair
4. Poor



Section III. Symptom Difficulties

The next set of questions is designed to determine the frequency with which your child has certain respiratory difficulties, such as coughing or shortness of breath.

Please indicate how your child has been feeling during the past two weeks.

	A great deal	Somewhat	A little	Not at all
33. My child had trouble gaining weight	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
34. My child was congested	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
35. My child coughed during the day	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
36. My child had to cough up mucus	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
				<input type="checkbox"/> ↓ Go to Question 38
37. My child's mucus has been mostly:	<input type="checkbox"/> Clear <input type="checkbox"/> Clear to yellow <input type="checkbox"/> Yellowish-green <input type="checkbox"/> Green with traces of blood <input type="checkbox"/> Don't know			

During the past two weeks:

	Always	Often	Sometimes	Never
38. My child wheezed	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
39. My child had trouble breathing	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
40. My child woke up during the night because he/she was coughing.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
41. My child had wind	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
42. My child had diarrhoea	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
43. My child had abdominal pain.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
44. My child has had eating problems.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Please make sure you have answered all the questions.

THANK YOU FOR YOUR COOPERATION!



These questions are for children like you who have cystic fibrosis. Your answers will help us understand what this disease is like and how your treatments help you. So, answering these questions will help you and others like you in the future.

Please answer all the questions. There are **no** right or wrong answers! If you are not sure how to answer, choose the response that seems closest to your situation.

Please fill in the answer or tick the box that matches your response to these questions.

A. What is your date of birth?

Date

--	--	--	--	--	--	--	--

Day Month Year

B. Are you?

- Male Female

C. During the **past two weeks**, have you been on holiday or out of school for reasons **NOT** related to your health?

- Yes No

D. Which of the following best describes your racial background?

- White - UK
 White - other
 Indian/ Pakistani
 Chinese/ Asian
 African
 Caribbean
 Other [not represented above or people whose predominant origin cannot be determined/ mixed race]
 Prefer not to answer this question

E. What year are you in now at school?

(If summer, year you just finished)

- Year 6
 Year 7
 Year 8
 Year 9
 Year 10
 Year 11
 Not in school

Please tick the box matching your response.

In the past two weeks :	Very True	Mostly True	Somewhat True	Not at all True
1. You were able to walk as fast as others.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
2. You were able to climb stairs as fast as others.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
3. You were able to run, jump, and climb as you wanted	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
4. You were able to run as quickly and for as long as others	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
5. You were able to participate in sports that you enjoy (e.g., swimming, football, dancing or others).....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
6. You had difficulty carrying or lifting heavy things such as books, your school bag, or a rucksack.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Please tick the box matching your response.

And during these past two weeks , indicate how often:	Always	Often	Sometimes	Never
7. You felt tired	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
8. You felt mad.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
9. You felt grouchy	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
10. You felt worried	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
11. You felt sad	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
12. You had trouble falling asleep	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
13. You had bad dreams or nightmares.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
14. You felt good about yourself.....	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
15. You had trouble eating	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Please tick the box matching your response.

And during these past two weeks , indicate how often:	Always	Often	Sometimes	Never
	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>



16. You had to stop fun activities to do your treatments.....

17. You were forced to eat.....

Please tick the box matching your response.

During the past **two weeks**:

	Very True	Mostly True	Somewhat True	Not at all True
--	----------------------	------------------------	--------------------------	----------------------------

18. You were able to do all of your treatments

19. You enjoyed eating

20. You got together with friends a lot.....

21. You stayed at home more than you wanted to

22. You felt comfortable sleeping away from home (at a friend or family member's house or elsewhere).....

23. You felt left out.....

24. You often invited friends to your house.....

25. You were teased by other children.....

26. You felt comfortable discussing your illness with others (friends, teachers)

27. You thought you were too short.....

28. You thought you were too thin.....

29. You thought you were physically different from others your age.....

30. Doing your treatments bothered you.....

Please tick the box matching your response.

	Always	Often	Sometimes	Never
Let us know how often in the past two weeks :				
31. You coughed during the day	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
32. You woke up during the night because you were coughing	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
33. You had to cough up mucus	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
34. You had trouble breathing	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
35. Your stomach hurt	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Please make sure all the questions have been answered.

