
ASSESSMENT OF LUNG ALVEOLAR DEVELOPMENT IN CHILDHOOD AND ADOLESCENCE USING 3-HELIUM MAGNETIC RESONANCE

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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June 2014

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ABSTRACT:

The major functional units of the lung called alveoli, are located in the periphery of the lung. Despite their functional importance, it has been difficult to evaluate their structure and development. Until the advent of 3-Helium magnetic resonance ($^3\text{HeMR}$), it was not possible to directly assess peripheral lung structure in a living individual.

When this study commenced, the prevailing hypothesis was that human alveolarization was complete by 3 years. It was believed that preterm birth would lead to persisting alveolar damage. The role of other factors affecting human lung development were not clearly understood.

In this work, I describe the use of $^3\text{HeMR}$ to : 1. Examine the current hypothesis regarding normal alveolar development, 2. Determine whether birth at very preterm gestation leads to long-term alveolar damage and 3. Evaluate factors affecting human alveolar development.

First, we determined alveolar size using $^3\text{HeMR}$ in healthy subjects aged 7 to 21 years. Alveolar dimensions did not increase by the expected rate over this age range, despite lung capacity increasing nearly fourfold. The only plausible explanation is new alveoli forming throughout the period of lung growth.

Then, we compared alveolar size between children born very prematurely (<32 weeks gestation), including survivors of neonatal chronic lung disease (CLD) with term born children and children born mildly preterm (33-36 weeks gestation). Alveolar dimensions were nearly identical suggesting alveolar catch up growth in the very preterm groups.

In the third part of the study, we investigated the relationship between various risk factors and alveolar dimensions. Exposure to environmental tobacco smoke

(ETS) was found to be consistently associated with larger alveoli, suggesting its detrimental effect on alveolar development.

Our results imply that developing lungs have the potential to recover from early life insults. Conversely, the window for adverse environmental exposures to affect alveolar development may be wider than previously believed.

ACKNOWLEDGEMENTS:

Though one name appears in the title page, I humbly acknowledge the fact that this work would not have been possible without the combined efforts of a number of people, to whom I am indebted. The grant that supported this research was funded by the Wellcome trust, without which this work may not have been possible.

I wish to thank my supervisors Professor Michael Silverman and Dr. Caroline Beardsmore, for their unwavering support and expert guidance during the course of this research. They have been constantly approachable and accessible. I acknowledge their role in not just this research, but in my overall personal development, for which I am extremely grateful.

I have been lucky to have a very good team of research technicians who have helped me throughout the project. The efforts of Ms. Sian Williams, Ms. Jenny Phillips and Ms. Ketna Thakrar were instrumental in the smooth running of this research. I also should acknowledge Ms. Teresa McNally, our research nurse, who helped in innumerable ways throughout this project.

I am especially indebted to the team from the University of Nottingham, headed by Professor John Owers-Bradley, who were instrumental in setting up the technique of $^3\text{Helium}$ magnetic resonance and making it available to us. This research would not have been possible without their involvement. I would like to thank Dr. Marius Mada and Dr. Iain Ball for their support and expertise during the scans. I wish to thank Dr. Kuldeep Panesar for patiently explaining the MR sequences and simulations and Dr. Ruslan Garipov for teaching me the basics of MATLAB.

I am grateful to the team from the University of Berne, headed by Professor Claudia Kuehni, for sharing their expertise in epidemiology and statistics. The meticulous proof reads of the journal articles submitted on the basis of this work helped tremendously in getting the work published. I would like to thank Dr. Ben Spycher and Dr. Cris Dogaru, who cleaned the data and did a major part of the statistical work in this thesis. I also wish to thank Ms. Marie-Pierre Strippoli, who helped to format the questionnaires and the epidata data entry forms in the early stage of this study.

I wish to thank the other researchers in the department, Dr Erol Gaillard, Dr. Hitesh Pandya and Professor Chris O'Callaghan for their tips and advice.

Last, but in no way the least, I would like to place in record the sacrifices made by my family during the period of research and the ensuing period when I was writing up. First of all thanks to my wife, Dr. Rekha Pillai for being a constant source of support and pushing me forward to finish the write-up in my spare time, while she managed the home. I am indebted to my son, Aaditya Manjith, who sacrificed his 'daddy-time' on many occasions, and to my infant daughter, Aaradhya Manjith, who has missed many a cuddle from her father. I would like to thank my parents, for being the most supportive parents in the world and helping me become what I am now. I would also like to thank my parents-in-law, who stayed with us during a large part of the write-up period, and being a source of support for me and my family.

STATEMENT OF AUTHOR'S CONTRIBUTION TO THIS THESIS:

This work was made possible by the efforts of a team of people who have been acknowledged in page 4 of this work. I would like to place in record my personal contributions to this work .

I was involved in planning the individual aspects of the study, including selection and recruitment of the subjects, and design and validation of the questionnaires. I also planned and developed the standard operating procedures for the physiological measurements, primarily spirometry and whole-body plethysmography.

I supervised the research technicians who were recruited to help with this study. I was directly involved in practical measurements of lung function in the volunteers and managed data entry along with the research technicians. I was involved in developing the standard operating procedures for the $^3\text{HeMR}$ measurements in children along with colleagues from University of Nottingham. I was involved in data cleaning together with colleagues with epidemiological and statistical expertise from the University of Berne.

I planned the data analysis along with colleagues from the University of Berne and performed the preliminary analysis of data. I co-ordinated the collaborative efforts of research personnel from three different centres. I took the main role in interpretation of results. I presented the data in several national and international meetings, and was the lead author in the papers that have been published as a result of this work.

Manjith Narayanan.

TABLE OF CONTENTS

1. INTRODUCTION.....	12
1.1. Importance of peripheral lung structure	13
1.2. Measurement of lung periphery.....	14
1.3. Current anatomical concepts.....	23
1.4. Normal development of periphery of human lung.....	34
1.5. Factors affecting development of lung periphery.....	45
1.6. Unresolved questions, controversies and ways to solve them.....	55
1.7. introduction to helium magnetic resonance	56
2. Helium magnetic resonance	58
2.1. Principle of magnetic resonance.....	59
2.2. Hyperpolarised gas magnetic resonance	62
2.3. Validation of ADC as a measure of physical dimensions.....	71
2.4. Investigating alveolar development with ³ HeMR.....	74
2.5. Aims and objectives.....	74
3. Methods	75
3.1. Subjects.....	76
3.2. Visit of the subject.....	80
3.3. Assessment of risk factors.....	85
3.4. Helium Magnetic Resonance technique	88
3.5. Data entry and statistical analysis.....	95
4. Normal alveolar development in children.....	97
4.1. Background.....	98
4.2. Methods.....	99
4.3. Analysis and results	101
4.4. Interpretation	115
5. Influence of preterm birth on alveolar development	116
5.1. Background.....	117

5.2.	Methods.....	118
5.3.	Results.....	123
5.4.	Interpretation	139
6.	Other Factors affecting alveolar development	140
6.1.	Background.....	141
6.2.	Analysis	142
6.3.	results.....	145
6.4.	Summary and interpretation.....	154
7.	discussion.....	156
7.1.	Summary.....	157
7.2.	Validity of ³ HeMR as a surrogate for alveolar dimensions.....	157
7.3.	Lung growth by Continuous alveolarization.....	160
7.4.	Neoalveolarization in preterm survivors.....	165
7.5.	Factors affecting alveolar development.....	172
7.6.	Conclusions and summary	179
8.	Implications, controversies, future directions.....	181
8.1.	Implications	182
8.2.	controversies	183
8.3.	Future research directions	184
	Appendix 1 - Questionnaires.....	184
	Appendix 2 - Ethics Approval.....	195
	Appendix 3 - List of Publications.....	198
	References	201

LIST OF FIGURES

Figure 1-1.....	36
Figure 1-2.....	37
Figure 1-3.....	39
Figure 1-4.....	45
Figure 2-1.....	59
Figure 2-2.....	60
Figure 2-3.....	63
Figure 2-4.....	64
Figure 2-5.....	65
Figure 2-6.....	65
Figure 2-7.....	66
Figure 2-8.....	67
Figure 2-9.....	68
Figure 2-10.....	70
Figure 3-1.....	79
Figure 3-2.....	81
Figure 3-3.....	84
Figure 3-4.....	89
Figure 3-5.....	91
Figure 3-6.....	92
Figure 3-7.....	94
Figure 4-1.....	106
Figure 4-2.....	107
Figure 4-3.....	110
Figure 4-4.....	111
Figure 4-5.....	112
Figure 4-6.....	114
Figure 5-1.....	128
Figure 5-2.....	132
Figure 5-3.....	132
Figure 5-4.....	138
Figure 5-5.....	138
Figure 6-1.....	146
Figure 6-2.....	147

LIST OF TABLES

Table 1-1	16
Table 1-2	25
Table 1-3	30
Table 1-4	41
Table 3-1	78
Table 3-2	85
Table 3-3	93
Table 4-1	101
Table 4-2	104
Table 4-3	105
Table 4-4	111
Table 4-5	113
Table 4-6	115
Table 5-1	123
Table 5-2	124
Table 5-3	125
Table 5-4	126
Table 5-5	127
Table 5-6	129
Table 5-7	129
Table 5-8	131
Table 5-9	133
Table 5-10.....	134
Table 5-11.....	135
Table 5-12.....	137
Table 6-1	146
Table 6-2	148
Table 6-3	149
Table 6-4	150
Table 6-5	151
Table 6-6	152
Table 6-7	153
Table 6-8	154

LIST OF EQUATIONS

Equation 1-1	20
Equation 1-2	20
Equation 2-1	60
Equation 2-2	61
Equation 2-3	61
Equation 2-4	61
Equation 2-5	66
Equation 2-6	67
Equation 2-7	68
Equation 2-8	69
Equation 2-9	69
Equation 2-10	69
Equation 3-1	90
Equation 3-2	92
Equation 4-1	102
Equation 4-2	102
Equation 4-3	102
Equation 4-4	102
Equation 4-5	103
Equation 4-6	103
Equation 4-7	103
Equation 4-8	108
Equation 4-9	109
Equation 4-10	109
Equation 8-1	183

1. INTRODUCTION

1.1.IMPORTANCE OF PERIPHERAL LUNG STRUCTURE

The major function of the human lung is gas exchange (acquisition of oxygen from atmosphere and diffusion of carbon dioxide back to the atmosphere). Gas exchange takes place in the functional units of the lung called alveoli which are located in the peripheral zone of the lung.

Anatomically, the lung consists of a system of branching tubes (airways) beginning at the trachea and ending in alveoli (1). There may be up to 28 generations of airways from the trachea to the alveoli, though the exact number is variable (2). The first few generations of airways have a purely conductive role. Airways distal to the 16th to 18th generation begin to carry alveoli on their wall and start taking part in gas exchange. The first generation of airways where alveoli are present in the walls are called first order respiratory bronchiole and the last generation of airways where the walls are devoid of alveoli are called terminal bronchioles (1). Therefore, lung can be conveniently divided into the proximal zone and the peripheral zone at this junction.

The proximal zone, therefore, consists of airways branching from the trachea to the terminal bronchiole. The function of this part of the lung is mainly distribution of air to and from the peripheral zone. The peripheral zone consists of the airways and airspaces branching out from first order respiratory bronchiole, which are characterised by presence of alveoli on the walls (1). It is important to remember, however, that the peripheral zone is not a contiguous zone but consists of branching trees of airways fed by a terminal bronchiole. These individual pockets are called lung acini.

By definition, all airways and airspaces in the peripheral zone take part to some extent in gas exchange. This underlines the vital importance of the peripheral zone of the lungs. Understanding the structure, function, development and adverse factors affecting the lung periphery is vital. The ability to study the lung periphery is an important pre-requisite to understand normal lung function, growth and development of the lung, pathogenesis and effect of diseases of the lung (3).

In clinical practice, most of the standard lung function tests assess the proximal conductive zone of the lungs. Assessment of lung structure in a living individual (using techniques such as high resolution computed tomography) is possible only up to a resolution of a millimetre. However, most structures in the peripheral zone are smaller than this. Therefore, structure and function of the peripheral zone of the lungs has been difficult to evaluate. Indeed a bulk of knowledge about structure has come from histological studies of autopsy specimens. Therefore, the lung periphery has been termed the 'quiet zone' of the lung (4).

There is evidence that the peripheral zone of the lung plays a major role in many of the common diseases of the lung such as chronic obstructive pulmonary disease (COPD) (5), asthma (6) and cystic fibrosis. Furthermore, paediatricians have been interested in the development of the periphery of the lung because developmental disorders such as chronic lung disease of prematurity (CLD) and congenital diaphragmatic hernia (CDH) can affect its structure. There is new evidence suggesting that COPD may be a disease originating in infancy and childhood with multiple developmental factors influencing the long term outcome (7).

Because determining structure and function of the peripheral zone of the lung is difficult in life, it has been almost impossible to directly determine influences on development of this zone in humans. The current knowledge is based on animal models, autopsy specimens and extrapolation from clinical surrogate markers like oxygen requirement. However, it is important to determine the influences on peripheral lung development to

- determine the normal pattern of lung development and predict influences of various factors on normal lung development.
- delineate the long term consequences of developmental disorders of the lung
- develop appropriate therapeutic strategies to mitigate the influence of adverse factors on lung development.
- establish the outcome of various therapeutic interventions on these developmental disorders.

The last decade has seen the development of two new technologies that offer the ability to assess the structure and function of the periphery of the lung in-vivo. They include 3-Helium magnetic resonance and multibreath washout. This chapter focuses current knowledge regarding the structure and development of the lung periphery and its measurement by conventional methods.

1.2.MEASUREMENT OF LUNG PERIPHERY

The structure of the lung periphery was subject of numerous studies in the later part of the last century. The studies focussed on various aspects of the lung periphery including the size of various structures (eg. alveoli, alveolar ducts, alveolar sacs, respiratory bronchioles) and the pattern of branching within the lung acinus.

1.2.1. TECHNIQUES FOR MEASURING SIZE

The size of components of the acinar unit was determined by two major histological techniques. The first technique involves the inflation of lung and creating a lung cast using a suitable moulding agent (e.g. latex, silastic). The 3-dimensional structure of the peripheral airways and acini is better delineated using this technique. The second technique involves the technique of morphometry – using 2D sections of human lungs (appropriately inflated and fixed) to determine the 3-D characteristics such as volume and shape of the components.

1.2.1.1. CASTING TECHNIQUES

There are 2 main techniques of casting which are considered together. In the first technique, the lung is inflated with appropriate pressure with a material that forms casts of the airways and the alveoli, the organic material is digested with strong acid or alkali and the airway sizes are measured using a microscope. The volume of a component is determined by weighing the cast and dividing by specific weight of casting material. As examples, Haefeli-Bleuer (8) used silicone casts, Schreider (9) used a silastic mould making rubber and Pump (10) employed vultex moulage. Haefeli-Bleuer (8) visualised the casts using both a stereomicroscope and scanning electron microscope on these casts, while Pump (10) employed a Leitz stereomicroscope and Schreider (9) a dissecting microscope.

In the second technique, the lungs are fixed with formalin at an appropriate pressure, embedded and sectioned to thin slices. The slices are stained and projected (with appropriate magnification). These projections are used to reconstruct a 3-D model of the airways - either manually (e.g. Hansen (11)) or by using computers (e.g. Berend (12)). Hansen et al (11) traced these projections on polyurethane foam, cut them out and removed areas equivalent to lumen. These cutouts were placed serially to form a 3D model. Measurements were made and using the magnification factor, the actual size of the structures in the acini were estimated. Berend (12) photographed the sections and projected to a touch sensitive screen. The sections were traced manually on the screen and information stored digitally. Airways were reconstructed by a computer program.

A summary of the techniques involved in casting is mentioned in table 1-1.

TABLE 1-1

Examination of lung periphery by casting techniques

Author, year	Pump 1969 (10)	Hansen 1975 (1)	Schreider 1981 (9)	Haefeli-Bleuer 1988 (8)	Ciurea 1989 (13)	Berend 1991 (12)
Step 1: Casting material, technique	Latex injected to bronchial tree at 50 mm Hg	Formalin fixed at 25 cm, paraffin embedded.	Cast before fixation in situ. Type E silastic mold making rubber with catalyst injected at pressures needed to expand lung in thorax	Formalin fixed. Saline instilled at 20-25 cm H ₂ O. Silicone rubber instilled at 0.5-1.1 Kp/cm ² and polymerised in situ. Lung tissue digested with KOH.	Formalin fixed, paraffin embedded	Ethanol dried, formalin infiltrated and embedded in a polythene mould.
Step 2: Visualisation	Casts visualised under a stereoscopic microscope	Sections projected and polyurethane foam reconstructions made	Cast examined under dissecting microscope. No formal randomisation	Casts dissected out under stereo microscope. 6 of 209 dissected acini sampled by systematic random sampling	Tracings of images analysed by software	Sections photographed and 3D reconstructed using computer

Fixation pressure	50 mm Hg	Formalin at 25 cm H ₂ O pressure	In situ expansion as required to fill the thorax	Silicone rubber instilled at 0.5-1.1 Kp/cm ²	Formalin at 30 cm H ₂ O pressure	Formalin at 25 cm H ₂ O pressure
Nature of lung specimen	2 lungs(a 26y woman and a 70y man)	19y woman died of non respiratory causes	2 men(50y and 60y old) died of non respiratory causes	2 lungs from cadavers. No further description	2 70y men otherwise healthy, died of non-respiratory causes.	18y man died of non-respiratory causes

1.2.1.2. MORPHOMETRY

Morphometry is the technique of extrapolating the number and size of components of a 3 dimensional structure by counting the number of transections and the fractional area of these components in a random section of this structure. It is based on Delesse theorem (14), which states the fraction ρ_V of a unit volume occupied by a given granular component, which is randomly distributed, is equal to the fraction ρ_S of the unit area of a cut surface through this volume.

Weibel was considered to be the father of morphometry. In his seminal work 'morphometry of the human lung' (2), he describes the principles of morphometry and the mathematical method of deriving the number and volume of lung structures from data from cross sections using the Delesse theorem. Weibel used Rosiwal's extension of Delesse theorem (14) to state that the fraction of a line passing through a randomly distributed tissue component is approximately equal to the fraction of volume occupied by this component.

The fractional volumes of the structures in the acinar unit (e.g. alveoli and alveolar ducts) can therefore be determined by estimating the proportions of random lines that lie within alveoli and alveolar ducts. Weibel estimated that about 55-66% of the lung parenchyma is made up of alveoli, 28-37% by alveolar ducts, 6-7% by other tissue and about 1% by vessels. By estimating the volume of the lung section studied (by volume displacement), and after determining the number of alveoli in the human lung (see 1.2.3.2), he was able to give absolute values to the volumes of the acinar components.

1.2.1.3. NEWER TECHNIQUES

Though Weibel's technique has been used by many researchers following his description, there have been some criticisms, particularly over the past 2 decades. This is summarised in section 1.3.4.2. One of the main drawbacks of his method is the lack of randomisation in the sampling of sections. Methods to surmount this difficulty were developed by Hyde et al (15). As lung structures are not isotropically oriented, the first step involves isotropically orienting the tissue before sectioning using orientator (16) or isector (17) and then sampling with design based approach (18), which is known to avoid sampling bias. Measurement of volume is then carried out by the Cavalieri method which involves systematic sectioning of the lung into slabs of equal volume, determining the total area of cross section of the structure in question and multiplying the area by the average slab thickness. Similarly surface area is estimated after measuring surface density using

linear probes and length is estimated after measuring length density using surface probes (15).

Heyder (19) described a technique of estimating the size of airways using measurement of gravitational deposition of aerosols in airways during a breath holding after inhaling boluses of aerosols. This was based on the principle that settling velocity is related to the calibre of the airway and the velocity of the aerosol. This technique, known as aerosol derived airway morphometry (ADAM) was refined by Zeman et al (20) and Brand et al (21) to determine the smallest effective airway diameter (EAD_{min}) which was equivalent to measurements of alveoli.

1.2.1.4. OTHER ESTIMATIONS OF SIZE

In many clinical/pathological studies, the exact size of the alveoli may be less important than surrogate measures of its dimensions. Mean linear intercept (L_m), defined by Campbell and Tompkieff (22) is one of the most common methods used in histological studies. Here, a grid of parallel lines is superimposed on the section of lung periphery and the average distance between intercepts of the lines with an airspace wall is measured under the microscope. A further modification proposed by Dunnill (23) is the use of perpendicular lines, so that any deformation during processing is accounted for. As it is not possible to measure the L_m for all fields of the specimen, a sampling technique was recommended. Gillooly and Lamb (24) estimated the 'alveolar wall per unit volume (AWUV)' for a given histological specimen as $AWUV = 2 / L_m$. They further developed an automated image analysis system (fast interval processor) to determine this histological measure.

1.2.2. GEOMETRY AND PATTERN OF BRANCHING OF AIRWAYS

While the branching structure of the proximal airways was described in detail by many authors (2,25), the pattern of branching of peripheral airways was not frequently studied, perhaps because of the difficulties in methodology. Weibel predicted a regular system of branching of airways where each airway would give rise to 2 daughter airways (regular dichotomy) and therefore, the number of airways in each generation was $n=2^z$ where z is the generation number.

The casting technique described above is ideally suited for determining the branching structure. The geometry of the branching structure of the airways can be directly determined from the cast of the airways.

1.2.3. COUNTING COMPONENTS OF LUNG PERIPHERY

1.2.3.1. CASTING

Casting techniques are not suitable to count the number of alveoli, alveolar ducts or alveolar sacs in the lung. Casting techniques are by nature labour intensive and time consuming. While determination of size and branching structure involve measuring or describing a sample of the structures, this approach is not suitable for counting the total number of the various components of the lung. However, estimates of the number of acini were given by Hansen(1) and Parker (26) using casting techniques. Hansen (11) and Pump (10) also gave estimates of the number of alveoli in the lung by calculating the average number of alveoli in an acinus and multiplying by the estimated number of acini in the lung.

1.2.3.2. MORPHOMETRY

The principle of morphometry described above can be used to make quantitative measurements of the number of structures in the lung.

1.2.3.2.1. Theory on which morphometry is based:

Morphometry is based on Rosiwal's extension of Delesse theorem (see 1.2.1.2). This states that the number of structures in a given volume (N) bears a relation to the number of transections through these structures of a random section of the above-mentioned volume (n) as

$$N = k.n^{3/2} \quad \text{EQUATION 1-1}$$

where k is a constant depending on the shape of these structures. This principle was applied to random sections of the periphery of the lung by Weibel (14). He derived the specific form of the above equation for counting alveoli in a section of the lung periphery:

$$N = \frac{n^{3/2}}{\beta \cdot \sqrt{\rho}} \quad \text{EQUATION 1-2}$$

where β is the 'shape coefficient', which is a parameter that is related to the shape of the alveoli and ρ is the volumetric density (the fractional volume occupied by the alveoli in the unit volume that is being studied). For a given shape, β remains constant whatever the size.

1.2.3.2.2. Technique of morphometry

Weibel pioneered the technique of morphometry (2). Briefly, a random section through a formalin fixed lung was studied under a microscope. A field of known area was superimposed on the section and all transections lying entirely within the field and transections intercepted by the left and upper field border are counted, while those intercepted by the right and lower border are neglected - this was repeated for the whole section and 'n' was determined.

He then derived the shape co-efficient (β) for various regular structures (sphere, ellipsoid, cylinder etc.) from mathematical derivation. Later, using experiments with inanimate objects, he determined β for inanimate objects and showed that they corresponded closely to the derived value for the nearest regular shape. Using light microscopy, he concluded that alveoli were polyhedral and estimated its β to be 1.55. The volumetric density of alveoli (ρ) was calculated by measuring the fractional area occupied by cut sections of alveoli (Section 1.2.1.2).

1.2.3.3. OTHER TECHNIQUES

1.2.3.3.1. Radial alveolar count

In some cases the exact number of alveoli need not be measured. An estimate of the number of alveoli in an acinus may be all that is required, particularly for developmental studies and studies looking at factors affecting lung development. Emery and Mithal (27) proposed a technique to count the number of alveoli in a terminal respiratory unit(or acinus). This technique was called radial alveolar count (RAC) and it involves dropping a vertical line from the most distal respiratory bronchiole(defined as partly covered with squamous epithelium and partly with respiratory epithelium) to the nearest definite connective tissue septum. The number of alveoli traversed by this line was counted. The advantage of this technique was that it overcomes the problem of variation caused by variable degree of inflation of the specimen.

1.2.3.3.2. Disector technique

Hyde et al (28) introduced the disector technique of counting alveoli. The technique is given in detail in his work (28,29) and was applied to counting human alveoli by Ochs (30). In brief, this technique uses the mathematical concept of 'Euler characteristic' of a network. It involves estimating the number of free septal edges and 'islands' that appear in consecutive sections of random samples of the lung tissue. This

ensures that only new alveoli are counted in consecutive sections. It also ensures that all alveoli, irrespective of shape or size are counted. The advantages of this method is that there are no shape assumptions. It relies on the fact that alveoli have a single opening. This technique can be adapted for airways remembering that they have more than one opening.

1.2.4. DIFFICULTIES IN MEASURING LUNG PERIPHERY

1.2.4.1. RELIANCE ON HISTOLOGY

Most of the available knowledge of the structure of the human lung periphery has come from histological studies. However, the structures of interest are complex, with irregular geometry and are 3-dimensional. Histological studies, which are necessarily 2-dimensional give incomplete information about the lung structure (3).

The technique of making casts of the peripheral airways (8-11) gives information about the 3-D structure of the lung periphery and the relation between various structures. Unfortunately, it is time consuming and labour-intensive. It is therefore not applicable for measuring large numbers of lung specimens, and therefore, cannot be used to determine normal lung development and factors affecting lung development.

The technique of morphometry described above was a major advance as it vastly decreased the time taken for these measurements. However, there are some assumptions inherent in the technique. It does not correlate very well with the 3D structure (31).

1.2.4.2. LACK OF STANDARDISATION

Human acini were studied by many authors with the casting technique. However, as given in table 1, the method of preparation of the casts was not standardised. For example, Pump (10) used moulding material injected at pressures of 50 mmHg, Hansen (11) used pressures of 25 cm of water and Schreider (9) and Haefeli-Bleuer (8) instilled the moulding material in situ (table 1-1).

There were controversies in naming. An alveolus, as the basic gas-exchanging unit should have no septa, partitions or outlets. Hansen (11) however introduced the concept of 'complex alveoli' with very low septa or partitions.

1.2.4.3. PAUCITY OF LUNG SPECIMENS

The human lung consists mainly of air and only 10-15% of the lung volume is composed of tissues and blood. Moreover, the lung periphery is elastic and has a natural tendency to collapse if taken out of the thorax. Therefore volumetric information is lost in

lung biopsy specimen. Furthermore, unless properly fixed, taking a thin section of the lung distorts the architecture. In order to perform structural analysis of the periphery of the lung, the specimen must first be fixed by instillation of fixative through the airways or the blood vessels (3). Therefore a complete lung or lobe is necessary to determine lung structure.

The need for a complete lung or lobe from autopsy is one of the major constraints in studies of peripheral lung structure. The difficulty in obtaining specimen for scientific studies is increasing with increasing barriers from ethical demands.

1.2.4.4. DIFFICULTIES IN ASSESSING LUNG DEVELOPMENT

This problem becomes exacerbated in case of studies of peripheral lung development. This requires examination of autopsy specimens of children's lungs. Because mortality rates of children, beyond infancy are very low and because children usually die from diseases rather than accidents, a healthy childhood lung specimen is difficult to obtain. The restrictions from law and ethical principles mean that determining peripheral lung development through histological and/or morphometric methods is extremely difficult.

Assessment of peripheral lung development ideally requires serial measures in the same lung. The reliance on autopsy specimen means that serial measures of the anatomy of the lung periphery is impossible. Therefore, determining factors influencing growth and development of the lung periphery has only been possible by using animal models and surrogate markers.

1.2.4.5. CHANGES IN LAW/ETHICS OVER TIME

Currently available studies on the structure and development of lung periphery in children are all from at least 3 decades ago (the latest one being Thurlbeck's study in 1982 (32)), with the exception of studies in fetuses and infants. It is not co-incidental that the laws and ethics governing the use of human tissue for research have undergone enormous changes in the last 2-3 decades. Therefore, the studies done between 1960 - 1982 are still used to inform our understanding of the structure and development of the periphery of the lung.

1.3.CURRENT ANATOMICAL CONCEPTS

Here, I will attempt to review briefly what is currently known about the anatomy of the periphery of the lung from histological techniques. Briefly, this structure can be

visualised if the size of the components, the morphology of branching of the components and the number of each components are elucidated. The previous section has detailed difficulties regarding determination of peripheral lung structure. The lack of available data is complicated by widely varying nomenclature. In view of clarity, in this thesis, I have defined the structures in the lung periphery according to the definitions proposed by Schreider (9).

- Respiratory acinus or the acinus: the terminal unit of the respiratory airways comprising the terminal bronchiole and all the airspaces distal to the terminal bronchiole.
- Terminal bronchiole: the last conducting airway without gas exchange structures (like alveoli) on its walls.
- Respiratory bronchiole: the most proximal airway with some alveoli in the walls.
- Alveolar ducts: ducts with walls being comprised almost entirely of alveolar openings.
- Alveolar sacs: airways whose walls consists primarily of alveoli and terminate in alveoli and not another airway.

1.3.1. DIMENSIONS OF AIRSPACES

1.3.1.1. BY CASTING TECHNIQUE

The dimensions of the structures in peripheral airspaces, determined by the casting technique (1.2.1.1) are detailed in Table 1-2. The values given are for airspaces in adults (age range 18 y to 70 y, see table 1-1). Some measurements are similar between the various studies in spite of the differences in processing the histological specimen. For example, the following values are quite similar between studies : volume of the acinus - 150 -186 mm³ (except for Pump (10)), inner diameter of an average alveolar duct - about 300 - 450 microns, length of an average alveolar duct - about 600 -800 microns, inner diameter of an average alveolar sac - about 250 microns, diameter of an average alveolus -about 150 to 350 microns and depth of a typical alveolus - about 150 to 350 microns. On the other hand, examination of the table shows the wide variation between some of the measurements by different authors. While this labour intensive technique improved our understanding of the periphery of the lung, it is clear that it has not provided a definitive final value for these measurements.

TABLE 1-2

Size of structures in the periphery of the lung by casting technique.

Author, year	Pump 1969 (10)	Hansen 1 1975 (11)	Hansen 2 1975 (1)	Schreider 1981 (9)	Haefeli-bleur 1988 (8)	Ciurea 1989 (13)	Berend 1991 (12)
Volume of acinus	1.3 - 30.1 mm ³	183 mm ³	No data	No data	mean 186 mm ³ (SD – 79 mm ³)	53.6 - 178.6 mm ³	153 mm ³
AD/ acinus	28 to 85	No data	1200 to 1500.	No data	754	277	111
AS/ acinus	36 to 118	No data	2500 to 4500	No data	No data	No data	No data
Alveoli/ acinus	1535 to 4041	No data	14000 to 20000	7100	No data	No data	No data
AD diameter	0.131 - 0.875 mm	Extrapolation - internal diameter mean 0.202 mm, external 0.678 mm.	No data	RB and AD diameter 384- 411 (SD 34).	Outer diameter 699μ (SD 122) inner diameter 323μ (SD 60)	No data	No data
AD length	0.118 - 0.875 mm	0.22 to 0.66mm	No data	RB and AD length 700-800 μ (SD 140, range	730μ (SD 258)	No data	No data

				96 to 1600 μ)			
AD volume	No data	0.032 mm ³	No data	No data	No data	0.04 - 0.13 mm ³	No data
AS volume	No data	0.00454 mm ³	No data	No data	No data	No data	No data
AS length	0.465 to 0.789 mm	0.18mm	No data	300 μ (160 to 1280)	1012 μ (SD 323)	No data	No data
AS diameter	0.253 to 0.520 mm	orifice diameter 0.26mm	No data	170 μ mean	Outer diameter 656 μ (SD 127) inner diameter 251 μ (SD 40)	No data	No data
Alveoli / sac	20 to 29	No data	from 3 to 4 (see remarks)	5 to 50(10)	No data	No data	No data
Alveoli / duct	No data	No data	No data	11 (SD6.3)	No data	No data	No data
Diameter of alveolus	0.125 - 0.325 mm (mean 0.205)	No data	No data	160 to 352(mean 250 μ)	No data	No data	No data

Depth of alveolus	0.125 - 0.375 mm (mean 0.212)	0.238 mm	No data	160 to 352 μ (mean 250)	No data	No data	No data
Shape of alveolus	blind extremity dome shaped base could be hexagonal, cylindrical, quadrilateral or irregular	6 different shapes 3/4 spheroid, truncated cone, 1/4 spheroid, cylindroid - hemispherical bottom, flat-bottomed cylindroid, truncated long ellipsoid	No data	polygonal, as deep as wide	No data	No data	No data
Entry of alveolus, diameter	No data	0.199mm	No data	lesser than alveolar diameter	No data	No data	No data
Surface area of	0.170 mm ²	.101 mm ²	No data	No data	No data	No data	No data

alveolus							
Volume of alveolus	No data	.00064 to .016 mm ³ (mean 0.0048), mean 0.007 if definition2	No data	No data	No data	No data	No data
Remarks	nomenclature: all alveolated airways termed RBs	Concept of complex alveolus (partition < 10% of size) - values with and without this definition provided	Alveoli per sac calculated without using complex alveoli definition	No data	No data	No data	No data

Abbreviations: TB: transitional bronchiole, RB: Respiratory bronchiole, AD: alveolar duct, AS: alveolar sac, SD: standard deviation)

1.3.1.2. BY MORPHOMETRY

Weibel estimated sizes of acinar structures by morphometry. His study included 5 lungs and 40 sections in each of the 5 lungs studied, taking care that each section was either a cross-section or a longitudinal section of an alveolar duct. The estimated value of dimensions are as follows :

- Depth of alveolus - Mean 238.2 micron, (range 193-281, SD=39.6),
- Radius of alveolus - Mean 121.2 micron, (range 99-145, SD=21.1),
- Diameter of spherical analog of alveolus - Mean 260.4 micron, range 215-314, SD=41.1).
- Diameter of alveolar duct to range from 150 to 400 micron in children and 200- 600 micron in adults.

It is seen therefore that these measurements correspond quite closely to the measurements by casting techniques.

1.3.2. ORGANISATION OF PERIPHERY OF THE LUNG

1.3.2.1. PATTERN OF BRANCHING OF ACINAR AIRWAYS

The branching structure of the acinar airways has been described by many authors (Table 1-3) using the casting technique. The degree of discrepancy between authors in the description of branching of the acinar airways is quite pronounced, unlike measurements of size of acinar structures. The pattern of branching is broadly described as irregularly dichotomous (8,13) though trichotomy and polychotomy have been described (1,10). The other pattern that has been described is that of a parent airway giving rise to side branches without changing direction (9,12).

1.3.2.2. GENERATIONS OF BRANCHING

Similarly, the number of generations of branching within the acinus (i.e. from terminal bronchiole to the alveolar sacs) have been variously described as ranging from 3 to 11. Ciurea et al (13) counted between 6 to 10 generations in the acini they described. The cumulative number of branches subtended by each generation was highest in the middle generations. A similar pattern was found by Haefeli-Bleuer et al(8), who described terminations to alveolar sacs occurring between intra-acinar generation 6 to 11. The number of branches in each generation followed $n=2^z$ from generation 2 to 7, whereas the number of branches following this generation was 220, 210, 100 and 2 for generations 8 to 11 respectively. However, Berend (12) only describes a total 3 to 6 generations of intra-

TABLE 1-3

Morphology of branching of peripheral airways of the lung

Author, year	Pump 1969 (10)	Hansen 1975 (1)	Schreider and Raabe 1981 (9)	Haefeli-bleur (8)	Ciurea 1989 (13)	Berend 1991 (12)
Branching pattern	dichotomy, trichotomy and polychotomy	dichotomy trichotomy and polychotomy	dichotomy, polychotomy, side branches (parent duct not changing direction or diameter after giving off side branch), angle of division- 0 to 180 ⁰	irregularly dichotomous	irregularly dichotomous	two RBs / TB go to the periphery giving of 4 systems of AD on the way. ADs have assymetric branching
Generations in acinus	9 generations from TB to AS	3 generations of cylindrical RB, total up to 11 generations from RB to AS	11 generations if TB is gen 0	6-11 generations from RB	6 - 10 generations of branches taking RB as gen 0. More number of branches in the middle generations	AD systems have one to 5 generations. However if RB is taken as increasing in generation after each AD is given off, there are 3 to 6 generations of airways.

Abbreviations: TB: transitional bronchiole, RB: Respiratory bronchiole, AD: alveolar duct, AS: alveolar sac.

acinar airways. The most likely explanation of the differences is that the number of generations depend on the configuration of the acinus studied.

It is clear from the analysis of the studies regarding the periphery of the lung that there is no common pattern of branching and that the branching seems to depend on the anatomy and position of the acinus. For example the pattern would be different in acini which are relatively central and spherical compared to acini which are wedge shaped and borders the pleural surface of the lung. The airways seem to form a 3-dimensional space filling structure (1).

1.3.2.3. SHAPE AND CONFIGURATION OF ALVEOLI AND DUCTS

Alveoli have been described as having various different shapes by different authors. Most authors describe alveoli as being polygonal (9) with an inlet slightly smaller than the rest of the alveolar body. Hansen (11) has described 6 configurations of alveolar shape with a 3/4 spheroid being the most common (26%). Other common shapes reported were truncated cone (22%) and 1/4 spheroid (22%). Pump et al (10) have described alveoli as being dome shaped with an opening (base) which could be hexagonal, cylindrical, quadrilateral or irregular. These shapes would change with the degree of inflation of the lung. Alveolar ducts are variously described as cylindrical (13) to spheroidal (11).

Alveoli are both smaller and less numerous when they arise from more proximal generations (12). Respiratory bronchioles(RB) become progressively more alveolated towards the periphery. Walls of alveolar ducts and alveolar sacs are completely lined by alveoli. Alveoli arising from RB were smaller than alveoli from alveolar ducts(AD) by a factor of 0.5 to 0.66 (11). The mean outer diameter of ADs remain constant, while the mean inner diameter reduces from generation 1 to 10, while the mean depth of alveoli increases from generation 1 to 10 (8).

1.3.3. NUMBER OF VARIOUS STRUCTURES

As opposed to dimensions, counts of various structures within the acinus (table 1-2), measured using the casting technique are widely variable between studies. For example:

- Average number of alveolar ducts in an acinus (from 28-85 (10) to 1200 to 1500 (11))
- Number of alveoli in an acinus - (1535 to 4041 (10) to 14000 to 20000 (11))

1.3.4. CRITIQUE OF EXISTING TECHNIQUES TO MEASURE SIZE AND NUMBER

1.3.4.1. ASSUMPTIONS AND CRITICISM OF CASTING TECHNIQUE

The casting technique is probably the most accurate method to delineate the exact structure of the lung periphery and the inter-relationship between various parts of the peripheral lung. This is because it gives a representation of the 3-D structure of the lung. However, attempting to measure size and number of structures from casting is fraught with number of difficulties.

First, it is labour intensive and time consuming. Therefore, only a small sample of the lung can be studied. For example, Haefeli-bleur et al(8) studied 6 acini, Berend et al (12) studied one acinus and Hansen et al (11) studied only a portion of one acinus (40 sacs and 60 ducts). Differences in acinar structure depending on position (subpleural vs. intraparenchymal, lower lobes vs upper lobes) have not been adequately elucidated. The differences in description of acini between authors could be partially explained by sampling bias.

Second, the technique has not been standardised, so that it is difficult to compare between authors. The pressures employed to inflate the lungs before casting varies from 50 mm Hg (10) to 25 cm H₂O (11) (Table 1-1). Some authors have used variable pressures to inflate the lung while it is within the thorax (9). This may lead to differences in the degree of inflation of alveoli and sacs in the lung periphery. It is clear that the measured dimensions are not comparable between studies.

Third, inflation with a viscous liquid can lead to irregular expansion, which might distort the structure and shape of acinus and interfere with the measurements. The methods used to minimise this limitation have not been adequately described. Nevertheless, the casting technique has contributed quite a lot to our understanding of the structure of acini.

1.3.4.2. ASSUMPTIONS AND CRITICISM OF MORPHOMETRY

The morphometric equation relating the number of alveoli in a given volume to the number of transections of alveoli in cut sections (equation in section 1.2.3.2.1) contain the term β (shape coefficient) which is an estimate of the relation between volume and the average cross sectional area of the alveolus (33). It was estimated to be 1.55 for alveoli measured by Weibel. Clearly, the relationship will depend upon the degree of inflation of

the lung. Many authors using the technique of morphometry have used the same shape coefficient as Weibel despite different techniques for inflation, fixation and section(23,32). Results from these studies are open to debate. In studies involving analysis of alveolar numbers and dimensions in children at different ages, using the same shape coefficient for lungs from different age groups, while being inflated using the same pressures (32) opens up the possibility of bias because the pressure-volume relationship is likely to change with age. Also, it is well known that alveoli are not uniform and not of similar geometry. Therefore, usage of a single shape coefficient for the whole lung is open to criticism.. When Moreover, the average number of alveoli measured by Weibel (and others using a similar technique) is nearly 300×10^6 alveoli, which is less than figures obtained by unbiased techniques of $400-500 \times 10^6$ alveoli(30). It is clear that there were underestimations of alveolar number using morphometry.

The morphometric technique as such was based on the assumption that the structures to be measured are uniformly and randomly distributed with random orientation throughout the lung. Alveoli are not uniformly distributed (e.g. there are more alveoli towards the peripheries of the lung than towards the hilum). Furthermore, the orientation is not random and is related to the branching structure of the bronchioles and alveolar ducts.

Specimen preparation techniques introduce another level of bias. Measurements of size of the structure depends on the degree of inflation. Therefore assuring meticulous attention to standardising preparatory steps such as inflation, fixation and sectioning are important in morphometric studies. Unfortunately, many studies do not give details about how this was achieved.

Lungs should be inflated to a standard volume in order to compare the results from different specimens. The pressure-volume relationships may vary between specimen. Therefore utilising a standard inflating pressure for the lungs may be inappropriate.

Sampling bias is another problem. As Weibel himself acknowledges in a subsequent paper (34), the sample examined is only an infinitesimal portion of the whole organ. Given that alveolar dimensions vary through the lung, unless a rigorous protocol for sampling is followed, morphometric estimations of alveolar dimensions cannot be compared between two lungs.

The disparity between Weibel's description of an acinus (3 branches of alveolar ducts) to the 3-D reconstruction (8-11 branches) was examined closely by Hansen (31). The disparity was linked to the fact that the boundaries of acini, alveoli and alveolar ducts are not well defined in all directions (in case of alveolar ducts, there were no tissue

boundary at all - the boundaries were openings of alveoli and openings of incoming and outgoing alveolar ducts).

Despite all these limitations, morphometry was the method used to determine the alveolar number in developing human lungs (section 1.4.6.3).

1.4. NORMAL DEVELOPMENT OF PERIPHERY OF HUMAN LUNG

1.4.1. MEASUREMENT TECHNIQUES

Many of the details of lung development are actually extrapolated from animal studies (35). The phases of lung development is relatively conserved between animals though the developmental stage at the time of birth varies widely between animals (35). Human studies involve histological analysis of stillborn fetal lungs and in some cases analysis of autopsied lungs of infants. The techniques involve fixation, staining and serial sectioning of fetal lungs followed by either microscopy (36), manual graphical reconstruction (37) or casting with a plastic mould (38). Morphometric techniques were used by Hislop et al (39) to determine the development of alveoli in late-fetal life and early infancy. These methods have not been elaborated here because they are similar to those described in section 1.2.1.

1.4.2. PHASES OF HUMAN LUNG DEVELOPMENT

Burri et al (35) reviewed the development of the human lung. In brief, human lung development can be divided into 5 phases:

1. Organogenesis or Embryonic phase (1-7 weeks gestation): The lung appears as an outpouching from the primitive foregut and separates from the prospective oesophagus.
2. Pseudoglandular period (5-17 weeks gestation): The division of the bronchial tree occurs; the lung shows features similar to a secretory gland.
3. Canalicular period (16-26 weeks gestation): Prospective gas exchange tissues of the acinar zone become visible and the tubes representing future air-spaces expand.
4. Saccular phase (24 weeks gestation to about 40 weeks gestation): Canals distal to the prospective terminal bronchiole widen to form branching clusters called saccules; further branching of the saccules takes place till almost all the generations are formed at the time of birth.

5. Alveolar phase(36 weeks gestation onwards): Outpouchings develop from the primitive saccules that represent definitive alveoli; new alveoli form on the walls of all the acinar airways in a progressive fashion; alveoli are currently thought to form till about 2-3 years of life (see section 1.6.1)

For the purposes of this work, which focuses on the structure of the periphery of the lung, a brief review of the normal development of acinar airways and alveoli (canalicular phase onwards) is provided below .

1.4.3. DEVELOPMENT OF ACINI

1.4.3.1. CANALICULAR PHASE

The acini begin to form in the canalicular phase (35). The early acinus consists of an airway stem and a spray of short tubules lined by cuboidal epithelial cells arranged in a cluster surrounded by a mesenchymal sheath (FIG 1-1. A). Three main processes occur in the canalicular stage:

- Development of distal pulmonary circulation: Capillaries start forming in the mesenchyme eventually giving rise to a three dimensional loose network.
- Apposition between pulmonary circulation and airways: The expansion of the acinar airways into the mesenchyme reduces the distance between putative blood vessels and future airways(FIG 1-1. B). The cuboidal epithelium then becomes closely associated with the capillaries.
- Differentiation of epithelium into type 1 and type 2 cells: The cuboidal cells differentiate into type 1 and type 2 epithelial cells. This occurs between 20 to 24 weeks of gestation (40).

1.4.3.2. SACCULAR PHASE

Towards the end of canalicular phase, the ends of the canaliculi start dilating (FIG 1-1. C) (41). This brings capillary layers of adjacent airspaces in closer approximation.

In the saccular phase, the three major changes that occur are:

- Widening of terminal airspaces: the peripheral airways form typical clusters of widened (future) airspaces called saccules (FIG 1-1. D). This occurs by lengthening and widening of all airspaces distal to the future terminal bronchiole and further subdivision to form the last few generations of airspaces.

FIGURE 1-1

Development of pulmonary capillaries.

A: pseudoglandular stage, capillaries are randomly distributed in mesenchyme.

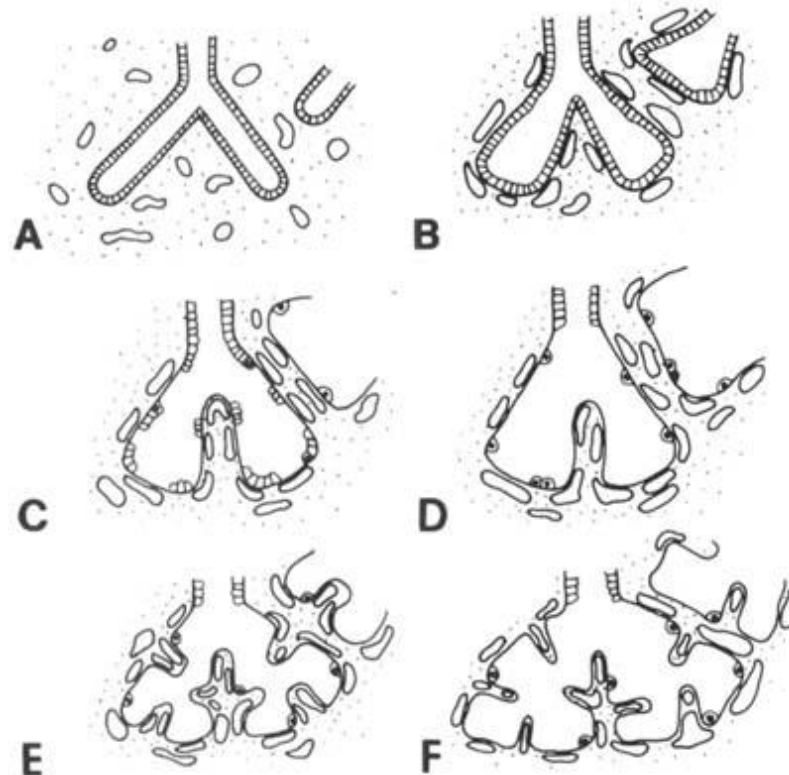
B: beginning of canalicular stage, capillaries start to arrange around epithelial tubes, which enlarge to canaliculi.

C: canalicular stage, capillaries establish close contact to lining epithelium, which flattens to form thin air-blood barriers. Widening of canaliculi reduces intervening interstitium so that capillary layers of adjacent air spaces lie closer to each other.

D: end of saccular stage, epithelium differentiated in type I and type II cells, intersaccular walls with 2 capillary networks.

E: alveolar stage, formation of secondary septa; all septa contain 2 capillary networks; further reduction of interstitial tissue.