THANK YOU FOR YOUR COOPERATION!

APPENDIX FOUR

CARDIOPULMONARY EXERCISE TESTING (CPET) PROTOCOL

Cardiopulmonary Exercise Testing (CPET) Summary of Protocol

Equipment

Cycle ergometer	Stadiometer
MedGraphics metabolic cart	Digital scales
SpO ₂ ear probe/temporal probe	Breath by breath gas analyser
Pneumotachograph	Sphygmomanometer
Stethoscope	

General Instructions

- Subjects asked to refrain from eating at least two hours before test and avoid strenuous exercise for at least 24 hours before CPET.
- Subjects asked to wear comfortable clothing and running shoes suitable for exercise.
- It should be made clear to subjects that sensations such as shortness of breath, tiredness and muscle fatigue are 'normal'.
- Subjects should be informed that the CPET will be terminated at any such time that they choose not to continue.
- However, it should be emphasised that data collected throughout the test will be of 'most' use if the participant works to near maximal capacity by the end of the CPET.
- The CPET should be terminated at the point of voluntary exhaustion, when the participant is unable to continue despite strong verbal encouragement.
- Experienced technicians should monitor the subject for any undue signs of distress including severe wheezing, chest pain, lack of coordination, and marked decrease in SpO₂ throughout the CPET.

Protocol

- Subjects currently taking bronchodilators should be administered usual bronchodilator i.e. 200 µg Salbutamol or 500 µg Terbutaline at least 10 minutes prior to CPET.
[NB: Cold air may precipitate bronchospasm (Consideration at altitude)].
- Spirometry and direct measurement of Maximal Voluntary Ventilation (MVV), i.e. 12 seconds of maximally deep and rapid breathing should be made prior to CPET
- Predicted peak workload (Watts) calculated as 3 x bodyweight (in kg) for girls, and 3.5 x bodyweight (in kg) for boys. This may need to be adjusted for subject's fitness level, and if any reduction in predicted lung function.
- The target cadence should be approximately 60 - 80 rpm.

- Ergometer ramp protocol adjusted to reach the predicted peak Watts within 8 - 12 minutes of exercise.

Example for a 50 kg girl. $3 \times 50 = 150$ Watts.

$150/10 = 15$ Watts/minute for an estimated 10-minute exercise test.

Therefore, a 15 W workload could be selected, or otherwise the closest appropriate option offered by the software.

NB: The predicted workload should be lowered if a patient's diagnosis suggests impaired aerobic fitness.

The exercise test should include:

- 5 minutes at rest (while connected to the 12-lead ECG and breathing through the PNT or mass flow sensor)
- 3 minutes of load-less cycling
- 8 – 12 minutes of exercise, or until volitional exhaustion
- 4 minutes active recovery (slow pedalling)

Blood pressure measurements should be made every 1-2 minutes at rest, throughout the test and during active recovery.

Analysis

The fulfilment of at least three of the following four criteria should be required to meet the definition of a 'maximal' exercise test: 1) a maximal heart rate similar to the theoretically predicted maximal value i.e. $>85\%$ of the participant's predicted maximal heart rate, where predicted maximal heart rate is calculated as $210 - (0.65 \times \text{age})$ (Spiro 1977); 2) a peak V_E close to the predicted target; 3) a plateau in oxygen uptake despite an increasing workload (i.e. final increase in $VO_2 < 200 \text{ mls.min}^{-1}$ for an increase in work of 5 to 10%); 4) an inability of the participant to maintain cadence despite encouragement (Crapo *et al.*, 2000, Wasserman *et al.*, 2004).