F: mature lung, capillary layers in primary and secondary septa have fused; at a few places double row may stay; septa have lengthened and narrowed. (from Burri, PH (35))



- Increased number of generations distal to the terminal bronchiole: This occurs by sequential branching, whereby the distal blind ending saccule divides dichotomously so that the original saccule now becomes a tube and the newly formed airspaces are now blind ending. Increasing of surface area by formation of primary septa: At the beginning of the saccular stage, the saccules are relatively smooth walled and cylindrical (40). Ridges then start to form in the walls of the saccules – they consist of elastic fibers

with collagen and proteoglycans (40). Discrete bundles of elastin are seen at the luminal edges of the crests (39) forming an uneven saccular wall. The epithelium of the saccules then bud into surrounding connective tissue – these may be called subsaccules or primary alveoli.

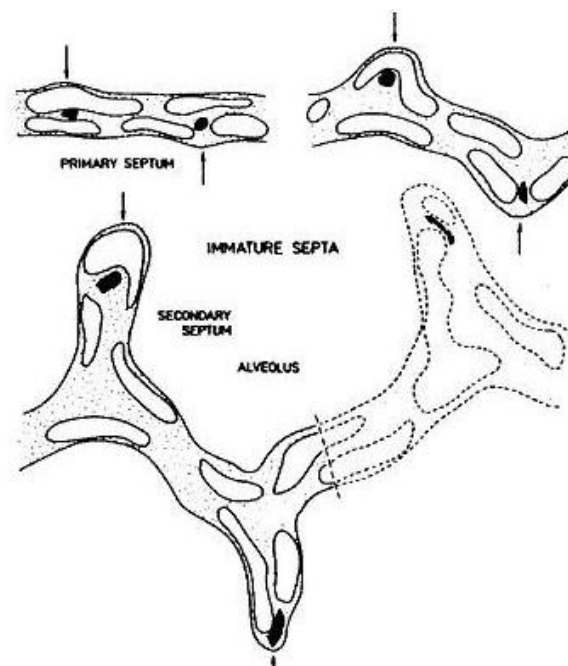
1.4.4. DEVELOPMENT OF ALVEOLI

This budding of the epithelium of the saccules into surrounding connective tissue heralds the alveolar stage.

- Formation of secondary septae: Early in the alveolar stage, the crests with elastin at their free margin have elongated sufficiently to subdivide the saccular airspaces to primitive alveoli. Secondary septae arise from primary septae to form true alveoli (Figure 1-1. E). These septae are initially thick walled with dual capillary layer. Primary septae can be differentiated from secondary septae by examination of the capillaries in them. The two layers of capillaries in primary septae have many interconnections because they originate from the three dimensional capillary network whereas the capillary layers in secondary septa are interconnected only in the free edge, because they are formed by folding of the primary septum.

FIGURE 1-2

Formation of secondary septa: top right panel: formation of ridges preferentially along elastin fibres (black circle). Bottom panel: Ridges elongate to form the secondary septa which have a capillary bilayer. (from Burri, PH (35))



- Merging of double capillary layer to single capillary layer (also called stage of microvascular maturation): The double capillary layer is thought to be an essential requirement to formation of new septa (called secondary septation (35))(Fig 1-2). In the stage of microvascular maturation the double capillary layers in the septae mature to form single capillary layer. This is thought to be due to thinning of the secondary septum mainly due to enlargement of the airspaces and also due to developmental processes which lead to thinning of the mesenchyme, which brings the capillary layers in close apposition and then result in fusion. Fusion is associated with apoptosis of some of the capillary endothelial cells. Maturation of the capillary layer results in capillaries being exposed to alveolar air on both sides rather than on one side only and greatly improves the efficiency of the air-blood barrier.

1.4.5. GROWTH OF AIRWAYS AND AIRSPACES

The number of generations of airways that form in the saccular stage is still a matter of debate. According to Hislop (41), by 28 weeks of gestation, 3 generations of respiratory bronchioles(RB) are seen followed by a number of generations of saccules, which are each so short that they give the appearance of an irregular cluster of airspaces. Until term, these saccules increase in length, possibly with further addition of generations. These saccules which undergo elongation and further remodelling represent the future alveolar ducts (AD). The last generation of saccule (terminal saccule) forms the future alveolar sac (AS). Alveoli form from all these peripheral airways. At 2 months of age, there are 3 generations of RB, followed by 4 generations of AD and one AS. The RB, AD and AS are all lined by alveoli by that stage (Fig 1-3).

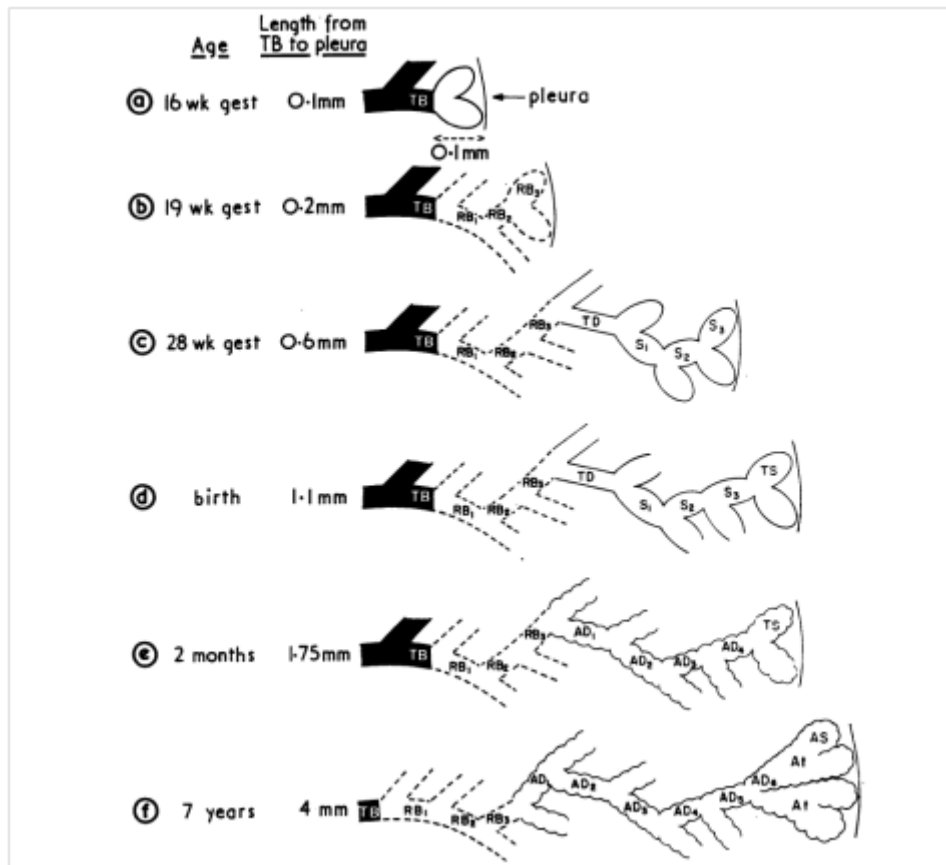
Using morphometric methods, Dunnill (23) estimated the number of acinar airways (respiratory bronchioles, alveolar ducts) from birth to 8 years of age. He found that the total number increased with age and seemed to reach adult values by 8 years of age (number of acinar airways - 1.5 million at birth, 4.5 million at 1 year, 7.9 million at 4 years and 14 million at 8 years and adulthood).

In the same study, he gives an estimate of increase in alveolar diameter with age. He selected 60 'typical' alveoli from cross sections and measured it using a micrometer eyepiece. Because, not all alveoli are bisected in cross-section, he used an arbitrary correction factor of 1.25 to estimate the mean alveolar diameter. Alveolar diameter was

found to remain nearly constant up to 8 years of age (210 to 220 μ) but the value found in adulthood was nearly 30% higher (280 μ) (also see 1.4.6.3).

FIGURE 1-3

Acinar development - diagrammatic representation (From Hislop (41))



The increase in length and diameter of the peripheral airways and alveoli as a function of age was estimated by reviewing previous literature by Menache et al (42). They give a comprehensive table of estimated measurements of the number, length, diameter and volume of 25-26 generations of airways in 9 children from 3 months to 14 years and 2 adults of 18 and 21 years. There were two major assumptions in the model -

- they assumed strict dichotomy and equal path length
- they assumed alveoli to be perfect spheres

However, this gives some estimation of airway growth with age and could be applied to studies on particle and airway deposition in children.

1.4.6. FORMATION OF NEW ALVEOLI IN CHILDREN

1.4.6.1. MORPHOMETRIC METHODS

Many studies done about 3- 5 decades ago attempted to determine the timeline of formation of new alveoli in children (Table 1-4). Most of the studies used morphometric techniques.

Weibel (33) estimated the total number of alveoli (N_A) in 5 subjects (including a 8 year old boy and a 16 year old woman) who died of non- respiratory causes. The estimated N_A in the young people was nearly the same as that in the adults (296×10^6 as against 294×10^6).

Dunnill (23) examined the lungs of 10 children (birth to 8 years) who were term born and died of non-respiratory causes and compared them with adult lungs. He examined the lungs using both Weibel's technique and the technique of Campbell and Tomkeieff (22). He found that N_A increases with age rapidly at first and then gradually up to at least 8 years of age. By 8 years of age the average number of alveoli (280 million) nearly approaches the average number in an adult (296 million).

Davies and Reid (43) counted N_A in lungs of 5 children who died of non-respiratory causes from birth to 11 years. The alveolar count increased rapidly from birth (17.3×10^6) to 3 years (196×10^6), gradually increased to 303×10^6 at 5 years and 336×10^6 at 11 years. They tried to quantify change in alveolar surface area with age using their 'index of alveolar complexity' which increases with increasing complexity and decreases with increasing size of the alveoli. The index of alveolar complexity was 4.5 at birth, decreased to 3.4 at 4 months, remained nearly constant at 3.8 at 3 years and gradually increased to 4.1 at 5 y and 5.7 at 11 years. They suggest that alveolar surface area increases more rapidly than increase in alveolar number with age.

TABLE 1-4

Increase in alveolar number from birth to adulthood - by morphometric methods

Author	Dunill (23)	Davies (43)	Hislop (39)	Thurlbeck (32)	Angus (44)	Weibel (33)
Subjects	10 subjects, birth to 8 years	5 children birth to 11 y	19 neonates including 7 preterms + 10 stillbirths.	56 children (36 m, 20 f) 6 wks to 14 y	14 subjects under 19	5 subjects, two less than 16 (8 and 16)
Cause of death	Non-respiratory causes of death (not documented)	Non-respiratory cause of death (documented)	Non respiratory cause of death (documented)	Trauma-31, infection-20, others-5	Free from disease and came from patient who died acutely?	Cerebral hemorrhage and splenic rupture
Inflation	Lungs inflated as near as possible to internal diameter of thoracic cage	Lung inflated with buffered formalin at a pressure of 75 cm of water	Distended through airways with formol saline at a pressure of 30 cm water.	Inflated with 10% formalin at 25 cm water	Inflated to transpulmonary pressure of 25 cm of water	Inflated by negative pressure maintained at 10 cm of water
Fixation	Fixed with formalin vapour	Fixed in liqd formalin for one week	One week of fixation in formalin	No information	Fixed with formalin for 18-24 hours	Fixed with formaldehyde steam 2-3 hours
Fixation constant	Estimated experimentally	Not estimated	Not given	Changes during fixation estimated by before and after photography	Not given	Fixation shrinkage factor estimated at 0.55
Volume proportion of alveolar air	Zeiss point counting integrating eyepiece		Point counting technique		Random point counting technique of Anderson and Dunnill	Point counting technique

Enumeration of alveoli	Weibel's technique	Weibel's technique	Dunnill's technique (which was based on Weibel)	Weibel's technique	Weibel's technique	Weibel's technique
Shape constant	Alveoli – 1.55 Alveolar duct -2.5		Not given	Alveoli – 1.55 for all age, distribution variable assumed to be 1	Distribution variable assumed to be 1. Shape constant not given	1.55
Shape of alveoli	Truncated cone surmounted by a cone	Used an 'index of alveolar complexity' – intercept count $\times 10^3$ alveoli/cm ²	Not given	No shape given, but shape constant assumed to be 1.55		Small polyhedral bodies – but approximated to truncated cone surmounts by cone
Additional points		Say that alveolar complexity increases from 4 months to 11 years even though alveolar size increases.		Number of alveoli per unit volume is constant between 7 years and 14 years		

Angus and Thurlbeck (44) examined the lungs of 14 subjects under the age of 19 years and 32 adult subjects. Their study was the first to look specifically at the problem of determining when human alveolarization finishes. They recognised the large scatter in N_A in human lungs. They approached the question about age of completion of alveolarization by looking at the number of alveoli per unit volume (N_A/V) against age. They postulated that N_A/V will not change during alveolar multiplication and then decrease rapidly when alveoli just increase with volume with no new alveolarization. Therefore, they postulated that there will be an inflection point which demarcates the age of completion of alveolarization. They were not able to demonstrate such an inflection point and therefore could not demonstrate completion of alveolarization.

Hislop et al (39) examined the lungs of 29 infants from 29 weeks of gestation to 18 weeks of postnatal life. They used both the morphometric technique of Weibel (14) and the radial alveolar count technique of Emery and Mithal (27) to measure N_A and the number of alveoli per unit volume (N_A/V) and surface area (N_A/S) and measured the diameters of at least 50 alveoli and 20 alveolar ducts per lung. The aim of this study was to determine the early development of alveoli. They note the following regarding alveolar development: N_A increased rapidly from about 20 million at 29 weeks of gestation to 288 million at 12 weeks of age. The rate of increase was seen to be fastest in fetal life. According to this study, N_A/V increased up to term and then decreased. This implies that alveolarization proceeds more rapidly than lung volume growth up to term and less rapidly after birth.

Thurlbeck (32) did the most influential study regarding human alveolarization to date. He examined the lungs of 56 children from 6 wks to 14 years of age dying of non-respiratory causes. They examined the cut sections microscopically and estimated N_A/V and N_A in the lung from Weibel's formula. These morphometric measures were compared with age, body length and body weight. They confirm the wide scatter in N_A in different individuals of similar age. Therefore, they grouped the individuals by age and estimated the average N_A in each group. They found that N_A was similar in the 2-4 year olds and 7-8 year olds and concluded that alveolarization is complete by 2 years of age.

1.4.6.2. OTHER TECHNIQUES

Zeman and Bennett used the technique of aerosol derived airway morphometry (ADAM) to determine the minimum effective airspace diameter (EAD_{min}) which was postulated to correspond to the alveolar dimensions (20). They measured these dimensions at total lung capacity (TLC) in 53 children and young adults aged 6-22 years

and 59 adults (45). They found that the EAD_{min} changed with age and the TLC varied as the third power of EAD_{min} . They postulated that between age of 6 and 22, alveoli do not increase in number but expand to cause lung growth.

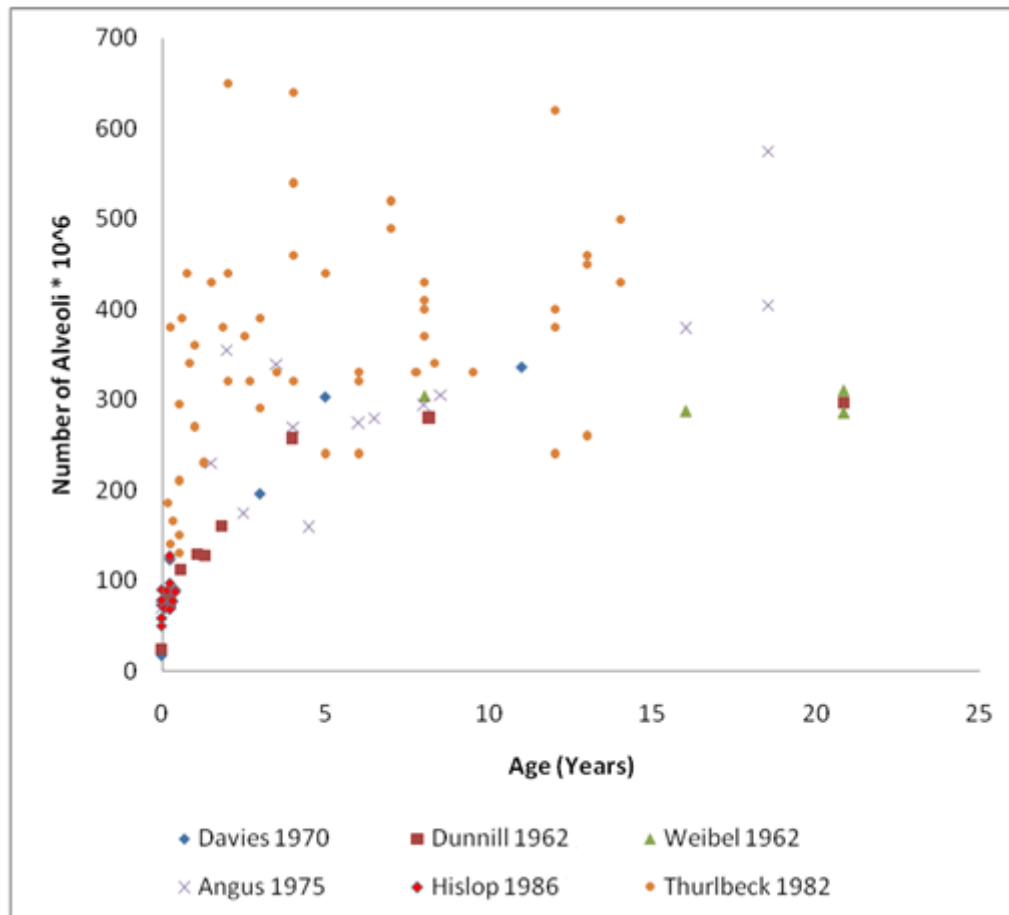
Brown et al (46) used a completely different approach, Electrical Impedance Tomography, to determine changes in alveolar number with maturation. Their approach consists of calculating the absolute resistivity of the lung using a high frequency alternating current passed between pairs of 8 electrodes placed around the chest wall and determining the changes in tissue resistivity of the thorax with breathing. The resulting measurements were combined for different frequencies of alternating current to reconstruct an image of change in tissue resistivity within the thorax. This was combined with a finite differences model of the thorax to determine alveolar number. Using this approach, they were able to demonstrate that the number of alveoli at about 3 years was only about 90 million as against the calculated adult value of 300 million. They did not, however try to determine the age of completion of alveolarization.

1.4.6.3. THE AGE OF COMPLETION OF HUMAN ALVEOLARIZATION

It is clear that prior knowledge the age of completion of human alveolarization was based on studies done using older histological morphometric methods between the 60s and 80s (Section 1.4.6.1). Attempts to delineate this using other techniques have given conflicting results (Section 1.4.6.2). There have been no studies specifically looking at the age of completion of human alveolarization using newer bias free techniques of morphometry. There are number of flaws in the older methods (1.3.4.2). Figure 1-4 below shows a summary of the studies evaluating the number of alveoli in the lung in children of different ages using the older morphometric techniques. This shows the wide disparity between these studies both in the measured alveolar number and in the estimated age at which alveolarization is complete. There was no consensus between the studies. However, later authors have mostly quoted Thurlbeck's study(32) as proof that alveoli develop up to age 2-3 years.

FIGURE 1-4

Number of alveoli in the developing human lung estimated by morphometry from previously published studies.



Studies quoted in the above figure: Davies 1970(43), Dunnill 1962(23), Weibel 1962(33), Angus 1975(47), Hislop 1986(39) and Thurlbeck 1982 (32).

1.5.FACTORS AFFECTING DEVELOPMENT OF LUNG PERIPHERY

It is clear from the above section that normal development of lung acinar structures is not easy to study in humans. This is particularly the case for normal postnatal development.

Therefore, exploration of factors affecting the normal development of the lung periphery has been an extremely difficult task. As with normal development, antenatal factors are easier to study than postnatal factors. The current knowledge about factors affecting development of the periphery is extrapolated from postmortem histological studies of stillborn fetuses, neonatal deaths of term or preterm born babies (48-51),

animal models (52-55) and correlation from clinical parameters like oxygen requirement (56).

1.5.1. STUDIES OF DEVELOPMENT OF LUNG PERIPHERAL STRUCTURES

A summary of techniques used to determine influences of various factors on lung acinar development is given below:

1.5.1.1. HISTOLOGICAL STUDIES

Standard light microscopy of the lungs was used by various authors to determine the impact of various factors influencing lung development. While light microscopy is suited to describe changes in peripheral lung, it is not a quantitative tool, and cannot be used to determine impact on size and number of the structures in lung periphery. As an example, the association of preterm birth with deranged acinar structure was first described by Northway et al in 1967 (57) in a group of preterm infants who developed pulmonary disease following ventilator and oxygen therapy for respiratory distress syndrome (RDS) of prematurity. Histological techniques are used wherever the changes are grossly visible. It is however, not suitable for determining subtle changes on the size and structure of the lung periphery.

1.5.1.2. MORPHOMETRIC METHODS

Formal morphometric methods like those used by Weibel (Section 1.2.3.2) were used to determine alveolar size and number following preterm birth by a number of authors including Hislop, Husain, Sobonya and Margraf (48-50,58). The advantage of the technique is that it is quantitative and subtle changes can be demonstrated. However, as described before (section 1.3.4.2), standardisation of the measurement technique is paramount to avoid measurement errors.

1.5.1.3. ANIMAL MODELS

Massaro's group have used rat and mice model to determine the influence of various factors on peripheral lung development, including effect of oxygen therapy, hypoxia, nutrition, corticosteroids, retinoids (59-62). Similarly, Maritz used a rat model to determine impact of antenatal tobacco smoke (63). Avdalovic (64) used monkey models to determine influence of nicotine and smoking. Coalson's group used the preterm baboon model to the influence of various factors involved in care of preterm born children (mechanical ventilation vs. CPAP, gentle ventilation, arterial duct ligation, antenatal infections, caffeine therapy, etc.) (65-69). Animal models give a good indication of the

factors affecting lung development, but there are limitations in extrapolating the findings to human lung development.

1.5.1.4. CORRELATION FROM PHYSIOLOGICAL MEASURES

In many clinical studies, physiological measures are used as surrogate measures of peripheral lung development. The most commonly used measure is the need for supplementary oxygen to maintain oxygen saturation in the bloodstream. This forms the basis of the definition of chronic lung disease of prematurity (CLDp) (70). In children born before 32 weeks of gestation, the current definition of CLDp is based on oxygen requirement at 2 time points - 28 days of life (mild CLDp) and 36 weeks postmenstrual age (moderate CLDp) (70). Here postmenstrual age (PMA) is defined as the sum of gestational age at birth and age after birth, expressed in weeks.

Oxygen requirement is theoretically related to the overall alveolar surface area available for gas exchange. Though there are many associated/ confounding factors affecting oxygen requirement, incidence of CLDp has been used as an endpoint in many studies determining the effect of various neonatal interventions and risk factors (71,72) with the implication that it is a surrogate marker for deranged alveolar development (56).

The disadvantages of using these clinical parameters as surrogate measures are that they are not specific to alveolar development and not sensitive to subtle alterations of lung development. Also, decision to provide oxygen therapy in preterm born infants is a clinical decision often based on different guidelines and protocols followed by each neonatal unit.

The pulmonary diffusion capacity of carbon monoxide has been used by some authors as an outcome measure (73-75). While this test is more specific to the alveolar-capillary barrier, it is nonetheless dependent on many factors such as haemoglobin concentration, presence of carboxy-haemoglobin etc. The manoeuvre is difficult for younger children.

1.5.2. SUMMARY OF FACTORS AFFECTING PERIPHERAL LUNG DEVELOPMENT

1.5.2.1. PREMATURITY AND FACTORS ASSOCIATED WITH CARE OF PRETERM INFANTS

Most of the critical steps in peripheral lung development take place in later stages of gestation. Therefore it is natural to assume that preterm birth is associated with altered peripheral lung development. As described above, Northway (57) first described the association between care of preterm born infants and deranged peripheral lung development using histological techniques. In that era, the care of the preterm neonate involved high pressure ventilation and oxygen therapy with nearly 100% oxygen. The preterm lung, devoid of surfactant, developed respiratory distress syndrome (RDS). High pressures were needed to inflate the lung and high concentrations of oxygen were needed for survival. It was unusual for infants born less than 28 weeks of gestation to survive. These infants were often left with bronchopulmonary dysplasia (BPD), as described by Northway. In the ensuing decades, several developments have transformed clinical management of preterm newborns. The evidence for these advancements have come from clinical studies and histological studies.

BPD, strictly speaking, is a histological diagnosis. However, this term has been used in the clinical context by many authors (70). In this thesis, I have preferred to use the alternative term chronic lung disease of prematurity (CLD), which, though not as specific for damage sustained after preterm birth as BPD, does not imply histological confirmation (76). Also, in this thesis, I have preferred to use very preterm born infants when referring to children born at <32 weeks gestation (preterm birth in humans is defined as birth <37 weeks gestation) and extreme preterm born infants when referring to children born at <28 weeks gestation (as per WHO: <http://www.who.int/mediacentre/factsheets/fs363/en/>).

1.5.2.1.1. Evidence from clinical neonatology

Introduction of surfactant therapy (77) and antenatal corticosteroid administration to pregnant women (78) have improved survival of preterm babies at lower gestations by reducing the incidence and severity of RDS (79). Higher pressures were no longer used to ventilate the newborn lungs and it was increasingly recognised that oxygen could contribute to lung injury and thereby increase the risk of CLDp. The STOP-ROP trial (56) showed higher incidence of BPD in preterm infants managed with

higher target of oxygen saturation. Refinement in ventilatory strategies to reduce lung injury were summarised by Ambalavanan and Carlo (80).

1.5.2.1.2. Histological studies:

In parallel, histological and morphometric studies demonstrated the damage to the lung due to preterm birth and associated neonatal care. The earliest studies conducted by Northway group (81) showed features of necrotizing bronchiolitis, bronchiolar fibrosis, marked widening of the alveolar septa, alveolar fibrosis and atelectasis in the preterm born lungs. At this point, neonatal care consisted of aggressive ventilation strategies with hyperoxia. It was surmised that high pressure ventilation and hyperoxia was detrimental to the developing lung.

In the later studies, derangement of alveolar development was the major feature. Hislop (48) compared histological and morphometric appearance of the lungs in 3 groups of preterm born infants (non ventilated 31-36 week gestation, low pressure ventilated 26-38 week gestation and high pressure ventilated 25-33 week gestation infants) with term born infants who died of non-respiratory causes. The ventilated group had the lowest alveolar number, highest mean linear intercept (Lm) and lowest total surface area (TSA) of alveoli. Slope of change of alveolar number with age was reduced in the ventilated infants. The age of death of these infants were from 1 day of life to 14 months of life.

Margraf (58) compared 8 children born at 24-32 weeks who were ventilator dependent for 2-12 months (BPD group) with 6 children who were term born but died due to non-respiratory causes (Control group). Lm was increased and alveolar number was reduced in the ventilated group. The age of histological analysis in case of the BPD group was from 2 months to 28 months.

Husain et al (49) compared peripheral lung histology of 14 infants who had surfactant treated BPD (S-BPD) with 8 infants who had non-surfactant treated BPD (NS-BPD) and 15 age matched controls using standard histology and morphometric methods. They showed that S-BPD had little alveolar septal fibrosis, but almost complete arrest of acinar development (resulting in larger, simpler and fewer alveoli). Of the 22 subjects who had BPD, only one was older than 2 years of age at the time of autopsy (7.75 years).

1.5.2.1.3. Animal studies:

The histological study of BPD in human subjects suffers from the flaw that the specimen necessarily reflects the severe end of the spectrum (subjects who died due to BPD). Also, histological studies can only give circumstantial evidence regarding influences of associated factors on incidence and severity of deranged lung development. Animal models therefore, have been used to delineate factors associated with deranged lung

development in the preterm infants. Coalson et al (52) developed the most realistic animal model of BPD with baboons delivered prematurely. Coalson first replicated the 'old BPD' of Northway et al. by delivering baboons at 140 days (term = 185 days), which corresponds to about 30 weeks of gestation in human infants (82). These baboons were then ventilated with high pressures and high oxygen concentrations of 100% for 7 days and 80% for 14 days (53) while the control group was ventilated with just enough ventilator pressures and oxygen to ensure survival. The lungs were analysed at 21 days and in 33 week survivors. The BPD group showed overexpanded areas with fibrosed walls alternating with normally inflated and atelectatic parenchyma. In 33 week survivors, the major histologic feature was scattered focal areas of irregularly enlarged airspaces. The total alveolar counts (determined morphometrically) and TSA of the airways were significantly less in this group. This mirrored the changes seen in Northway's original study. The control group showed nearly normal well alveolated lungs with thin alveolar walls at 33 weeks.

They then developed the borderline viability model by delivering baboons at 125 days, which corresponds to 26 weeks gestation in human infants. With these baboons, they used antenatal corticosteroids, exogenous surfactants, gentle ventilation and appropriate oxygenation to replicate the lesions seen with the 'new BPD' (83). They then analysed the lungs after 27-71 days of survival. The pattern of overinflation and atelectasis seen in classic BPD was not a feature in this group of baboons. Rather, they had simplified distal saccules and decrease in alveoli (associated with increased Lm and decreased total internal surface area). In 8 month survivors, the Lm and TSA approached the 156-day gestation controls, suggesting some catch-up alveolarization occurred over the survival period.

Using further experimental work, Coalson's group have tried to determine influence of various factors on acinar development in the preterm lung. The highlights of these work include :

- a. the finding that early extubation to CPAP with caffeine therapy in very preterm baboons (delivered at 125 days) preserved peripheral lung structure (i.e., similar to 156 day gestation baboon controls) when compared to baboons of similar gestational age which were ventilated longer (84).
- b. PDA ligation did not seem to have any effect on the histological evolution of chronic lung injury (66).
- c. Infection with ureaplasma was associated with profibrotic, proinflammatory responses (69) which are associated with BPD.

1.5.2.1.4. New BPD

It is clear that the pulmonary histopathological features in the preterm infants who were born in the era of modern neonatal care (i.e., antenatal corticosteroids, surfactant therapy, gentle ventilation and judicious use of oxygen) differ from the features in the classical BPD as described by Northway et al (57). These features were called 'new BPD' (85), and predominantly involve an arrest in acinar development characterized by larger, simpler and fewer alveoli (49).

1.5.2.1.5. Summary:

The complex interplay of being born preterm, and neonatal care involved in survival of these preterm infants can be summarised as follows:

1. Being born preterm exposes infant to the risk of deranged acinar development manifesting as larger and fewer alveoli (49). This risk increases with degree of prematurity. This is corroborated by clinical data showing increased risk of death and BPD with decreasing gestation age (86,87).
2. Use of higher ventilator pressures and higher oxygen concentrations can increase the incidence of BPD as shown by studies by morphometric studies by Hislop (section 1.5.2.1.2) and animal studies by the Coalson group (section 1.5.2.1.3).
3. Modern neonatal care (i.e., antenatal corticosteroids, surfactant therapy, gentle ventilation and judicious use of oxygen) can mitigate lung damage (section 1.5.2.1.3). Overall the incidence of BPD doesn't change (88) because of increased the survival of infants of even smaller gestational age, but the pattern of BPD changes (85).
4. Experimental work from baboons have shown that early extubation to nasal CPAP seemed to preserve alveolarization and lung infection, particularly with ureaplasma spp., tended to worsen lung injury (section 1.5.2.1.3).

1.5.2.2. SMALL FOR GESTATIONAL AGE AT BIRTH

In very premature infants, being growth restricted is known to exacerbate lung disease at birth, increasing the risk of neonatal respiratory distress syndrome. It is also associated with increased risk of developing bronchopulmonary dysplasia (89,90). The mechanisms by which intra-uterine growth restriction may contribute to deranged lung development may include compromised repair of lung injury, inability to resist hypoxia, barotrauma and infections (91). Nutritional restriction has been shown to be associated with reduction in number of alveoli by morphometry in near term rabbits (92). In an experimental model of IUGR in sheep, Rozance et al (93) found that near term IUGR lambs had significantly less radial alveolar count and less pulmonary vascularisation compared to near term control lambs.

There is evidence for long term effects of IUGR on lung function. Infants born small for gestational age are known to have decreased lung function in early infancy (94) and aged 5-11 (95), after adjustment for parental smoking and socioeconomic factors. The impact of fetal growth restriction on lung function measurements have been reviewed by Maritz and Morley (96).

1.5.2.3. NUTRITION

Nutrition is known to affect peripheral lung structure not only in the intra-uterine period. Decreased caloric intake can be associated with emphysema like changes in adulthood. Coxson (97) compared Computed Tomographic images of 21 young adults with anorexia nervosa with that of 16 age matched controls (all females). CT measures of attenuation confirmed that the adults with anorexia nervosa had changes similar to emphysematous lungs.

Massaro et al (60) showed by morphometric methods that calorie restriction leads to alveolar loss in mice . Following this, ad libitum refeeding of the starved mice resulted in regeneration of alveoli. This was despite both groups getting equivalent dose of retinol.

1.5.2.4. ENVIRONMENTAL TOBACCO SMOKE

Tobacco smoke is well known to be detrimental to the lungs, and is associated with adult COPD and emphysema (98,99). Exposure to ETS is associated with multiple problems in infants including intra-uterine growth restriction, poor infant and adult lung function. Emerging evidence suggests that it exposure to environmental tobacco smoke can damage peripheral lung development.

Avdalovic et al (64) determined peripheral lung structure using non-biased histological techniques in 3 groups of 5 infant rhesus monkeys at 13 months of age. The first and second groups were exposed to either filtered air or environmental tobacco smoke (ETS) throughout gestation and postnatal life while the third group was exposed to ETS in postnatal stage alone (from 6 months to 13 months of age). They found that the alveolar number was decreased in the ETS exposed groups compared to the non-exposed group and alveolar volume was highest in the group exposed to ETS both antenatally and postnatally.

Sekhon (100) exposed pregnant rhesus monkeys to nicotine using subcutaneous pumps. They delivered the fetus at 132 days (equivalent to 32 week gestation in humans) and analysed the peripheral structure of the fetal lung morphometrically. The mean linear intercept was higher and the alveolar surface area was lower in the nicotine treated group

compared to the controls group, suggesting that fetal nicotine exposure is associated with larger and fewer alveoli.

Elliot et al (101) examined the lungs of 32 infants who died of sudden infant death syndrome. They assessed the distance between alveolar attachments to intraparenchymal airways (a surrogate measure of alveolar development) by histological methods. Pre-existing questionnaire based data on ETS exposure was available in these infants. Multiple regression analysis showed that ETS exposure and decreased gestational age were associated with greater distance between alveolar attachments (i.e. deranged alveolar development). The effect was greater for in-utero exposure than postnatal exposure.

There is also a reported link between early life exposure to smoking and risk of emphysema in adults. Lovasi et al (102) studied emphysematous changes on CT scan in 1781 adult non-smokers and related this to retrospective, reported childhood ETS exposure. Early emphysema on CT chest was associated with greater childhood ETS exposure in these non-smokers.

1.5.2.5. OXYGEN

The effect of hyperoxia on lung development could be extrapolated from the effect on preterm born babies (section 1.5.2.1). In these situations, however, there are two other associated factors (immature lungs and positive pressure ventilation), that cannot be disentangled. However, animal evidence has clearly shown that hyperoxia in itself is detrimental to alveolar development.

Bucher and Roberts(103)exposed term born neonatal rats to varying concentrations of oxygen (21%, 40%, 80% and 95%) for 6-12 days. Some of the hyperoxia exposed rats were allowed to then grow in air for 1- 2 weeks (recovery period). They assessed lung development by determining the number of fully formed and partially formed secondary septae within fixed lung assessed by light microscopy. Septation was reduced in oxygen exposed rats. This tends to recover following growth in air. Oxygen exposure was also found to be associated with increased levels of oxygen protective enzymes (superoxide dismutase, catalase etc.). This study was able to show the dose-response relationship between oxygen exposure and impaired alveolar development in newborn rats.

Randell et al (104) exposed 6 male rats to 95% oxygen for first seven days of life and then allowed growth in 21% oxygen till 40 days of life. They compared alveolar structure in these rats against controls using bias-free disector techniques. Mortality was

greater in the 95% oxygen group. The number and volume of alveoli was similar in both groups, but the hyperoxia exposed group had greater heterogeneity in alveolar size. On light microscopy, the mean linear intercept (Lm) was higher in the exposed group.

Massaro and his group determined developmental responses of alveoli to hypoxia. First, they exposed groups of fetal and neonatal rats to 10% oxygen, at varying stages of their prenatal/postnatal life (55). The group exposed to hypoxia at late gestation and early neonatal life was found to have larger and fewer alveoli compared to the control group which was never exposed to hypoxia. The groups exposed to hypoxia in either early neonatal period only or in fetal life only did not show any detrimental effect due to hypoxia on peripheral lung structure. These experiments suggest that exposure to hypoxia in fetal and early neonatal life can cause decreased alveolar development.

Then they (105) exposed pregnant rats to 13% oxygen throughout their pregnancy. The term-born neonatal rat pups were exposed to 13% oxygen for 2-40 days, with a subgroup being exposed to 13% oxygen for 15 days and then removed to room air for 15-25 days (hypoxia- air group). The hypoxia only group had significantly lower body weight and higher lung volume/body weight ratio. They had significantly higher mean linear intercept (Lm) and lower surface area/volume ratio (S/V) compared to the control rats. The group that had been exposed to hypoxia for 15 days and then placed in room air (hypoxia-air group) had similar body weight and lung volume/body weight ratio compared to controls (in contrast to hypoxia only group). However, they had significantly higher Lm and lower S/V compared to controls (similar to the hypoxia only group). This indicates that exposure to 13% oxygen during fetal and neonatal life diminishes septation and impairs alveolarization. While body weight and lung volume may recover after the hypoxic stimulus ceases, this study suggests that impairment in alveolarization does not recover. The same group also exposed rats to 13% oxygen between 23 and 44 days of life and compared the structure of the peripheral lung in these rats to control rats (62). They found that the rats which were exposed to hypoxia between 23-44 days had larger lungs, larger alveoli and alveolar ducts compared to controls. It is clear from the above studies that both hypoxia and hyperoxia particularly in the early neonatal periods have a potential to impair alveolar development.

1.5.2.6. CORTICOSTEROIDS

1.5.2.6.1. Antenatal corticosteroids

The role of corticosteroids in antenatal lung development has been extensively studied. Antenatal corticosteroids, by upregulating the expression of surfactant proteins, help in decreasing incidence of respiratory distress syndrome in premature infants (106). They are accepted part of antenatal management of expected preterm delivery (78).

However, they are also known to have a detrimental effect on alveolar development. Massaro et al (61) administered glucocorticoids to pregnant rats at days 17-19 (gestation duration 21-23 days) and found that alveolar surface area and number were decreased at day 14 in the offspring compared to control animals. A similar effect of antenatal corticosteroids on postnatal lungs was found in rhesus monkeys (107) and lambs (108,109).

1.5.2.6.2. Postnatal corticosteroids

Corticosteroids have also been used in infancy in clinical practice, particularly in situations such as 1. Multitrigger infantile wheeze, 2. Croup and 3. Severe chronic lung disease (as rescue therapy).

The effect of postnatal corticosteroids on alveolar development in rat pups was studied by Massaro's group. They first treated newborn rats from day 4 to 13 with dexamethasone and determined the effect of this on alveolar development at day 14 and 60 by three dimensional reconstruction (110). They found profound effects on alveolar development. There were twice as many alveoli at 14 days of life in control rat pups than dexamethasone treated rats. This effect on alveolar development continued throughout period of body growth and there were 2.4 fold more alveoli at 60 days in control group than the dexamethasone treated group.

1.6.UNRESOLVED QUESTIONS, CONTROVERSIES AND WAYS TO SOLVE THEM

1.6.1. AGE OF COMPLETION OF ALVEOLARIZATION

It is clear from section 1.4.6 that the evidence regarding completion of alveolarization at the age of 3 years in humans is based on a few morphometric studies. Both the technique used in the studies and the comparability of the studies are open to

question. Repeating these studies in humans with newer histological techniques is impractical due to ethical constraints and difficulty in obtaining adequate specimen in children. On the other hand, recent animal studies indicate emerging evidence that alveolar development proceeds up to completion of physical growth (i.e. adulthood) in mammals (111-113). Post-pneumonectomy alveolarization has been shown to occur in mature animals (114). Non-invasive techniques are the only realistic options to answer the question regarding age of completion of alveolarization in humans.

1.6.2. INFLUENCE OF PRETERM BIRTH ON ALVEOLAR DEVELOPMENT

Histological studies of influence of preterm birth on human alveolar development have a flaw that it represents the severe end of the spectrum of disease. Studies using animal models were perfected to address this situation. However, animal studies do not look at survivors beyond the human equivalent of 3 years of life. Emerging evidence of continuing alveolarization in animal lungs suggest that there is a potential for repair of any damage sustained to the peripheral lung structure beyond 3 years of life(60,114). Again, non-invasive techniques are the only realistic option to determine whether preterm birth causes irreversible damage to alveolar structure in the human lungs.

1.6.3. FACTORS AFFECTING LUNG ALVEOLAR DEVELOPMENT

There has been limited research into factors affecting lung alveolar development in humans. There is little histological data from human studies. The current knowledge is derived from extrapolation from animal studies. In contrast to preterm birth, some of the other risk factors affecting alveolar development may act potentially throughout childhood and adolescence. Replicating this effect in animal studies is difficult. Non-invasive techniques may be the only way to clarify the effect of various risk factors on human alveolar development.

1.7.INTRODUCTION TO HELIUM MAGNETIC RESONANCE

It is clear that non-invasive techniques are need to resolve the unanswered questions and controversies in human alveolar development. The technique in question needs to be sensitive enough to resolve structures of the dimensions of human alveoli. In addition to being non-invasive and sensitive, application on healthy children dictates that they should not have the potential to cause harm.

Over the past 15 years, one such novel technique has been described and perfected. This depends on the measurement of self-diffusion of hyperpolarised helium-3

(^3He) in the lung periphery during a brief breath-hold using magnetic resonance (MR). A parameter known as apparent diffusion coefficient (ADC) can be determined, which is related to the dimensions of the enclosing structure, in this case the lung peripheral airspaces including alveolar ducts and alveoli. Recently it has also become possible to determine the average linear dimensions of the peripheral airspaces and alveoli by $^3\text{HeMR}$. The next chapter introduces $^3\text{HeMR}$ and the physical principles behind it.

2. HELIUM MAGNETIC RESONANCE

2.1. PRINCIPLE OF MAGNETIC RESONANCE

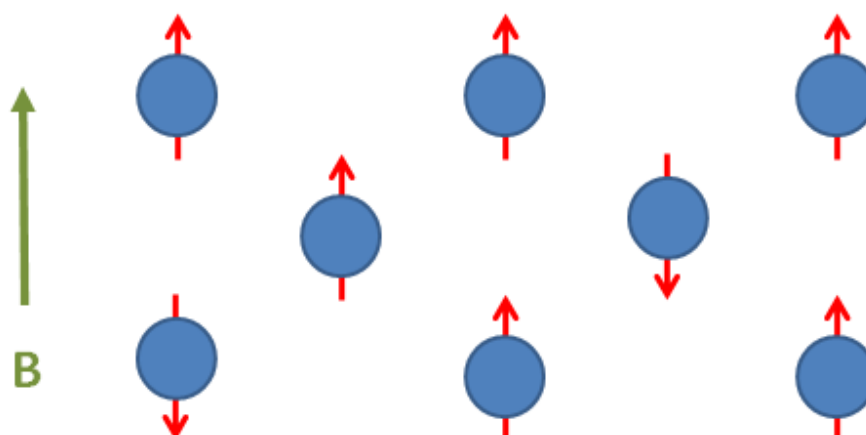
2.1.1. NUCLEAR SPIN AND ALIGNMENT IN EXTERNAL MAGNETIC FIELD

All atoms consist of nuclei, which are in turn made up of nucleons (positively charged protons and neutral neutrons). Nucleons have a fundamental quantum property called spin, which is related to magnetic force. Spin of a nucleon can be either $+\frac{1}{2}$ or $-\frac{1}{2}$. The overall spin of the nucleus depends on the spins of the constituent nucleons. Many elements have no net nuclear spin (e.g. Helium 4, the abundant isotope of helium) because the spin of the nucleons cancel each other out. However, many elements have a non-zero net spin (e.g. Hydrogen 1, Sodium 23 etc, Helium 3), typically those elements which have an odd number of nucleons.

Magnetic Resonance (MR) is based on the interaction of such atomic nuclei with an external magnetic field (115). In an external magnetic field, they orient themselves in one of two directions – Parallel, spin up or N^+ (lower energy state) and antiparallel, spin down or N^- (higher energy state) (115). Usually, there is a slight excess of the N^+ than the N^- state at equilibrium, of the order of 3ppm (at room temperature and high magnetic field) (116). In other words only three in a million nuclei contribute to the signal of the MR(115). The resultant of the tiny magnetisation caused by all the atoms aligning themselves up in this way is called the net magnetisation vector (Figure 2-1). This lies in the direction of the external magnetic field of the scanner (This direction is denoted as the z-direction or z-axis).

FIGURE 2-1

Representation of nuclei aligning to external magnetic field (B = external magnetic field). Image courtesy: http://bio.groups.et.byu.net/mri_training_b_Alignment_in_Magnetic_Fields.phtml



2.1.2. TIPPING BY EXTERNAL RF PULSE

When a MR scan is done, these nuclei are perturbed from their position of equilibrium by a short lasting external radiofrequency signal (RF excitation pulse or tipping pulse). The tipping pulse brings about a change in the direction of the net magnetisation vector due to a change in the overall magnetic field experienced by the nuclei (see figure 2-2 b and c). A specific frequency (known as the resonating frequency or Larmor frequency) is needed for interacting with a particular nucleus(117) , given by

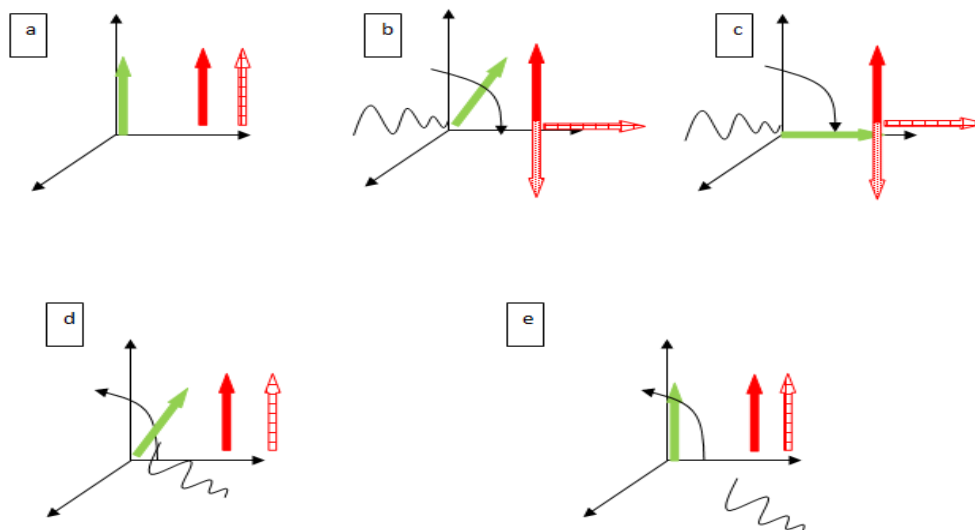
$$\nu = \gamma B \quad \text{EQUATION 2-1}$$

where ν is the frequency of external signal, B the strength of the external magnetic field it is placed in and γ a property of the nucleus called the gyromagnetic ratio.

After the termination of the signal, the nuclei tend to return back to the equilibrium state. This can be represented as the return movement of the net magnetisation vector to the original position (See figure 2-2 d and e). This return movement results in the emission of radio waves (also with the same frequency), which are the basis of the MR signal (spin relaxation).

FIGURE 2-2

Figure showing the sequence of Magnetic resonance: a: aligned spin in an external magnetic field. b): tipping pulse tips the spin in the direction of the resultant magnetic field. c): completely tipped state. d): after tipping pulse stops, spin reverts back to earlier alignment producing RF. e): end of relaxation.



(legend - 1: solid red arrow: external magnetic field. 2. Green arrow: direction of net magnetic vector. 3: dotted red arrow: magnetic field due to RF pulse. 4. Dashed red arrow: resultant magnetic field. 5: waveform: radiofrequency waves (External pulse - horizontal, emitted pulse - oblique))

2.1.3. SPIN RELAXATION – T1 AND T2 PROCESSES

The return of the net magnetization vector to the equilibrium position is governed by two processes called T1 and T2 process respectively. The net magnetization vector has two components – the z-component along the z-axis (same direction as the magnetic field of the scanner) and the xy component along the transverse axis (perpendicular to the magnetic field of the scanner). Expressed as a vector equation, the net magnetization vector,

$$M = M_z + M_{xy}$$

EQUATION 2-2

Before the RF pulse is applied to the nuclei, the net magnetization is entirely along the z axis, i.e., $M_0 = M_z$. Consider the case when the RF pulse causes the net magnetization to be completely transverse as shown in figure 2-2c above (i.e. Longitudinal magnetization at this time τ , $M_z = 0$). After the tipping pulse, the longitudinal magnetization gradually increases and the transverse magnetization gradually decreases (back to equilibrium state- figure 2-2e). The time constant which describes how M_z returns to equilibrium value is called the T₁ relaxation time (and the process is called T₁ process). The equation governing this is given as follows:

$$M_z = M_0(1 - e^{-t/T_1})$$

EQUATION 2-3

where t is the time after the end of the tipping pulse. The parameter T₁ (T₁ relaxation time or spin-lattice relaxation time) is the time to reduce the difference between longitudinal magnetization and equilibrium value by a factor of e.