Key measurements

Heart Rate	Blood Pressure
Respiratory Rate	Tidal volumes
Oxygen utilisation (VO_2)	Carbon dioxide elimination (VCO_2)
End-tidal CO_2 ($etCO_2$)	Oxygen saturations (SpO_2)
Work Rate (Watts)	

Outcomes

$VO_{2\text{max}}/ VO_{2\text{peak}}$	Anaerobic thresh-hold
Exercise desaturation	Maximal exercise ventilation $V_{E\text{max}}$

APPENDIX FIVE

HARDY-WEINBERG CALCULATION FOR ACE POLYMORPHISM

The ACE polymorphism – Hardy Weinberg equilibrium calculation

I allele frequency= 0.5625

D allele frequency = 0.4375

p=0.5625, q=0.4375

	II	ID	DD	Total
Observed	13	19	8	40
Expected	p^2	$2pq$	q^2	
	0.31	0.49	0.19	1
Number expected	p^2N	$2pqN$	q^2N	
	12.6562	19.6875	7.6562	
Deviation	0.3438	0.6875	0.3438	
Chi-Squared [Deviation squared divided by number expected]	0.009	0.024	0.0154	0.0484

$$\chi^2 = 0.0484$$

With 1 degree of freedom, $p \gg 0.50$.

APPENDIX SIX

PUBLICATIONS ARISING FROM THIS THESIS

Appendix 6

Publications arising from this thesis

- Review articles (3)
- Book Chapters (1)
- Abstracts (8)

Review articles:

1. **Urquhart DS**, Narang I, Jaffé A.
Assessment and interpretation of arterial oxygen saturations in children with CF.
CF Worldwide 2008; **10**: Published online January 2008
2. Prasad SA, **Urquhart DS**.
Exercise and Cystic Fibrosis.
HealthEx Specialist 2006; **10**: 9-14.
3. **Urquhart DS**, Montgomery H, Jaffé A.
Assessment of hypoxia in childhood cystic fibrosis.
Arch Dis Child 2005; **90**: 1138-1143.

Book Chapters:

1. **Urquhart DS**, Montgomery H, Jaffé A.
Assessment, definition and treatment of hypoxia in children with cystic fibrosis.
In: 2008 Yearbook of respiratory care clinics and applied technologies. Eds: Esquinas A, Volsko T.
Tipografia San Francisco S.A., Murcia, Spain. ISBN: 978-84-612-5901-4. ps. 769-774.

Abstracts:

1. **Urquhart DS**, Lavery A, Stanojevic S, Odendaal D, Derrick G, Scrase E, Bryon M, Montgomery H, Narang I, Jaffé A. Association of nocturnal hypoxia and clinical status in childhood cystic fibrosis.
Thorax 2009; **64 (Supplement 4)**: A114.
2. **Urquhart DS**, Stanojevic S, Lavery A, Montgomery H, Narang I, Jaffé A.
Selection of an optimal definition of clinically-significant hypoxia in children with cystic fibrosis.
Pediatr Pulmonol 2009; **44 (S10)**: 348-349.
3. **Urquhart DS**, Lavery A, Stanojevic S, Odendaal D, Derrick G, Scrase E, Bryon M, Montgomery H, Narang I, Jaffé A.
Association of nocturnal hypoxia and adverse clinical status in children with cystic fibrosis – cause or effect? *Pediatr Pulmonol* 2009; **44 (S10)**: 349.
4. **Urquhart DS**, Field B, Bryon M, Jaffé A.
Effects of lung function and exercise capacity on quality of life in CF using the United Kingdom Cystic Fibrosis Questionnaire [CFQ-UK]. *Thorax* 2007; **62 (Supplement 3)**: A103-A104.
5. **Urquhart DS**, Scrase E, Narang I, Montgomery H, Jaffé A.
Respiratory muscle pressure measurement in children with cystic fibrosis. *Thorax* 2006; **61**: ii97.
6. **Urquhart DS**, Odendaal D, Lavery A, Scrase E, Narang I, Derrick G, Montgomery H, Jaffé A.
Predicting nocturnal hypoxia in children with CF. *Pediatr Pulmonol* 2006; **41(S29)**: 346-347.
7. Selvadurai H, Montgomery H, **Urquhart D**, Wilkes D, Schneiderman-Walker J, van Asperen P, Jaffé A. Angiotensin converting enzyme gene polymorphism and exercise capacity in children with cystic fibrosis. *Pediatr Pulmonol* 2006; **41(S29)**: 352.
8. **Urquhart DS**, Odendaal D, Narang I, Derrick G, Montgomery H, Jaffé A.
Exercise prescription in CF – A therapeutic modality for all? *Pediatr Pulmonol* 2006; **41(S29)**: 354.