With regard to horizontal magnetization, this gradually decreases back to the equilibrium value of 0 after the tipping pulse is turned off. The horizontal magnetization decreases according to an equation shown below:

$$M_{xy} = M_{xy,\tau} e^{-t/T_2}$$

EQUATION 2-4

where t is the time after displacement of the net magnetization vector to a completely transverse position and $M_{xy,\tau}$ is the baseline value of M_{xy} at the time the relaxation starts. The parameter T₂ (T₂ relaxation time or spin –spin relaxation time) is the time taken to reduce the transverse magnetization by a factor of e (117). This relaxation of transverse magnetization is due to inter-nuclear interactions between the individual atoms/molecules.

The horizontal component of magnetization, apart from decreasing as per equation 4, also will rotate (precess) about the z axis at a frequency equal to the Larmor frequency (equation 2-1). In practice, small variations in the magnetic field of the scanner

can cause slightly different precession for different nucleons, which then interact to cause a more rapid decay in the transverse magnetization than predicted by the T_2 parameter. This results in an apparent T_2 which is known as T_2^* .

2.1.4. MANIPULATING SIGNAL – GRADIENTS AND SEQUENCES

A single RF pulse in a uniform magnetic field causes all excited atoms to contribute to the MR signal equally. This is not useful for clinical or research application because spatial information is lost.

However, if the nuclei are subjected to different magnetic fields based on location, a certain population of nuclei can be selected for each frequency of the tipping pulse (because the Larmor frequency is dependent on the strength of the resultant magnetic field). This is achieved by a magnetic field gradient applied at the same time as the tipping pulse (Slice select gradient). Similarly, gradients applied at the time of relaxation cause nuclei at different positions to precess with different frequencies (giving out signals of a mixture of frequencies). These signals can be analysed by mathematical techniques called Fourier transform to deduce spatial information from the frequency of emitted radio waves (phase encoding gradient). A typical MR imaging system uses gradients of magnetic field (phase encoding gradient and slice selection gradient) to encode the MR signal so that a picture of the tissue in question can be built up.

A co-ordinated set of radio-frequency pulses and gradients with fixed time-intervals - also known as the MR sequence - is used to maximize the information obtained from the MR scanner. They are selected in such a way as to increase signal, improve contrast and reduce noise in the minimum time possible (118).

2.2. HYPERPOLARISED GAS MAGNETIC RESONANCE

Currently, clinical MR scanners use the proton (Hydrogen nuclei) as the basis for image generation. Hydrogen nuclei are found in abundance (as water) in the body. However, imaging the lung needs a different approach, because most of the lung is a cavity filled with air, which is not suitable for proton-based MR. Inhaled gases are too sparse of nuclei to give a meaningful conventional MR image.

Hyperpolarisation of gases is a technique to surmount these difficulties. Instead of relying on the externally applied magnetic field to orient the nuclei, this orientation is carried out externally by a technique called optical pumping by lasers (116). This typically results in the $N^+ : N^-$ ratio reaching the region of 1.08-1.15 rather than 1.000001, increasing

signal strength obtained about 100,000-fold. The gases typically used for hyperpolarised lung magnetic resonance are Helium-3 and Xenon-129. They have the advantage of being chemically inert, though xenon has some anaesthetic properties in high concentrations. Helium-3 is relatively more expensive. The following account deals with hyperpolarised Helium-3 MR ($^3\text{HeMR}$).

2.2.1. DIFFUSION WEIGHTED HYPERPOLARISED 3-HELIUM MR

Generation of conventional images using conventional sequences is very difficult with $^3\text{HeMR}$. Firstly, because the polarization is done externally, it is not recoverable. The maximum length of the whole sequence is limited by the T_1 property of Helium, which is about 25 seconds. Also, the spatial information encoded by the gradients is lost because of the diffusion of the helium nuclei. Again, the breath-hold time limits the duration of the sequence. On the other hand, sequences which can be used to determine information regarding diffusion of helium molecules have been developed in the last decade. This in turn can be used to determine the characteristics of the periphery of the lung. There are 2 diffusion-weighted sequences that are used in this study.

2.2.2. MODIFIED RARE (RAPID ACQUISITION WITH REFOCUSED ECHOES) SEQUENCE:

FIGURE 2-3

RARE sequence

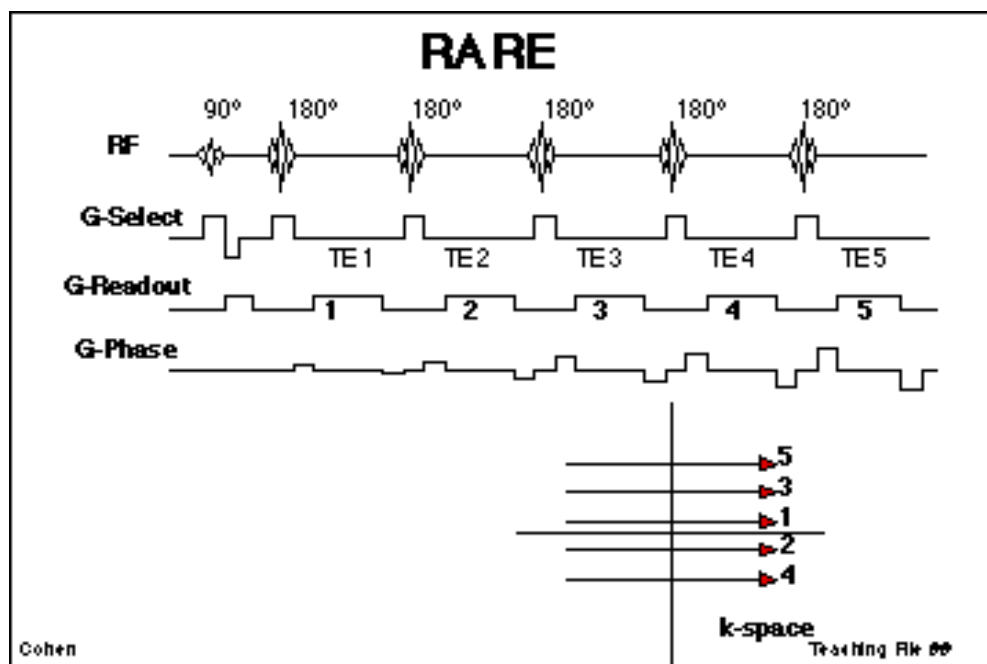
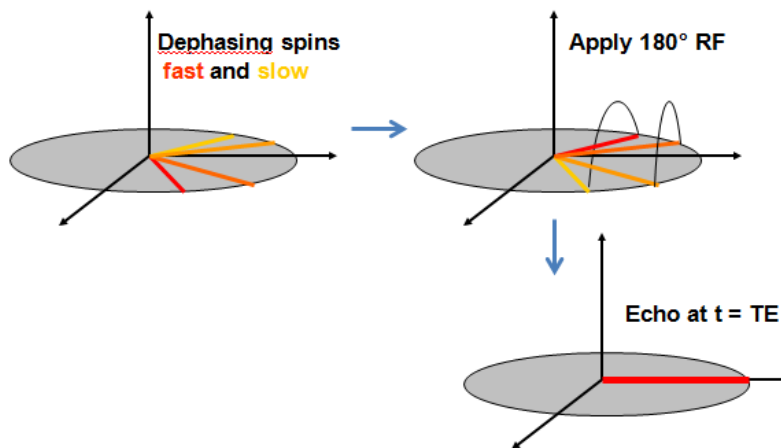


Image courtesy Dr Mark Cohen, PhD. <http://airto.hosted.ats.ucla.edu/BMCweb/SharedCode/slides/SlideFiles.html>)

The most common sequence used for capturing diffusion data is the modified RARE sequence. The RARE sequence consists of a 90° RF pulse followed by a train of 180° pulses. The effect of the 180° pulses are to effectively 're-phase' the signals. This helps to negate the effect of $T2^*$. If there were a number of nuclei that are precessing at different rates, the 180° degree pulse effectively inverts their rotation in the XY plane (Figure 2-4). Therefore after the application of the 180° pulse at a given time say 't', the nuclei which precess faster are behind the nuclei which precess slower and they catch up at time '2t'. Phase encoding and slice select gradients are applied between each 180° pulse. The signal readout takes place between the 180° pulses.

FIGURE 2-4

Refocussing of echoes.

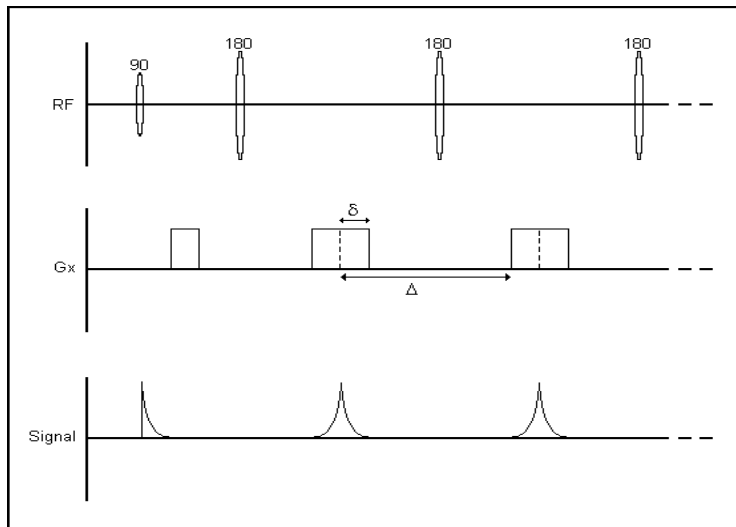


In modified RARE sequence employed by our team, the phase encoding and slice select gradients are switched off (Figure 2-5). A positive read out gradient lobe is employed before and after each 180° pulse. The effect of the repeated 180° pulses and the read-out gradient is to reduce the signal from nuclei which move or diffuse but maintain signal due to stationary nuclei(119). Therefore, they are termed 'diffusion weighting gradient'. Signals will be refocussed only if they are stationary. If they are mobile, the movement causes a change in the frequency of precession due to the gradient lobe and signal decay is faster. Signal can thus be weighted according to the degree of diffusion.

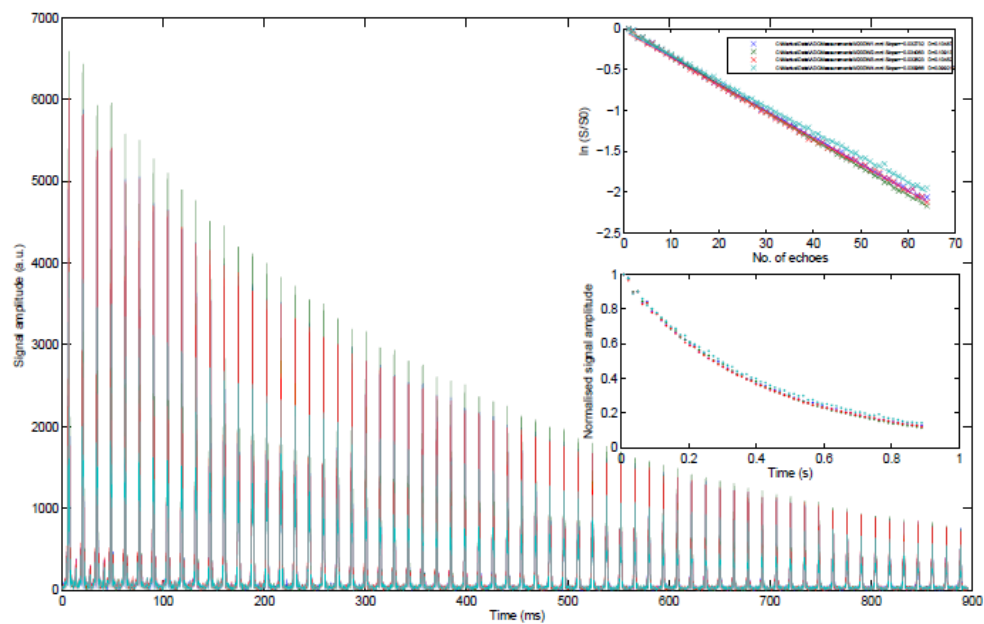
The gradient strength of the diffusion weighting gradient in our case is $b=0.3\text{s.cm}^{-2}$. The sequence (Figure 2-5) results in a signal similar to the one depicted in figure 2-6 below.

FIGURE 2-5

Modified RARE sequence: image courtesy Dr Marius Mada, PhD. Thesis submitted to Nottingham University.

**FIGURE 2-6**

Signal vs. time: exponential decay. Train of echoes following RARE sequence. Inset, Top right - semilog plot of $\log(\text{signal})$ vs. echo number with linear fit (3 different measurements with one showing the mean of the 3 measurements)



It is seen that the signal due to each pulse decays very rapidly (the T_2^* property, see 2.1.3). The 180° pulse 'refocuses' stationary nuclei so that they are in the same phase

(117). The mobile nuclei experience varying magnetic field due to the READ gradient and dephases differently to the stationary nuclei. Therefore, in case of stationary nuclei, the exponential decay of the envelope of the signal is related to the T_2 property of the nuclei. In case of mobile nuclei, decay is faster depending on their net movement (characterized by the diffusion coefficient (D)) by the equation described by Torrey (120)

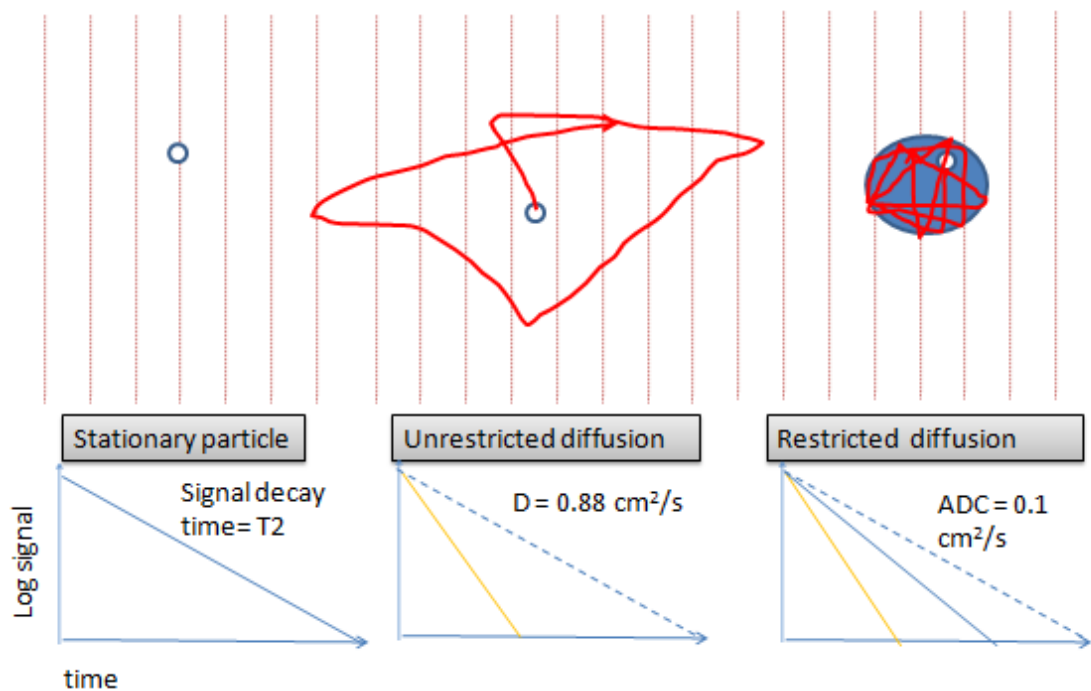
$$\frac{1}{T_{2,apparent}} = \frac{1}{T_2} + \frac{1}{3} D \gamma^2 G^2 t_1^2 \quad \text{EQUATION 2-5}$$

where D is the diffusion coefficient, γ is the gyromagnetic ratio, G is the gradient of the magnetic field and t_1 is half the time interval between successive 180° pulses. In free space the diffusion coefficient of pure ^3He is $D = 1.8 \text{ cm}^2 \text{ s}^{-1}$ and that of ^3He in Nitrogen is $D = 0.798 \text{ cm}^2 \text{ s}^{-1}$ at 324K and 1 atmosphere.

Nuclei of ^3He in an enclosed space diffuse less readily than in free space due to the constraints imposed by the walls. In this case, the measured value of D differs from the diffusion coefficient in free space and is called the apparent diffusion coefficient (ADC). ADC is a measure of restriction of diffusion and can be used as a surrogate for the physical dimensions (figure 2-7).

FIGURE 2-7

Diffusion weighted signal. The diffusion coefficient (D in the case of unrestricted diffusion and ADC in the case of restricted diffusion) is a measure of the degree of constraint experienced by the individual molecule.



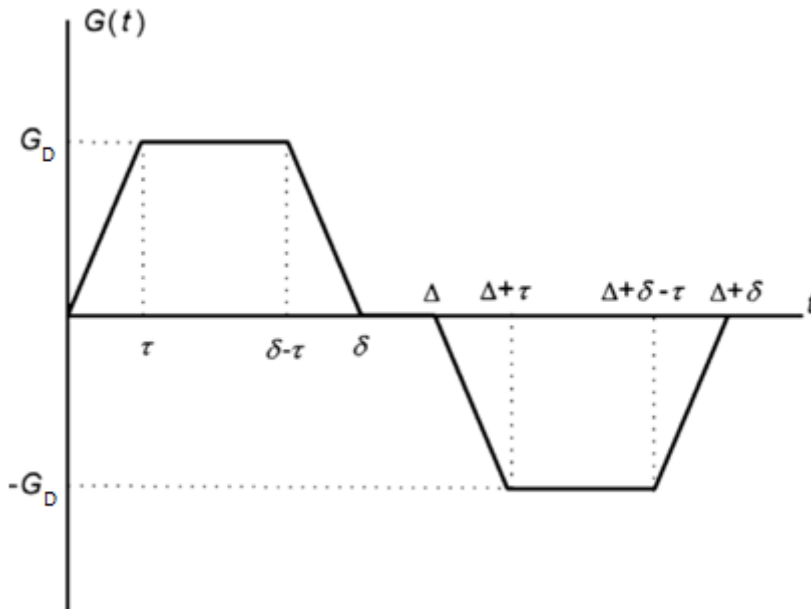
In the case of the lungs, therefore, where the majority of the nuclei are constrained by the alveolar wall, it is a measure of alveolar dimensions.

2.2.3. THE Q-SPACE TECHNIQUE

The second sequence used in our studies is characterized as the q-space technique (figure 2-8). In essence this technique enables us to derive a probability density function of displacements of the helium nuclei, thus giving information about the degree of restriction of movement (and therefore about the microstructure of the lung acinus) of the nuclei.

FIGURE 2-8

Sequence diagram for the diffusion sensitizing signal of the q-space technique



In q-space technique the diffusion sensitization of the nuclei (characterized by the b-value) are varied for each acquisition. The variation in b-values changes the proportion of signal arising from relatively slow moving and relatively faster moving nuclei, thus giving better insight into anisotropic diffusion coefficients. The b-value depends on gradient time, diffusion time and gradient strength. In our case, the b value is changed by varying gradient strength. Because the gradient lobes are biphasic and trapezoidal in our case (figure 2-8), the b value is given by (121):

$$b = \gamma^2 G_D^2 \left[\delta^2 \left(\Delta - \frac{\delta}{3} \right) + \frac{\tau^3}{30} - \frac{\delta \tau^2}{6} \right]$$

EQUATION 2-6

where γ is the gyromagnetic ratio of helium, G_D is the diffusion sensitizing gradient, τ is the ramp time of gradient, δ is the duration of one lobe of the bipolar gradient and Δ is the diffusion time. The signal from this sequence can be analysed in two ways.

2.2.3.1. DIFFUSION WEIGHTED SPECTROSCOPY

The signal strength for each acquisition is normalized and plotted against the respective q-value, which is defined as

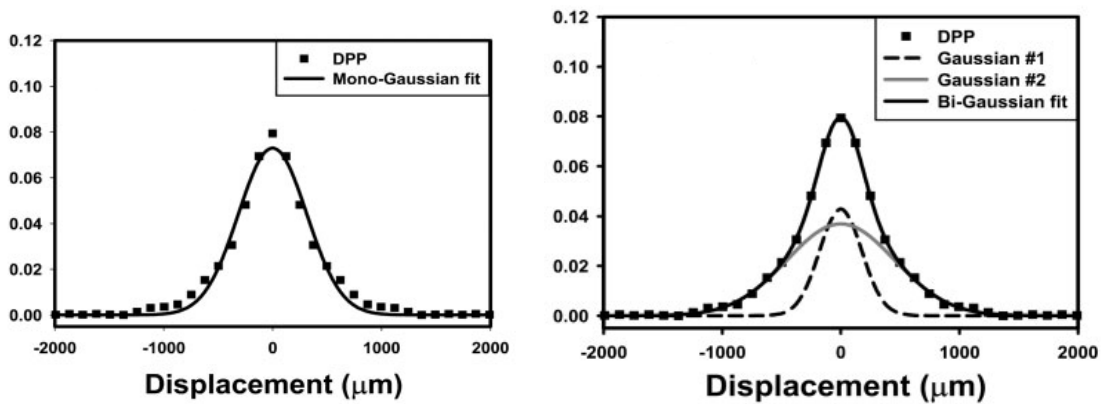
$$q = \frac{1}{2\pi} \gamma \delta G_D \sqrt{\frac{\Delta - \delta/3}{\Delta + \delta}} \quad \text{EQUATION 2-7}$$

q is a measure of the degree of dephasing experienced by the nuclei during application of the gradient pulse. Fourier transform of the q-space curve is carried out to give the displacement probability profile of the helium nuclei. Details of the mathematical process behind this is beyond the scope of this work, but further details can be found in the paper by Shanbhag et al and the thesis by our collaborator Mada (121,122).

The diffusion probability profile (DPP) is a frequency distribution curve with displacement on the x axis and probability on the y axis. It can be thought about as a mathematical illustration of the probability of helium molecules moving a certain extent within the diffusion time.

FIGURE 2-9

Monogaussian and bigaussian models of the diffusion probability profile.



In case of uniform restraint in all directions, the DPP will fit a Gaussian distribution. In this case, the RMS width of the displacement is known as X_{RMS} , the root-mean squared displacement of the ^3He gas. This gives a measure of the linear dimension of

the airways which restrict diffusion and is characterised as the mean peripheral airspace dimension.

In some cases, mono-Gaussian fit does not explain all the data. In these instances, a multi-Gaussian fit is used to represent the data. This model regards the helium molecules as belonging to different families based on their displacements (e.g. In the bi-exponential case, slow/ short diffusing family of helium and fast/longer diffusing family of helium). This can be represented by the models expressed in terms of signal and DPP for m number of families of helium nuclei as proposed by Shanbhag (121) and adapted by Mada (122) for the sequence employed in our study as summarised below:

$$S = \sum_{n=1}^m S_n e^{-bD_n} \quad \text{EQUATION 2-8}$$

where S is the cumulative signal and S_n is the signal from each family of ^3He molecules and D_n , the respective ADC.

and

$$DPP(x) = \sum_{n=1}^m Z_n e^{-0.5(\frac{x}{X_{RMS,n}})^2} \quad \text{EQUATION 2-9}$$

where DPP(x) is the displacement probability for a given displacement x, Z_n represents the zero DPP ($DPP(x=0)$) for the nth family, and $X_{RMS,n}$ the rms displacement of the nth family.

Shanbhag et al (121) determined that their data most closely approximates a bi-Gaussian distribution and found two populations of helium atoms with the majority having a shorter RMS displacement $X_{RMS,1}$, and a minority having a longer RMS displacement, $X_{RMS,2}$. They propose that the shorter scale was similar to the size of alveolar sacs and the longer scale approximates the size of the transitional and respiratory bronchioles. Our data was originally fitted to the bi-gaussian model of Shanbhag. However, $X_{RMS,2}$ did not satisfy the criteria for restricted diffusion (123) (i.e., it was within confidence interval for free diffusion of helium molecules). Therefore, our data was fitted to a simplified mono-exponential distribution.

$$DPP(x) = Z e^{\frac{-x^2}{2(X_{RMS})^2}} \quad \text{EQUATION 2-10}$$

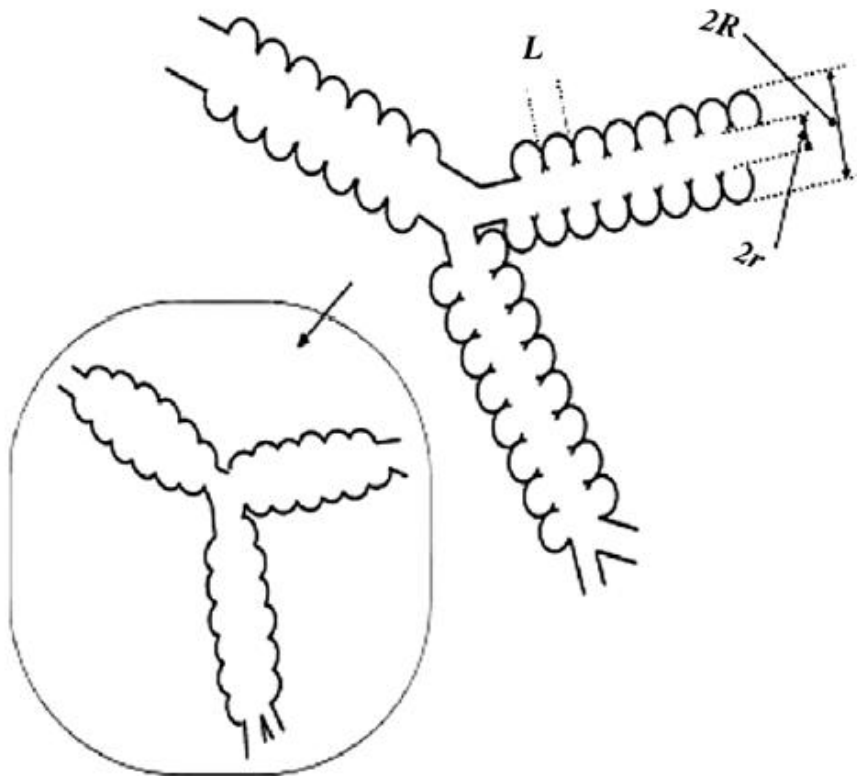
Here, the term X_{RMS} is a measure of the mean displacement of the helium atoms in the restricted environment of the alveoli and alveolar ducts within the diffusion time Δ , and is a measure of the mean peripheral airspace dimensions.

2.2.3.2. YABLONSKIY'S ACINAR MODEL

Yablonskiy et al (124) tried to give a physical meaning to the anisotropic diffusion coefficients. They constructed an acinar model which consists of sets of branching tubes (alveolar ducts) surrounded by alveoli. Because 95% of gas in the lungs is in the acini, they give rise to the majority of signal. They propose that there are two populations of helium nuclei, one travelling along the axis of the alveolar duct and the other travelling across. These two populations have two distinct diffusion coefficients characterized as D_L and D_T respectively. At low b values, all airways contribute equally to the measured signal, whereas at higher b values, signal will be generated by gas diffusing the shorter distance (i.e. D_T). Though Yablonskiy used a different sequence to determine D_L and D_T , these parameters can also be determined by the q -space sequence as well. In a further paper, Yablonskiy et al (125) used Monte Carlo simulations to determine the geometrical parameters r (internal radius), R (external radius i.e. radius including alveolar sleeve) and L (distance between two alveolar walls) from the values for D_L and D_T .

FIGURE 2-10

Yablonskiy's acinar model (124). Yablonskiy characterises acinar model as branching cylinders (representing alveolar ducts) surrounded by spherical alveoli.



2.3.VALIDATION OF ADC AS A MEASURE OF PHYSICAL DIMENSIONS

2.3.1. VALIDATION OF ADC AGAINST MICROSCOPY

Chen et al (126) compared the apparent diffusion coefficient (ADC) in two groups of rats, one treated endotracheally with elastase and the other treated with saline in the same way. They showed that the elastase treated rats had higher ADC than the saline treated rats at end expiratory volume. They then demonstrated by histology that the elastase treated rats had higher mean linear intercept (L_m) than the saline treated rats. They did not do a direct comparison because the volume of fixation was different to the volume at which ADCs were obtained.

Peces-Barba et al (127) compared ADC obtained by MR imaging against L_m and alveolar internal area(AIA) in two groups of rats, a control group and one with elastase-induced emphysema. The ADC measurements were done ex-vivo in a lung inflated with nitrogen and the histological measurements done were done at inflating pressure of 25cm water. Significant correlation was found between regional ADC and corresponding L_m and AIA(correlation coefficient = 0.7 and 0.71 respectively).

Woods et al (128) compared histology with ADC derived by ^3He MR in explanted human lungs, using lungs of 6 patients who had undergone bilateral lung transplantation for advanced COPD. 3 lungs of unsuitable donors were used as controls. Both regional and global comparisons were done. They showed a strong negative correlation between the global ADC of the 9 lungs and their morphometrically measured surface area/volume ratio(SA/V) (correlation coefficient =0.96). Regional correlations were also significant, with higher regional ADC corresponding to higher regional L_m ($r=0.62$) and lower regional SA/V($r=0.61$).

Mata et al (129) evaluated the progression of elastase induced emphysema in a rabbit model. They conducted serial measurements of ADC in rabbits using both hyperpolarised Helium and Xenon MR at baseline, 2 weeks, 4 weeks, 6 weeks and 8 weeks after instillation of elastase or saline(in control group) intratracheally. They then euthanized the rabbits and measured mean chord length(MCL) histologically. MCL was found to correlate extremely well with both global Helium ADC(correlation coefficient = 0.65, 0.66) and global Xenon ADC(correlation coefficient = 0.79, 0.80). The regional measurements were also closely correlated with regional ADC. The features of the study were the demonstration of correlation of in-vivo ADC against histology and the demonstration of the ability to non-invasively assess progress of alveolar destruction by elastase.

2.3.2. VALIDATION OF ADC AGAINST OTHER IMAGING

Tanoli et al (130) induced emphysema in 4 dogs using pancreatic elastase. Serial measurements of ADC in 2 dogs were made. The final ADC was compared with the CT image of the lungs. There was good correlation between ADC and both the derived parameter V, specific volume of gas per volume of tissue and the raw parameter Hounsfield units (negative correlation).

2.3.3. VALIDATION OF ADC AGAINST LUNG INFLATION

Waters et al (131) report measurement of ADC at different degrees of inflation in the same lungs. A tight linear relation was found between fractional change of volume and fractional change of ADC. ADC was found to change by 28% for a doubling of resident volume. This figure is similar to that expected for a change of linear dimension for a doubling of volume because $2^{1/3} = 1.26$

2.3.4. VALIDATION OF Q-SPACE AND YABLONSKIY PARAMETERS

Jacob et al (132) determined newer histological parameters and MRI parameters on two sets of rats, an elastase induced emphysema group and a saline control group. The histological technique used the method of Parameswaran et al (132) after euthanasia 3 weeks after elastase/ saline instillation. This consisted of first measuring individual airspace areas (A) from histological samples, determining the equivalent diameter ($D_{eq} = \sqrt{4A/\pi}$), and then determining the mean (μ), first (D_1) and second (D_2) moments of the distribution of airspace sizes. Applying Yablonskiy's acinar model (124) on a 3D diffusion weighted $^3\text{HeMR}$ sequence obtained at 1, 2 and 3 weeks after instillation, they determined first the longitudinal and transverse components of apparent diffusion coefficients (D_L and D_T respectively) and derived the diffusion anisotropy, $D_{AN} (= D_L - D_T)$, average diffusivity, $D_{ave} (= (D_L + 2D_T)/3)$ and apparent airway radius, R_{mean} . Here D_{ave} is equivalent to the apparent diffusion coefficient, ADC. They found very strong association between the histological parameters and the $^3\text{HeMR}$ parameters derived from Yablonskiy's model. They found significant correlations between mean airspace size (μ) and D_{ave} , D_{AN} and R_{mean} ($R = 0.86, 0.87$ and 0.8 respectively). The first moment of distribution of airspace size (D_1) also showed significant correlation with D_{ave} , D_{AN} and R_{mean} ($R = 0.85, 0.86$ and 0.79 respectively). The correlation coefficient between the second moment of distribution of airspace size (D_2) and D_{ave} , D_{AN} and R_{mean} were $R = 0.85, 0.88$ and 0.77 respectively.

In 2009, Yablonskiy et al (125) published their own histological verification of their $^3\text{HeMR}$ technique and parameters derived from it. They used 6 human lung specimen, 2 of which were normal (from unmatched lung transplant donors), 2 with mild to moderate emphysema (resected for peripheral lung cancers) and 2 with severe emphysema (diseased lungs from recipients of lung transplants). They determined the parameters D_L , D_T , R , r and L from the $^3\text{HeMR}$ measurements (see 2.6.3.2.2). Using an automated algorithm, they were able to determine L_m from digital photomicrographs of formalin fixed specimen. They showed that D_L , D_T , ADC and R increased with L_m from histology. They also found that h , depth of alveolar sleeve ($=R-r$) decreased with increasing emphysema, as has been shown in previous histological studies. Using mathematical methods, they were able to estimate L_m from $^3\text{HeMR}$ measurements of R , r , h and L . The correlation between the $^3\text{HeMR}$ derived and the histologically determined L_m was found to be 0.985.

In 2010, O'Halloran (133) et al, published measurements of X_{RMS} by the q-space technique and demonstrated its increase with lung inflation with various boluses of gas (500, 1000 and 1500 ml) in a human subject. They noted that inflation from 500 ml to 1000 ml causes more change in the X_{RMS} than inflation from 1000 ml to 1500 ml. This is probably because the fractional change of lung volume is greater for the former change in bolus volume. It may also be because of recruitment of closed alveoli at volumes near the TLC.

2.3.5. REPEATABILITY OF ADC

Morbach et al (134) measured the ADC using $^3\text{HeMR}$ in 5 healthy volunteers and 6 patients of COPD. They assessed repeatability by measuring the parameter twice in the same subject without repositioning the subject - the interval between the measurements being 20 minutes. They found a repeatability coefficient of 5.1% between volunteers and 6.1% between subjects.

Mata et al (129) found that pairwise ADC s done using the same magnetic parameters varied by a mean of 1.7%(range 0.26 -7.6%) in rabbits and only one out of 16 paired measurements had variation of greater than 5%.

2.4. INVESTIGATING ALVEOLAR DEVELOPMENT WITH $^3\text{HeMR}$

Therefore, it is clear that $^3\text{HeMR}$ is a non-invasive technique that has been validated as a measure of dimensions of peripheral lung structures, including alveoli. It involves only a single breath hold of up to 10 seconds (and less than 3 seconds in case of ADC). In addition, the technique does not depend on ionising radiations like X-Rays unlike other imaging techniques like CT scan. It does not involve radiation emitting isotopes. Therefore it is a very safe technique. It is repeatable with a very low coefficient of variation. Being non-invasive, longitudinal studies are possible. Moreover, there are no sampling issues within the lung as it can provide the average measurement across the whole lung. It is clear that this is an ideal technique to try to answer unresolved questions about human alveolar development (Section 1.6).

2.5. AIMS AND OBJECTIVES

The main aim of this study was to investigate lung alveolar development in children using $^3\text{HeMR}$. There were 3 main objectives:

- To test the hypothesis that alveolar development in humans is complete by 3 years of age.
- To determine whether alveolar catch-up is possible following birth <32 weeks of gestation and in survivors of chronic lung disease of prematurity.
- To identify the effects of various factors that could potentially affect human alveolar development.

The methods used in the study are discussed in the following chapter (chapter 3). The 3 chapters following the methods chapter (chapters 4, 5 and 6) discuss the results of the study pertaining to the above 3 objectives. A full discussion of the results follows the results section.

3. METHODS

3.1.SUBJECTS

3.1.1. SOURCES OF SUBJECTS

The study protocol had full approval from the research ethics committee (LREC number 04/Q2501/114 (appendix), Sponsorship number UHL 09580). Children were selected from three sources.

Leicestershire Respiratory Cohorts (LRC):

Leicestershire respiratory cohorts (LRC) are 2 community based cohorts of children who were followed up from early childhood. They are a random sample of population who were recruited from the Leicestershire Health Authority Child Health Database. The LRC is composed of a total of 10350 subjects (1650 young adults born between 1985 and 1990 and 8700 children born between 1993 and 1997). They have been followed up by frequent questionnaires, interviews and measurements. Maternal and environmental factors and early respiratory health of these children have therefore been well characterized prospectively (135). A stratified random selection of subjects from the LRC cohorts (stratified by year of birth and presence or absence of risk factors known apriori associated with alveolar damage (see below)) was identified for inclusion in this study. The current study commenced in 2007 and recruitment was complete in 2009. These subjects were aged between 10 and 23 years at recruitment.

Trent Neonatal Survey (TNS):

Trent Neonatal Survey (TNS) holds data regarding all infants born in Trent region (Leicestershire and Nottinghamshire) at or below 32 weeks of gestation from February 1990 onwards (136). It includes detailed information about the antenatal period, birth history and stay in the neonatal unit for these children. Children who were born between August 1996 to August 1998 and resident in the Leicestershire were identified by stratified random selection (stratified by year of birth and presence of chronic lung disease of prematurity) for inclusion in this study.

Community Health Services(LSCCHS) database:

Children between 7 and 11 years old were recruited from the Leicestershire Specialist Community Child Health Services (LSCCHS) database. This database stores records of all children in Leicestershire. This group was recruited because the youngest child belonging to the LRC was 11 by the time this study started. The younger age limit was designed to reflect the lowest age of compliance with complex respiratory

manoeuvres needed for the study. Children were identified by stratified random sampling (stratified by year of birth, 1997-2001) from LSCCHS database for inclusion in this study.

3.1.2. RECRUITMENT

The protocol for recruitment of subjects is described in this section. Potential subjects were identified by stratified random sampling from the various databases as described above. For subjects identified from the TNS and the LCCHS cohorts, prior information on the suitability for this study was not available. We sent letters to the general practitioner (GP) of each potential subject asking them to report back if they considered that the person could not do standard lung function measurements or considered that the subject was unsuitable for the study for other reasons. We enclosed a self addressed envelope to facilitate the process. If the GP did not respond to the initial letter within an interval of about 3 weeks, a second letter was sent out. The potential subject was excluded only if the GP replied in the negative to one of these letters. We did not write to the GP for the potential subjects identified from the LRC.

We sent out invite letters to most of the subjects who were identified as above. The invitation package contained the information leaflet to parent, information leaflet for child or young adult (in age appropriate language) and a reply slip with a self addressed envelope. Again two sets of letters were sent with an interval of about 3-4 weeks. All the documents and the protocol of invitation were approved by the research ethics committee. Subjects who consented to the study were contacted by telephone and a mutually convenient appointment was made.

We identified 633 subjects from the LRC cohort by stratified random selection (stratified by age and presence or absence of risk factors associated with alveolar damage (see below)). This included 274 children with one or more of the following risk factors: maternal smoking, late preterm birth, low birth weight or early childhood wheezy illness and 359 children without these factors. Of these, 404 were invited and 149 agreed to take part in the current study. Of these children, 119 children participated in the study (3 failed to attend appointments and 27 were not contactable or withdrew consent after initial agreement) (See Table 3-1).

In case of the LCCHS cohort, we identified 347 potential participants. A total of 347 GP letters were sent out and in 12 cases, the GP replied that the children were unsuitable for the study. Of the 335 children who were potentially contactable, 265 were invited to the study, of who 63 agreed to take part. 54 children took part in the study, 2 did not

attend the appointment and 7 were not contactable or withdrew consent after initial response (Table 3-1).

In case of the TNS cohort, a total of 147 GP letters were sent out and in 7 cases, the GP replied that the children were unsuitable for the study. Of the 140 children who were potentially contactable, 116 were invited to the study, of who 46 agreed to take part. 36 children took part in the study, 1 did not attend the appointment and 9 were not contactable or withdrew consent after initial response (Table 3-1).

TABLE 3-1

Recruitment table for the study.

	LCCHS	LRC	TNS	Total
GP Letter	347	-	147	
GP no	12	-	7	
GP yes	203	-	78	
GP no reply	132	-	62	
Potentially contactable	335	633	140	1108
Invited	265	404	116	785
Agreed	63	149	46	258
Refused	45	66	13	124
No reply	157	189	57	403
DNA	2	3	1	6
Not contacted	7	27	9	43
Participated	54	119	36	209

Volunteers for the bolus effect study

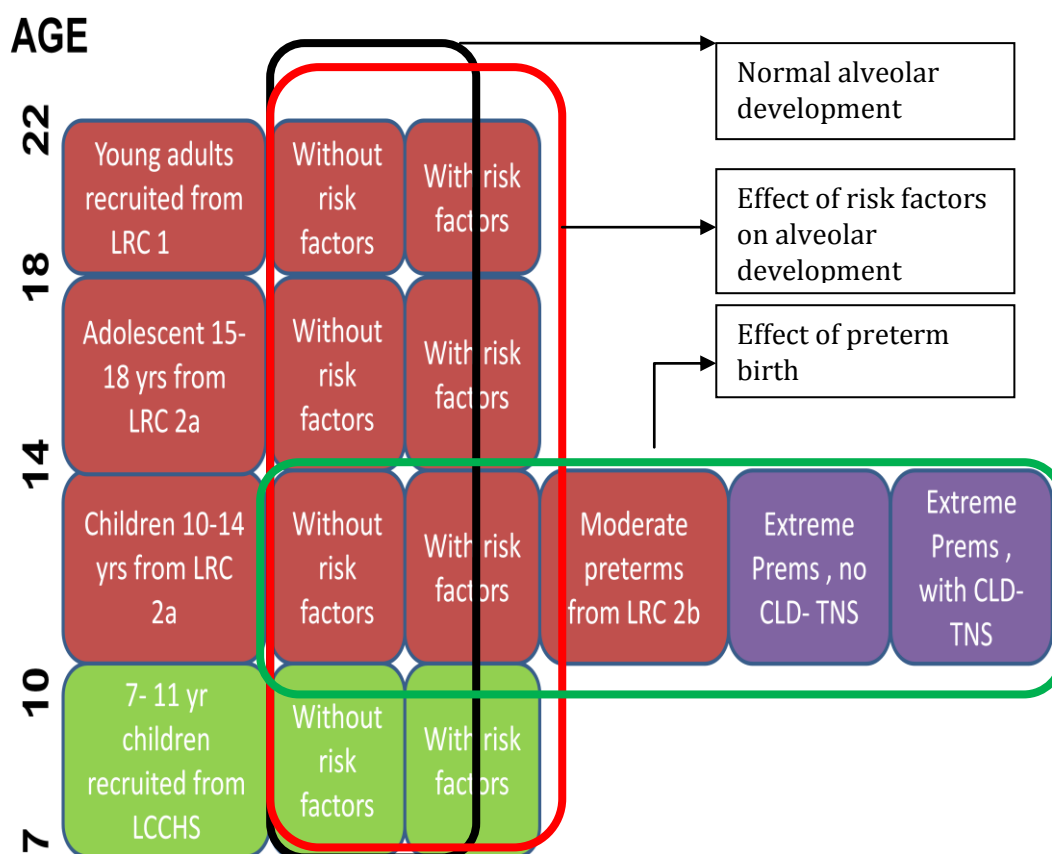
Measurements were also done on 4 children who were not part of either of the three abovementioned cohorts. These volunteers had heard about the study from our subjects (2 were siblings of subjects and 2 were classmates) and wanted to participate. The data from these children were not included in the main study but only as a part of determination of change of ADC by volume of helium inhaled (bolus-effect study)

3.1.3. SELECTION OF SUBJECTS FOR THE INDIVIDUAL ASPECTS OF THE STUDY

Selection of the recruited subjects for the various aspects of the study will be covered in detail in the individual chapters. Figure 3-1 gives the plan of recruitment. Children who were born at greater than 37 weeks gestation were stratified based on presence or absence of the following risk factors, if known a-priori (small for gestational age at birth, exposure to environmental tobacco smoke and pre-school wheeze).

FIGURE 3-1

Schematic diagram showing the recruitment sources of the children invited to the study and the selection of these subjects for answering the various objectives raised by the study.



Legend :

LRC: Leicestershire respiratory cohorts (LRC 1 - young adults born between 1985 -1990; LRC 2a - children and adolescents born between 1993 and 1997; LRC 2b- moderate preterms (gestation 32-36 weeks - see chapter 5- were sampled by stratified random selection from children born between 1996-1997).

TNS: Trent Neonatal Survey

LCCHS - Leicestershire Specialist Community Child Health Services

CLD - chronic lung disease of prematurity

Initially, we planned to determine normal alveolar development using just children who did not have any risk factor. However, the number of children who did not have any risk factor was quite low and this affected the power of the study. Moreover, because children who did not have any risk factors affecting peripheral lung development actually form a minority, it was decided that presence of these risk factors should not be regarded as an exclusion to the study on normal alveolar development. Therefore, with the exception of children born <37 weeks, those who had current respiratory illness and those who had a significant chronic respiratory disorder, all other children were analysed for the purpose of determining normal alveolar development. The risk factors were then controlled by multivariate analysis.

Term born children who were aged 10-14 from the LCCHS and LRC2a formed the control group of the study regarding alveolarization following preterm birth. Mildly preterm born children (born at 32-36 weeks gestation) were recruited from LRC2b and very preterm born children (born at <32 weeks gestation, subgrouped into with or without CLD) were identified from TNS. These 4 groups were compared to inform alveolarization in survivors of preterm birth.

The study looking at risk factors to alveolar development analysed all subjects who were born >32 weeks of gestation who had no significant chronic respiratory illness of note. Details of the inclusion and exclusion criteria are covered in the individual chapters.

3.2.VISIT OF THE SUBJECT

3.2.1. WELCOME AND CONSENT

The subject usually had one single appointment lasting for 5 hours. The subject went through a set protocol summarised in Figure 3-2. They usually came to the Leicester Royal Infirmary (LRI) Children's Lung Function Lab. The process started with explanation of the study, going through the information sheets and obtaining consent for the study. Consent was obtained from the parent or person with parental responsibility in children who were under the age of 18. Children between 10 and 18 years who were deemed to be able to understand the process were encouraged to take part in the consenting process. Consent was directly taken from young adults above 18 years old.

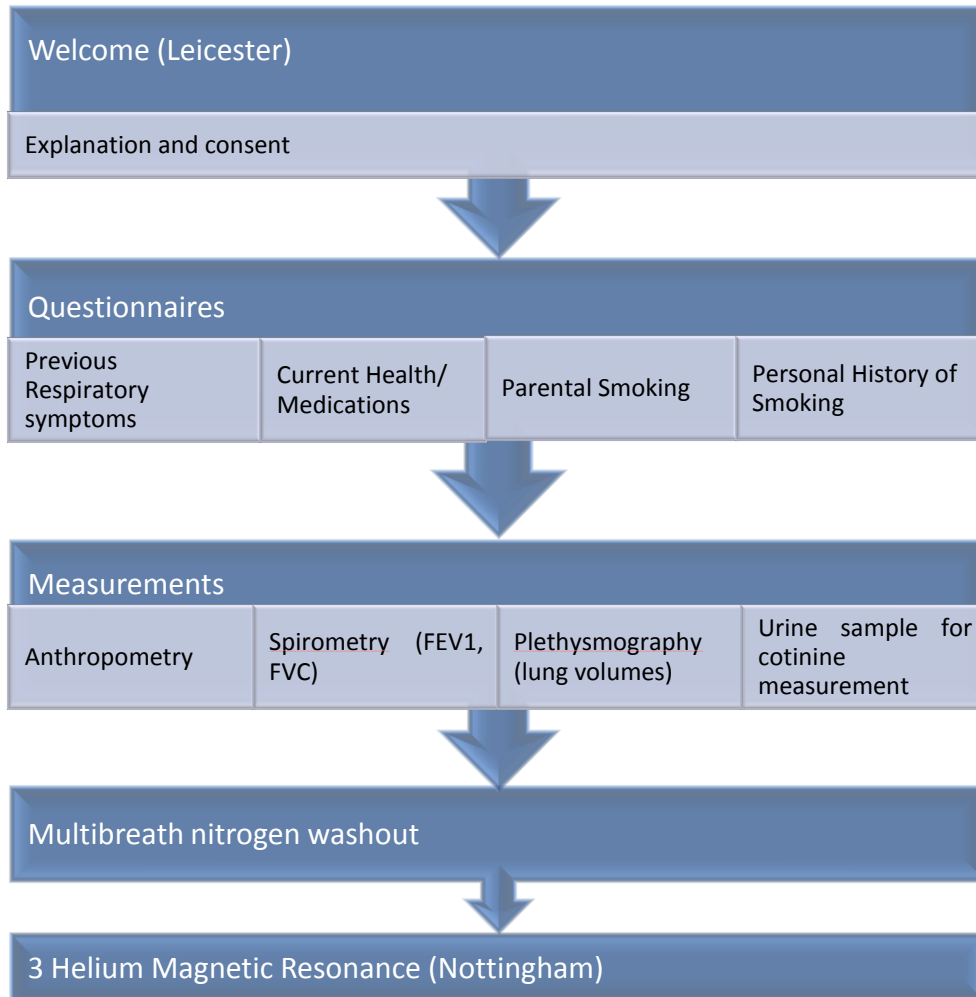
3.2.2. ADMINISTRATION OF QUESTIONNAIRE

Questionnaires regarding perinatal and postnatal history, exposure to environmental risk factors in early and late childhood, health status and personal habits

were administered. The administered questionnaire is discussed in detail in section 3.3 and sample questionnaires are included in the appendix.

FIGURE 3-2

Flow chart of the process followed by subjects during the day of the study. The measurements done with Multi-breath nitrogen washout are not analysed in this thesis.



3.2.3. PHYSICAL MEASUREMENTS

Anthropometric measurements included height and weight. Height was measured by the Leicester Height Measure (Seca, Birmingham) according to the policy of national child measurement programme to an accuracy of 0.1 cm (137). Care was taken to

- ensure that 4 points of the child's body touches the scales - heel, buttock, shoulder, back of head.
- ensure that head is positioned in the Frankfurt plane (i.e. external auditory meatus should be level with the base of the orbit).

- legs are placed close together and flat on the measuring surface.

Weight was measured by digital scales (Seca, Birmingham) to the accuracy of 0.5 kg.

3.2.4. LUNG FUNCTION TESTS

This was followed by spirometry and plethysmography. Measurements were done on Jaeger Masterscreen Body (Wuerzburg, Germany) by one of four trained personnel (JP, SW, KT, MN). The theory and principles of these measurements are well established (138,139).

3.2.4.1. SPIROMETRY

The primary parameters measured include forced expiratory volume in 1 second (FEV₁), and forced vital capacity (FVC). FEV₁ is the maximum volume of air expelled in one second after maximum inhalation. FVC is the total volume of air that can be expelled after maximum inhalation. Other parameters measured during spirometry include forced expiratory flow at 25%, 50% and 75% of FVC (FEF₂₅, FEF₅₀, FEF₇₅) but they were not analysed for the purposes of this study. At least 3 valid runs of spirometry are performed (138). The best FEV₁ and best FVC from technically acceptable blows are recorded (138). For younger children, animated screens with visual incentives (blowing down the candles, toaster catching a floating bread slice, bowling alley) designed to encourage rapid and prolonged blows were used at the discretion of the operator.

3.2.4.2. PLETHYSMOGRAPHY

Spirometric techniques cannot be used to measure the residual volume of air in the lungs following either tidal exhalation (functional residual capacity, FRC) or maximal exhalation (residual volume, RV). A full body plethysmograph measures these lung volumes by indirect means. In brief, the subject sits in an enclosed chamber and breathes through a pneumotachometer (normal tidal breathing). While quiet tidal breathing is taking place, a shutter closes the airway transiently. Changes in pressure at the airway opening, pressure of the air in the chamber and volume of air in the chamber are measured during this manoeuvre. Using Boyle's law ($P_{\text{lung}} \times V_{\text{lung}}$ is constant under isothermic conditions) in this situation, the volume of the lung can be calculated from the known parameters. This gives the thoracic gas volume (TGV). Following this, the subject is instructed to do a full inspiratory effort followed by deep exhalation to residual volume. Calculated parameters include:

- Functional residual capacity, FRC: volume of the air remaining in the lung at the end of a tidal expiration.

- Residual Volume, RV: volume of the air remaining in the lung at the end of maximal expiration
- Total lung capacity, TLC: volume of the air in the lung after maximal inspiration

At least 3 valid runs are performed. FRC is calculated as the mean of the technically acceptable values (139). The rest of the parameters are calculated from the measurement with the maximal forced expiratory effort (139).

3.2.4.3. SETTING UP, CALIBRATION AND QUALITY CONTROL

Set up and parts of the equipment (Fig 3-3): The Masterscreen body consists of a glass chamber with an air tight door within which the subject can be seated. The height of the chair is adjustable. The pneumotachometer and mouthpiece assembly is mounted on an adjustable handle. Infection control is ensured by including disposable bacterial filters in the circuit. The computer controlled shutter valve is connected to the rear of this assembly (for closing the airway during plethysmography).

Calibration: The equipment is calibrated on every day that it is used (this included volume calibration and box calibration: see below). The ambient temperature, humidity and atmospheric pressure is entered at machine warm-up to ensure adjustment for BTPS. The equipment is serviced annually by manufacturers.

Volume calibration : A 3 litre calibration syringe is used to ensure the accuracy of the volume measured by the device. Air is blown into the device using the calibration syringe at 5 different flow rates. The volume measured by the device is ensured to be within 0.5% of the known volume delivered (3 L).

Box calibration: This is done to ensure that the Masterscreen 'body box' is sensitive to the minute changes in box pressure and volume during a plethysmograph manoeuvre when the door is shut. This part of the calibration is automated and involves a motor which pumps in and out about 50ml of air to the box after its is shut. The measurements are checked for linearity.

Quality control: The spirometry and plethysmography traces were independently verified by an experienced paediatric respiratory physiologist (CSB).

In most children younger than 10 years and in children older than 10 where it was felt necessary, incentive spirometry was done. All measurements conformed to the specifications of the ATS/ERS task force on standardisation of spirometry and plethysmography(138,139). The data were checked by an experienced paediatric respiratory physiologist(CSB) before data-entry.

FIGURE 3-3

Subject sitting in the Masterscreen body.



3.2.5. ASSESSMENT OF PERIPHERAL LUNG STRUCTURE

Children then had assessment of ventilation inhomogeneity by multi-breath nitrogen washout. This measurement does not form part of the current thesis. Following this, they were transported to Nottingham University for 3-Helium magnetic resonance measurement (section 3.4 and Chapter 2).

3.2.6. SAMPLES - URINE AND SALIVA

In the midst of this procedure, depending upon the convenience of the subject, two samples were taken. Urine was collected in an universal container and a saliva sample was collected using the Oragene DNA ® (DNA Genotek, Ontario, Canada) kit. The urine was analysed in batches for urinary cotinine, a metabolite of nicotine, in the biochemistry laboratory of the University Hospitals of Leicester NHS trust. The concentration of cotinine was standardised for concentration of urine using urinary creatinine. Measurements were done using a standardised high performance liquid chromatography(HPLC) technique (140). Urinary cotinine is a sensitive marker of environmental tobacco smoke (ETS) exposure with a half life of 16 hours (141). We used the following values for classifying exposure (141)

TABLE 3-2

Levels of cotinine against exposure status (141).

	Cotinine Level	Exposure status
1.	< 2 ng/ml	No evidence of exposure
2.	2-10 ng/ml	Possible evidence of passive smoking
3.	10- 30 ng/ml	Consistent with passive smoking
4.	30 – 50 ng/ml	Passive smoking/ smoker
5.	>50 ng/ml	Smoker

Saliva was collected with prospective consent for future genetic analysis. Genetic analysis was not part of the current work.

3.3.ASSESSMENT OF RISK FACTORS

The assessment of exposure to risk factors was done using the following methods

3.3.1. PRE-EXISTING DATA

We were fortunate to have access to prospectively collected data regarding the antenatal and perinatal risk factors of the Leicestershire Respiratory Cohorts (LRC). They had been followed up in the past with numerous questionnaires, interviews and measurements (135,142). The data collected in the past included assessment of parental wheeze/ atopy, antenatal and perinatal tobacco smoking, socio-economic indicators (parental occupation, highest qualification of parents, crowding index (number of rooms in the house/ person living in the house)), childhood wheeze/atopy, respiratory infections

in childhood and treatment including inhaled corticosteroid therapy assessed at a minimum of 2 points in childhood. The questionnaires were previously validated (135,143).

For children belonging to the Trent Neonatal Survey (TNS)(i.e., those born very preterm), details of antenatal factors and factors associated with care in the neonatal intensive care unit (NICU) were available from their records. These included: antenatal corticosteroids, spontaneous pre-labour rupture of membranes, presence of fetal distress, APGAR score at 5 minutes, surfactant therapy, total days on oxygen, total days on CPAP, total days on ventilator, details of respiratory status in first 12 hours of life (highest FiO₂, lowest FiO₂, highest base excess) and length of stay in neonatal unit. Thus data regarding the most important risk factors of neonatal chronic lung disease were available.

Birthweight and gestational age at birth were available from records children from all 3 sources (LRC, TNS and LCCHS). 'Small for gestational age' was defined as birth weight less than the 10th centile for gestation, using gestation specific birthweight centiles (144).

3.3.2. QUESTIONNAIRES.

As described above, questionnaires were administered to children taking part in this study to explore exposure to antenatal, perinatal and later risk factors related to alveolar development. 3 questionnaires were filled in by the subjects of this study.

3.3.2.1. FIRST QUESTIONNAIRE - EARLY LIFE AND ETS EXPOSURE

The first questionnaire (Q1, see appendix), explored neonatal history, past medical history and exposure to environmental tobacco smoke in detail. This questionnaire was designed to be an administered questionnaire and was administered to parents when the child came for the appointment.

In case of the TNS cohort, questions regarding neonatal exposures were supplementary to the data collected from the TNS. They included questions regarding antenatal corticosteroids and duration of oxygen, CPAP and ventilator therapy but excluded details not likely to be known to parents such as surfactant therapy, fetal distress, APGAR scores etc. They also included questions regarding common serious complications associated with preterm birth including intraventricular haemorrhage, patent ductus arteriosus, retinopathy of prematurity, necrotising enterocolitis and severe lung infections. Where data was available from both the TNS records and the questionnaire, data from TNS records were used for analysis. In case of children not belonging to the TNS, these questions were used to exclude unforeseen cases of neonatal

disease/neonatal intensive care among the cohort/LSCCHS population (who were selected from the general population).

Q1 also included questions about the duration of breast milk feeds in infancy. In addition, Q1 also explored past medical history of wheezy illness or other chest diseases and past medical treatment, in particular corticosteroid therapy. The timing of exposure to these risk factors (whether they occurred antenatally, between 0-3 years or after 3 years of age) was also checked. History regarding exposure to environmental tobacco smoke was gathered in detail from this questionnaires. Data collected include person who smoked in the household (mother, father, other), timing (antenatal/0-3 years/ 3-10 years/current exposure) and dosage of exposure (average number of cigarettes per day). The questionnaire explored risk factors before and after 3 years of age because we wanted to ascertain the timing of exposure in relation to the age when alveolarization would be complete as per the existing paradigm at the time of the study.

Data from urinary cotinine measurements and pre-existing data regarding parental smoking in case of subjects belonging to LRC were used to validate the questionnaire data regarding current exposure to tobacco smoke.

3.3.2.2. SECOND QUESTIONNAIRE - CURRENT HEALTH AND SOCIOECONOMIC DETAILS

The second questionnaire (Q2: see appendix) was designed to be self administered and was sent out along with the invitation letter (detailing the date, time and venue of their appointment for the study). There were 2 versions of the first questionnaire - a. adolescent version (over 14 year old), designed to be filled in by the subjects themselves and b. child version (under 14 year old), designed to be filled in by the parents. We requested that the subject/their parent complete the questionnaire at home and bring the completed questionnaire when they came for their appointment. Q2 explored current symptoms including wheeze over the past 12 months, chronic cough, and treatment for asthma. This questionnaire also evaluated demographic data such as ethnicity, household, environment and social factors. History of parental respiratory illness was also checked. Questions on current clinical status, treatment, environment and social factors in Q2 and breast milk feeds and exposure to tobacco smoke in Q1 were used in previous validated questionnaires employed for the Leicester respiratory cohorts surveys (135).

3.3.2.3. THIRD QUESTIONNAIRE - PERSONAL HISTORY

The third questionnaire (Q3: see appendix) explored personal history and pubertal stage. There were 4 versions of the questionnaire (under or over 14 years, male

or female). Questions regarding personal history of smoking, physical activity levels, diet, sleep position and playing wind musical instruments were answered by all children. Questions regarding experimentation with smoked recreational drugs (in particular, cannabis) were asked to adolescents over 14 years old. Questions regarding pubertal status were asked, based on gender. These questions were based on Petersen's pubertal development scale (145,146). This has been validated for self administration previously (147). Q3 was filled in by subjects privately while they came for their appointment, after verbally confirming from parents whether they agreed to the children filling in these questionnaires themselves. These answers were regarded as confidential and therefore probably led to more honest responses from the children.

3.3.3. DATA FROM MEASUREMENTS

BMI z-scores (calculated from the measured height and weight on the day of the study) was used as a measure for current nutritional status. Z-scores were calculated from international reference ranges (148,149).

3.4. HELIUM MAGNETIC RESONANCE TECHNIQUE

3.4.1. PRE-SCAN PREPARATIONS- SETTING UP, SHIMMING AND POLARIZATION

Hyperpolarised ^3He was prepared by metastable optical pumping using a custom-made laser optical polarisation system in a separate room with no access to volunteers (122). Standard laser safety precautions (class IIIB) were followed during the production process. The gas was then stored in a vacuum container at 4×10^{-2} mBar until the subject was ready for the manoeuvre.

Measurements were undertaken in a 0.15 Tesla permanent magnet system (Intermagetics General Corporation, New York, NY) with a Surrey Medical Imaging Systems Console (Surrey, UK). The system was prepared on the day by shimming, a term widely used in magnetic resonance physics which means tuning the frequency of coils in the magnet to counteract imperfections in the magnetic field. A scout image was obtained to ascertain the fidelity of the sequences used. A customised induction coil (the helium coil) was attached to the scanner and the gating was verified. The depolarisation of ^3He in the magnetic field induces a signal in the coil which is the basis of measurements. Further details of the pre-scan preparation is available from the work of physicists at Nottingham university (122,123).

3.4.2. SUBJECT MANOEUVRES

The procedure was explained to the volunteers. Trial runs were done before the actual scan to ensure that they were familiar with the procedure and to check whether they could comply with the breath hold time. After a magnet safety check (including removal of metallic items), they were placed supine in the magnetic field of the scanner with the helium coil centered around the chest.

Six hundred ml of gas containing 15-30 ml of hyperpolarised ^3He mixed in Helium-4 was transported to the MR scanner in a tedlar bag (SKC limited, Blandford Forum, UK) with a disposable one-way valve and mouthpiece attached to it. The subject inhaled the gas mixture from FRC through a disposable one-way valve and held their breath for 2-10 seconds, depending on the nature of the measurement. The gating button next to the magnet was activated by the operator once he was satisfied that the subject was adequately breath-holding. This initiated the sequence and completes the measurement.

FIGURE 3-4

Volunteer getting ready for the scan (inhaling air from Tedlar bag in the hand - trial run)



3.4.3. SEQUENCES

3.4.3.1. DIFFUSION WEIGHTED IMAGING TO DETERMINE ADC

In the first technique, the global apparent diffusion coefficient (ADC) was obtained using a 64-echo, rapid acquisition with refocused echoes (echo time = 14 ms, acquisition time = 896 ms) MR sequence (RARE sequence) with fixed gradient strength ($b=0.3 \text{ s.cm}^{-2}$) and slice select and phase gradients turned off (131). This gives a series of 64 echoes with incrementally decreasing amplitude depending on diffusion weighting (See section 2.2.2). The ADC value is calculated by fitting to the exponentially decaying echo-train. Values of ADC were obtained from 64 planes of the lung, parallel to the sagittal plane during a single breath hold. The mean value of the 64 measurements, the global apparent diffusion coefficient (ADC_m), is a measure of average dimension of alveoli within the lung. The standard deviation of these values (SD_{ADC}) is an estimate of the uniformity of ADC within the lung. At least three technically satisfactory ADC values were obtained in all subjects and the mean was taken as the raw ADC.

3.4.3.2. Q-SPACE TECHNIQUE OF MR

The second technique (q-space technique) became available in the later part of the study. In this sequence diffusion weighted free induction decay (FID) signals are acquired following the application of a radiofrequency pulse of small tipping angle, and a bipolar magnetic field gradient pulse of variable amplitude G_d , in the right-left direction (figure S1). For each scan, 40 diffusion weighted FIDs were acquired, with decreasing G_d , corresponding to a range of q values between 0.02 and 1.23 mm^{-1} (see section 2.2.3). ' q ' is a measure of the degree of dephasing experienced by the nuclei during application of the gradient pulse. Different q values were achieved whilst keeping timing parameters fixed. Every four diffusion-weighted acquisitions ($G_d > 0$) were followed by a single non diffusion weighted acquisition ($G_d = 0$), to allow measurements to be corrected for attenuation due to longitudinal relaxation (T_1) and RF depletion.

The magnitude of each FID signal was normalised and plotted as a function of q . The q -space curves were then Fourier transformed to give a displacement probability profile (DPP). The DPP is a probability density function of the average displacement of the helium atoms during t_{exp} . We fitted the DPP to a mono-Gaussian model (123)

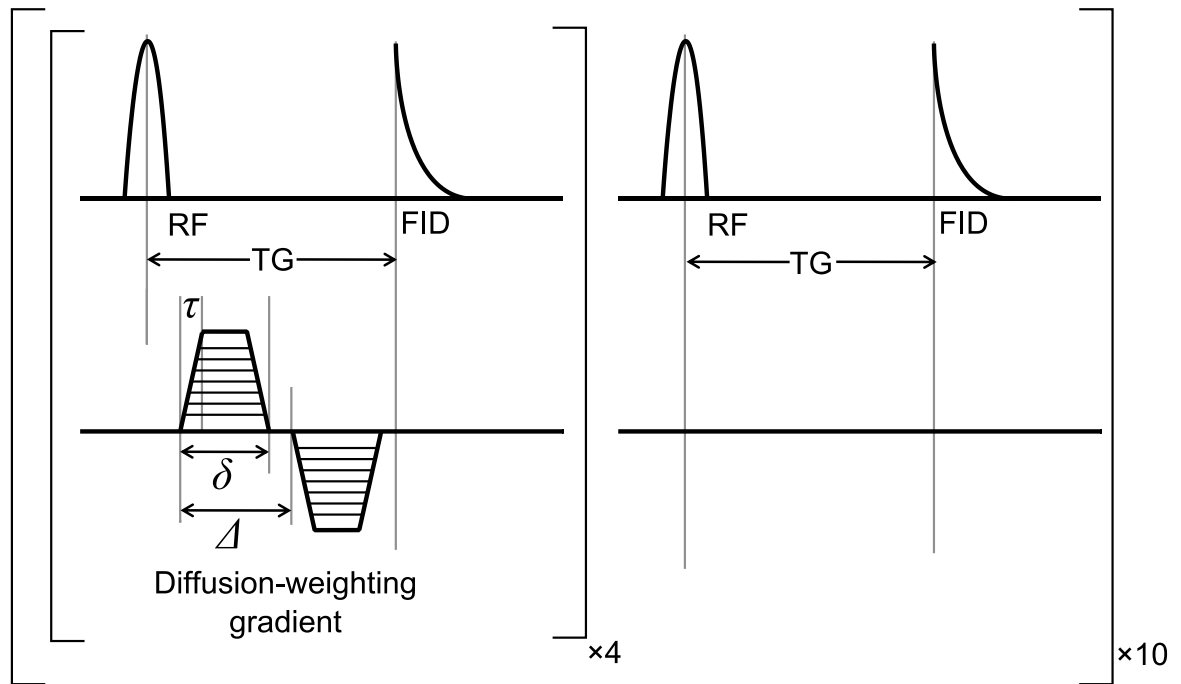
$$\text{DPP}(x) = Z \text{EXP} \left(-\frac{x^2}{2X_{\text{RMS}}^2} \right) \quad \text{EQUATION 3-1}$$

The width of the Gaussian function is determined by X_{RMS} , the root-mean squared displacement of the ^3He gas. This gives a measure of the linear dimension of the airways

which restrict diffusion. The parameter Z is interpreted as the zero-displacement probability. We also applied Yablonskiy's acinar model(125) on the q -space data and obtained the values for mean alveolar duct diameter, R (including alveolar sleeve) and mean alveolar sleeve depth, h .

FIGURE 3-5

Diagram of variable-gradient pulse sequence used for q -space MR spectroscopy measurements. The sequence comprises 50 acquisitions in total: 40 diffusion weighted acquisitions (lobe width $\delta=4.7\text{ms}$, ramp time $\tau=0.150\text{ms}$, diffusion time $\Delta=5.2\text{ms}$), interleaved with 10 non-weighted acquisitions, which are used to correct diffusion-weighted signals for radio-frequency depletion and longitudinal relaxation. Spoiler gradients (not shown) are applied after the acquisition of each free induction decay (FID). Relaxation time (TR)=200ms, corresponding to a total scan time of 10s; TG (see figure)=10.4ms.



3.4.4. SCALING OF ADC

3.4.4.1. RATIONALE FOR SCALING

All volunteers were given a 600ml bolus of a $^3\text{He}/^4\text{He}$ gas mixture (comprising 15-30ml hyperpolarised ^3He , depending on the type of scan) for each measurement. Because the FRC varied from 1L to nearly 3.6 Litres in our subjects, this meant that the final concentration of helium present in the lung after inhalation differed between

subjects. Also, similar sized boluses in different sized subjects will cause difference in relative lung expansion between subjects.

Therefore, the measurements have to be 'scaled back' to a similar level of concentration of helium and lung expansion in order to compare between subjects.

3.4.4.2. EFFECT OF CONCENTRATION ON ADC

The effect of ^3He concentration on expansion was determined by measuring ADC at a range of helium concentrations but at similar lung expansion in 3 adult male volunteers. This was achieved by having the volunteer expire a controlled amount of air, from FRC, before inspiring the same amount of a hyperpolarised $^3\text{He}/^4\text{He}$ mixture. ADC appears to increase systematically with helium concentration and the data are well described by a second order polynomial expression (123).

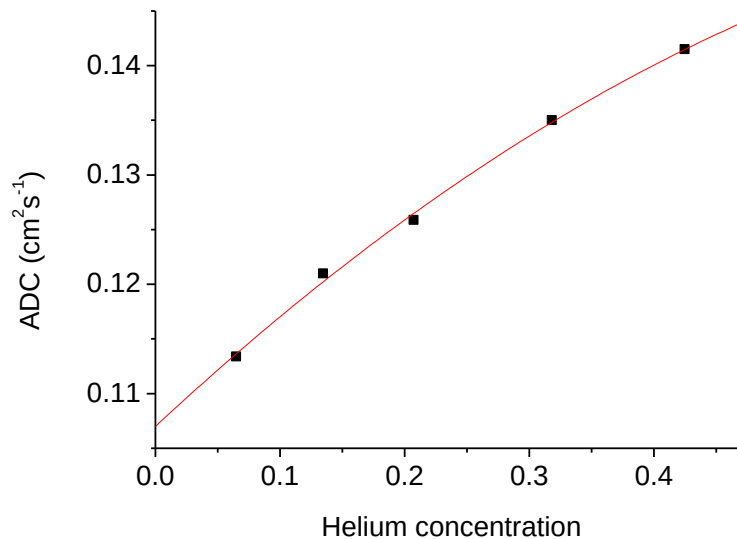
The ADC at a concentration 'x' (expressed as ratio) of helium in the lung

$$ADC_x = -0.059X^2 + 0.106X + ADC_0 \quad \text{EQUATION 3-2}$$

where ADC_0 is the ADC at 'zero helium concentration' (i.e. the diffusion coefficient for one helium atom in the lungs).

FIGURE 3-6

Apparent diffusion coefficient measured at FRC using the modified RARE sequence against helium concentration expressed as a ratio in one volunteer.



In the example shown in figure 3-6 above, the $ADC_0 = 0.107$ and the curve fits the equation $ADC[x] = -0.059X^2 + 0.106X + 0.107$

3.4.4.3. EFFECT OF LUNG INFLATION ON ADC: THE BOLUS EFFECT STUDY

The dependence of ADC on the degree of lung inflation has been previously reported for measurements in adults. Waters et al (131) measured ADC over a range of lung inflation levels and found that ADC increased linearly with inflation. We measured ADCs in a total of 9 children at different bolus sizes to determine the effect of lung inflation on ADCs.

5 children were measured with two different bolus sizes inhaled from FRC (both containing similar initial concentrations of helium - resulting in different final concentrations of helium). 4 children were measured with 3 different bolus sizes inhaled from FRC (but the initial concentration of helium in these cases was adjusted so that the final concentration of helium was the same at the end of inhalation). Each measurement was done in triplicate and the average values were taken as the final ADC in each case. (Therefore there were $5 \times 2 + 4 \times 3 = 22$ measurements, each in triplicate- see table).

Table 3-3

Raw ADC values measured at different boluses of helium. In the first 5 subjects, the final level of expansion and final concentration of helium are both different. In the next 4 subjects, though the expansion level is different, the final concentration achieved after each bolus was same. Each of these measurements was done in triplicate

Vol. No.	FRC	Bolus 1	ADC1	Bolus 2	ADC 2	Bolus 3	ADC 3
1	2.05	0.3179	0.1251	0.6297	0.1305	-	-
2	2.25	0.3323	0.095	0.645	0.1006	-	-
3	2.63	0.3296	0.095	0.6379	0.1025	-	-
4	1.73	0.3308	0.096	0.6341	0.0976	-	-
5	0.98	0.32355	0.1086	0.63155	0.115	-	-
6*	1.53	0.301	0.1106	0.609	0.1222	0.896	0.126
7*	1.52	0.354	0.0789	0.817	0.0915	1.312	0.979
8*	2.04	0.396	0.1043	0.708	0.1096	1.029	0.1144
9*	2.02	0.397	0.1606	0.709	0.1689	1.03	0.1691

The concentration corrected values (ADC_0) were calculated from equation 3-2. These values were analysed in the multilevel model described in the Chapter 4 to arrive at the equation to derive the final corrected value of ADC (ADC_{corr}).

3.4.4.4. VALIDITY OF ADC CORRECTIONS

The paired ADC_{corr} for the 5 children who had ADCs measured at different concentrations were compared using t-test. The mean difference was 0.00068 (0.72% of ADC) which was well within the coefficient of variation of 3.08% and the p-value for no difference between the values was 0.61.

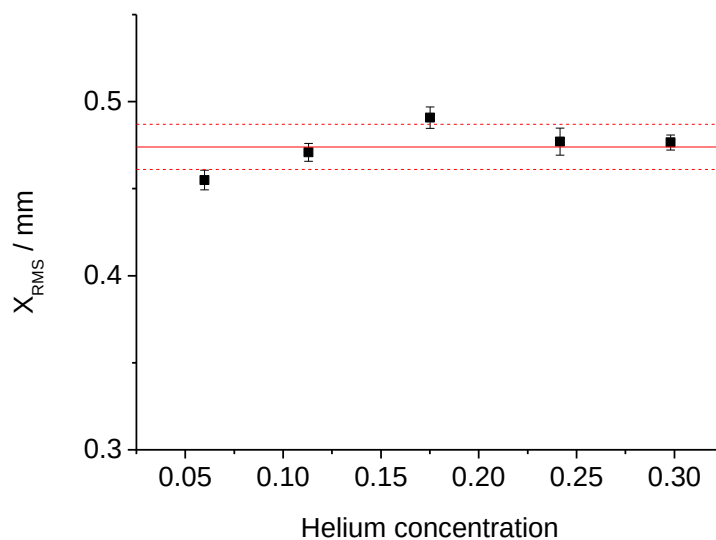
3.4.5. SCALING OF X_{RMS}

3.4.5.1. EFFECT OF CONCENTRATION ON X_{RMS}

X_{RMS} was measured in a healthy adult male (27 years) volunteer for a range of helium concentrations, at fixed lung volume, namely FRC. The concentration of helium in the lung was controlled using the method described above (section 3.4.4.2). Figure 3-7 shows that X_{RMS} remains constant across the range of helium concentration (defined as the volume of helium inspired, divided by FRC).

FIGURE 3-7

X_{RMS} measured at FRC, using the variable b-value sequence, versus helium concentration. Each data point is the average value of X_{RMS} based on two separate scans. Solid red line is the mean value, and dashed lines show the standard deviation. X_{RMS} does not depend on the concentration of helium.



3.4.5.2. EFFECT OF LUNG INFLATION ON X_{RMS}

The data obtained from the MR Spectroscopy sequence can be analysed using two methods, namely q-space analysis, and application of Yablonskiy's geometric model. We find strong correlation between parameters derived from the two analysis techniques, i.e. X_{RMS} and R ($r^2 = 0.51$, $p < 0.0001$) (123). Yablonskiy has found it reasonable to assume that R scales with the cube root of lung inflation (125). Because X_{RMS} is also a measure of length (RMS displacement of ³He), similar to 'R', we propose that X_{RMS} will scale in the same way. Therefore all measurements of X_{RMS} have been scaled as per the cube root of relative lung inflation.

3.5. DATA ENTRY AND STATISTICAL ANALYSIS

3.5.1. DATA ENTRY

The questionnaire data, results from physiological measurements and lung function tests were entered using a customized data entry software Epidata (150). The fidelity of data entry was assured by the following steps:

1. We tried to avoid errors at the data collection stage by going through the paper forms at the time of the subject visit (including the second questionnaire (filled at home) and the third questionnaire (filled privately by subject)) and double checking with the subject/parent if the responses seemed illogical
2. The data were entered into the customized computerized epidata form. Each field in the computerized form had appropriate limits to avoid inadvertent wrong entry (For example : yes /no questions were coded as 0- no, 1- yes, 9 - missing. Any other value would generate an error message. Importantly, it was not possible to skip entering a field: missing data had to be explicitly entered as '9'. In the case of numerical values, appropriate limits were entered. For example, it was not possible to enter height below 80 cm or above 200 cm and weight below 20kg and above 100kg. In case of numerical values, missing was coded as 99, 999 or 9999, depending on the maximum number of digits allowed in the field. For example, in case of height, the highest legal value had 3 digits and therefore, missing was coded as 999.).
3. Skip questions were built into the data entry file. For example, the section on birth and neonatal care in the first questionnaire starts with the question (question 1) , 'Immediately after the child was born, did he/she need to be admitted to the special care unit (the neonatal unit)'. If the answer was no, 6 questions about neonatal unit care are

skipped as they are not applicable (e.g., how long did the baby stay in the neonatal unit) and question on breastfeeding is asked (question 8). These skipped sections are built in to the epidata data entry form. The intermediate questions are coded as not-applicable appropriately.

4. The data were entered independently by two different personnel (either of the technicians involved in the study / myself) . The second data entry is done on the so called 'double entry' form in epidata, which verifies the original data in real time and alerts if there is a discrepancy in the entered data. This alerts the worker to cross check the paper form and either correct the original entry or the double entry as appropriate.

5. The above checks ensured that the likelihood of errors in paper form was minimal and the transcription of data from the paper form to the computerized database was error-free. The final step in ensuring the fidelity of the data collection is data-cleaning. The numerical data was plotted on a distribution and any outlying values were double checked on the paper file. Data from different parts of the questionnaire/ two different questionnaire were checked for consistency. As an example q12 in questionnaire 1(12.Has the child ever had wheezing or whistling in the chest at any time in the past?) and q1 in questionnaire 2 (1. Has your child ever had wheezing or whistling in the chest at any time in the past?) captured essentially the same information. Responses to these questions were checked for consistency.

The data from the Helium MR lab at Nottingham were not transcribed into paper format. They were directly exported from the Matlab program which analysed the Helium MR raw data to an Microsoft Excel spreadsheet.

The raw data from questionnaires, physiological measurements and lung function tests entered in epidata and the excel spreadsheet from the MR lab were exported to STATA version 11 (Stata Corporation, Austin, TX) for data analysis. The details of data analysis are given in the respective chapters.

4. NORMAL ALVEOLAR DEVELOPMENT IN CHILDREN

4.1.BACKGROUND

The prevailing theory about the timeline of human alveolar development at the beginning of this study was that alveolar development was complete by the age of 2-3 years of age. This theory was based on studies done between the 1960s and 1980s (section 1.4.6). These studies used histological morphometric methods to evaluate the number of alveoli in the lungs in children of different ages who died of non respiratory causes. These methods have since been superseded because of recognised flaws (section 1.3.4). Also, there is a disparity between these studies both in the measured alveolar number and in the estimated age at which alveolarization is complete (Figure 1-4). Despite these issues, later authors have quoted Thurlbeck's study (32) to conclude that human alveolar development is complete by 2-3 years of age.

However, in recent years there have been many studies in mammals (111-113), which use modern histological techniques to show that alveoli can develop beyond early life and even up to adulthood. There is evidence of neo-alveolarization following partial pneumonectomy in adult mammals (151). There is also evidence of calorie intake related neo-alveolarization following refeeding in starved mice (60). The possibility of alveolarization beyond early life in humans was recognised before this study (152), but no direct proof existed.

4.1.1. RATIONALE

Repeating histological studies on human subjects with modern techniques of morphometry is impractical because of severe ethical constraints in access to adequate specimens from children. In-vivo measurement methods are currently the only realistic options to study the timeline of human alveolarization.

Apparent diffusion coefficient (ADC) of 3-helium in the lung, measured by ^3He magnetic resonance ($^3\text{HeMR}$), is a safe non-invasive measure of the size of the lung peripheral airspaces including alveolar ducts and alveoli (Chapter 2). Newer techniques of $^3\text{HeMR}$ have made it possible to measure of the average linear dimensions of the peripheral airspaces and alveoli non-invasively (121). This study used these techniques to determine the timeline of alveolarization in children.

4.1.2. AIMS AND HYPOTHESIS

The primary aim of this study was to test the hypothesis that human alveolarization is complete by about 3 years of age and that growth of the lung after this

period takes place by expansion of pre-existing alveoli. Human lungs grow three to four fold in volume between 7 years of age and adulthood. If the hypothesis above was true, then individual alveoli should expand by the same extent as lung growth during this period. $^3\text{HeMR}$, which can reliably detect such an increase in alveolar dimensions (chapter 2), was used to test this hypothesis. The second aim was to describe the change in alveolar dimensions with growth in childhood and adolescence.

4.2.METHODS

4.2.1. RECRUITMENT OF SUBJECTS

Details about recruitment of the subjects for this study is given in the methods section (section 3.1). In brief, subjects over 11 years were recruited from the Leicestershire Respiratory Cohorts (LRC) and those between 7 and 11 years of age were recruited from the Leicestershire Specialist Community Child Health Services Database (LSCCHS). Overall, we contacted 669 subjects of whom 212 initially agreed to participate and 173 attended the laboratory: the remainder either subsequently decided not to participate or failed to attend. For the purposes of this part of the study, which was designed to look at normal alveolar development, we included only those children (120 children) who were born at more than 36 weeks of gestation, were never admitted to a neonatal unit and had no current respiratory symptoms or chronic respiratory illnesses. Eleven children were excluded because lung function data and/or helium MR data did not meet our criteria for validity (see below), leaving 109 children.

4.2.2. VISIT AND MEASUREMENTS

The protocol of the visit for each individual child is given in the methods section (section 3.2). In short, after welcome and consent, the subjects and their parents completed questionnaires and then performed measurements of lung function including spirometry and plethysmography. Urine specimen was collected for cotinine detection.

Helium magnetic resonance measurements were then carried out. Details of the technique are given in the methods chapter (Section 3.4). In brief, the global apparent diffusion coefficient (ADC) was measured after inhalation of a 600ml bolus of a $^3\text{He}/^4\text{He}$ gas mixture from FRC using a modified rapid acquisition with refocused echoes (RARE) MR sequence (Section 2.2.2 and 3.4.3.1). At least three technically satisfactory ADC values were obtained in all subjects and the mean was taken as the raw ADC. This value was corrected for different final concentrations of helium to give the concentration corrected ADC value, ADC_0 (section 3.4.4.2). Measurements were also done with different sized gas

boluses in 9 volunteers (bolus effect study) (section 3.4.4.3). These measurements were used to test our hypothesis regarding the age of completion of alveolarization and to arrive at the final concentration and volume corrected value of ADC (see below).

The second technique (q-space technique) (Section 2.2.3 and 3.4.3.2) was used in 46 subjects. The mean displacement (X_{RMS}) of ^3He atoms is derived from the displacement probability profile of the ^3He atoms obtained from this technique. This gives a direct measure of mean linear dimensions of peripheral airspaces. We also applied Yablonskiy's acinar model (124) to the q-space data and obtained values for mean alveolar duct diameter, R (including alveolar sleeve) and mean alveolar sleeve depth, h . Correction for the effect of helium concentration and bolus size on these parameters was done on similar lines as for ADC (see below and section 3.4.5).

4.2.3. STATISTICAL METHODS

We tested our hypothesis using a multilevel linear regression model of ADC_0 (the concentration corrected ADC) against volume using the combined data of measurements at a standard bolus volume in subjects of the main study sample (studied at FRC + bolus volume) and repeated measurements at varying boluses of helium (referred to as the bolus effect study). Under the model, absence of new alveolar formation requires that the slope of the ADC change with FRC be equal to the slope of ADC change with inflation volume. We tested this using a Wald test. The coefficients from this model can also be used to estimate the concentration and bolus-volume corrected ADC (ADC_{corr}) (See below).

In the second step of analysis, we assessed associations of log transformed ADC_{corr} with FRC, age, height and weight separately using linear regression models and single measurements in subjects of the main study sample only. In the regressions against FRC, FRC was also log transformed for compatibility with the multilevel model. All models were estimated with and without adjusting for ethnicity, gender, previous exposure to environmental tobacco smoke (ETS) at home, maternal smoking during pregnancy, active smoking, previous use of corticosteroid treatment (inhaled or oral) and z-scores for height, weight, FEV_1 , FVC and FRC. All tests were two-sided with a significance threshold of 0.05. Similarly we tested for trends in mean peripheral airspace dimension (X_{RMS}) with height, weight, age and FRC. We also checked X_{RMS} for potential confounding due to the above variables. Data were analysed using Stata® (Stata Version 11, StataCorp, Texas, 2009).

4.3. ANALYSIS AND RESULTS

4.3.1. DEMOGRAPHICS AND CONFOUNDERS

The study population consisted of 109 subjects (50 males) aged 7 to 21 years (median 12.8 years). Of these, 76 were from the Leicestershire Respiratory Cohorts and the rest from the Leicestershire Specialist Community Child Health Services Database (LSCCHS). Seventy nine (72.4%) were of white Caucasian ethnicity, 22 (20.2%) of south Asian origin and 8 (7.3%) of other or mixed ethnicity. Forty six participants (42.2%) had current or previous passive exposure to tobacco smoke (before birth and/or during childhood). Seven (6.4%) had tried cigarette smoking but none were current smokers. Previous inhaled corticosteroid treatment was recorded in 18 (16.5%).

4.3.2. PHYSIOLOGICAL AND LUNG FUNCTION MEASUREMENTS

TABLE 4-1

Anthropometric and lung function parameters of the population

Measurement	n*	Mean (SD)	Range
Anthropometry			
Height, cm	109	154.7 (14.9)	122.6 – 184.5
Weight, kg	109	49.7 (14.8)	22.5 – 99.7
Lung Function			
FEV ₁ †, L	108	2.8 (0.89)	1.34 – 5.16
FVC†, L	108	3.2 (1.02)	1.51 – 5.85
FRC†, L	109	1.98 (0.7)	1.02 – 3.95
FEV ₁ z-score	108	0.2 (0.94)	-2.5 to 2.9
FVC z-score	108	0.09 (1.00)	-2.4 to 2.6
FRC z-score	109	-0.55 (0.95)	-3.2 to 1.9

* - number of technically acceptable observations

† - FEV₁ – forced expiratory volume in one second, FVC – forced vital capacity, FRC – functional residual capacity.

Anthropometric parameters were appropriate to the age distribution of the population (Table 4-1). Measurements of FEV₁, FVC and FRC (Table 4-1) were approximately

normally distributed. The range of these lung function parameters were as expected given the wide age range. Linear regression of FRC on age gave a 3.4-fold increase in FRC from 7 (1.02 L) to 21 years (3.50 L).

4.3.3. MULTILEVEL REGRESSION MODEL

4.3.3.1. VARIABLE DEFINITIONS AND ASSUMPTIONS

For a given subject we defined the following variables:

- y : ADC corrected to He concentration of zero (ADC_0)
- x : FRC of the subject
- v : Inflation volume at ADC measurement, i.e. FRC + bolus volume
- w : Relative inflation volume compared to FRC, i.e.

$$W = \frac{v}{x} \quad \text{EQUATION 4-1}$$

- n : Number of alveoli
- s : Mean alveolar size, i.e.

$$s = \frac{v}{n} = \frac{wx}{n} \quad \text{EQUATION 4-2}$$

This ignores physiological dead space, but because the lung expansion is due to expansion of alveoli (and dead space remains constant), equation 4-2 holds true for the purposes of this analysis.

Two assumptions were made

- a.** For each subject, ADC is proportional to a power of the mean alveolar size, i.e.,

$$y \propto s^\beta \quad \text{EQUATION 4-3}$$

For the purposes of the analysis, we allow β to vary between subjects. According to this assumption, ADC depends only on alveolar size but not on alveolar geometry. Even if ADC is in reality affected by alveolar geometry, the assumption will still hold as long as alveolar geometry is constant within each subject. This equation does not assume any particular alveolar geometry because β can vary freely across subjects and ADC is derived with a constant diffusion time for all subjects.

- b.** Within our study population, the number of alveoli is proportional to a power of FRC, i.e.,

$$n \propto x^\gamma \quad \text{EQUATION 4-4}$$

This relationship is the mathematical representation of the hypothesis. The proportionality factor γ is not allowed to vary between subjects. The null hypothesis of no neo-alveolarization would be satisfied if $\gamma = 0$, i.e., the number of alveoli does not change with FRC.

Equations (4-3) and (4-4) imply (by differentiating)

$$\frac{dy/y}{ds/s} = \beta \text{ and } \frac{dn/n}{dx/x} = \gamma$$

respectively, where d denotes infinitesimal changes. The parameters β and γ can therefore be interpreted as follows: a change in mean alveolar size by 1% is associated with a change by $\beta\%$ in ADC and a change in FRC by 1% is associated with a change of $\gamma\%$ in alveolar number.

4.3.3.2. MATHEMATICAL DESCRIPTION OF THE MODEL

In terms of log-transformed variables, (iii) and (iv) imply

$$\ln y = \alpha_1 + \beta \ln s \text{ and } \ln n = \alpha_2 + \gamma \ln x$$

for some constants α_1 and α_2 . Using $s = \frac{v}{n} = \frac{wx}{n}$ (Equation 4-2), these equations can be combined to the model

$$\ln y = \alpha + \beta \ln w + \beta(1 - \gamma) \ln x \quad \text{EQUATION 4-5}$$

where α is a constant. The variables y, w and x in equation (4-5) are observable and the equation can be used to estimate the parameters β and γ from observed data. The formal notation of the multilevel regression model is then:

$$\ln y_{ij} = \alpha + \beta_i \ln w_{ij} + \theta \ln x_{ij} + \mu_i + \varepsilon_{ij} \quad \text{EQUATION 4-6}$$

$$\text{Where } \theta = \beta(1 - \gamma) \quad \text{EQUATION 4-7}$$

In the regression model (4-6), i indicates a given child ($i = 1, \dots, N$) and j indicates one of the measurements taken in that child ($j = 1, \dots, T_i$). In addition to the measurement specific random errors ε_{ij} , the model includes child specific random intercepts μ_i and random slopes β_i (because β was allowed to vary across subjects)(see figure 4.2). These random variables are assumed to be independent of each other and across subjects. Furthermore, they are assumed to be normally distributed(i.e, $\mu_i \sim N(0, \sigma_\mu^2)$, $\varepsilon_{ij} \sim N(0, \sigma_\varepsilon^2)$, and $\beta_i \sim N(\beta, \sigma_\beta^2)$ for all i and j , where σ represents the standard deviation of the concerned random variables). The parameters to be estimated include $\alpha, \beta, \theta, \sigma_\mu, \sigma_\varepsilon$ and σ_β . The parameter σ_μ harbours the between subject variability in ADC, while σ_ε accounts for residual variability within a subject over different measurements.

Once the model is fitted, γ can be estimated from the relationship $\gamma = 1 - \theta/\beta$ (from (4-7)) using the estimates of β and θ and the confidence intervals of γ can be estimated by the delta method(153). Also, testing the equivalence of β and θ is the same as testing the hypothesis that of $\gamma = 0$ i.e. that the number of alveoli remains constant as FRC increases.

Note that fitting the model (4-6) requires T_i (the number of measurements within subject i) to be greater than 1 in at least some subjects. The precision of estimates will increase with the number of subjects, N and with T_i . Reliable estimation of β requires measurements to be taken at a range of different bolus volumes and reliable estimation of θ requires measurements at a range of different FRC values.

4.3.3.3. MEASUREMENTS

The main study included 109 subjects where ADC was measured after inhalation of a 600ml bolus of the gas mixture from FRC. Measurement with different bolus volumes were carried out in 9 subjects (4 were part of the main study and 5 were recruited specifically for this purpose (section 3.4.4.3)). This included 4 measurement triplets (measurements of ADC in same subject (same FRC) but 3 different bolus volumes) and 5 measurement pairs (measurements in same subject at 2 different bolus volumes).

TABLE 4-2

Bolus volume as a percentage of FRC

	FRC (L)	Bolus volume (L)	Bolus volume /FRC (%)
Main study subjects	1.02 to 3.95	0.6	15.2 to 58.8
Bolus subject 1	2.05	0.32 and 0.63	15.6 and 30.7
Bolus subject 2	2.25	0.33 and 0.65	14.6 and 28.8
Bolus subject 3	2.63	0.33 and 0.64	12.5 and 24.3
Bolus subject 4	1.73	0.33 and 0.64	19.1 and 36.4
Bolus subject 5	0.98	0.33 and 0.63	33.6 and 64.2
Bolus subject 6	1.53	0.3, 0.61 and 0.9	19.6 to 58.8
Bolus subject 7	1.52	0.35, 0.81 and 1.31	23.0 to 86.1
Bolus subject 8	2.04	0.4, 0.71 and 1.02	19.6 to 50
Bolus subject 9	2.02	0.4, 0.71 and 1.03	19.8 to 51.0
All subjects	0.98 to 2.63	0.3 to 1.31	12.5 to 86.1

Bolus volumes ranged between 0.30 L and 1.31 L (Table 4-2) and FRC values ranged between 0.98 L and 3.95 L. The bolus volumes in the bolus effect study were selected to reflect the range of expansion of the lung due to the 600 ml bolus given to the main study subjects and to avoid distortion of alveolar geometry due to overexpansion of the lung alveoli at volumes near the total lung capacity.

4.3.3.4. FITTING THE MODEL

In summary, the multilevel regression model (Eq. 4-6) was fitted to data from 130 measurements from 114 children. We fitted the model using the xtmixed command in Stata version 11.1 (StataCorp, Texas, USA). The default estimation method by restricted maximum likelihood was used. Results are summarised in the table 4.3. A comparison of the random effects parameters shows that $\sigma_\epsilon < \sigma_\mu$, suggesting that the model fit is substantially better within subjects than it is between subjects. Also, the parameter σ_β is very small suggesting that the slope of ADC change with inflation volume varies negligibly between subjects. Model fit within subjects is illustrated in Figure 4-1.

TABLE 4-3

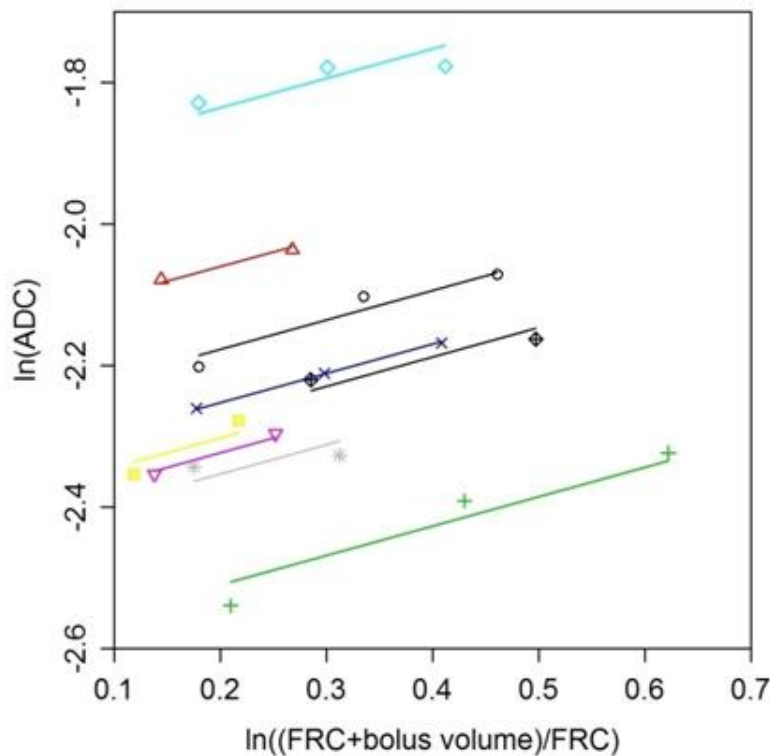
Estimated parameters of the multilevel regression model (equation 4-6)

Main model parameters	Estimate	SE	95%CI
θ	0.189	0.032	0.127 - 0.252
β	0.415	0.054	0.308 - 0.521
α	-2.483	0.033	-2.547 - -2.419
Random-effects parameters			
$\hat{\sigma}_\beta$	1.38×10^{-8}	3.23×10^{-8}	1.38×10^{-10} - 1.37×10^{-6}
$\hat{\sigma}_\mu$	0.122	0.009	0.106 - 0.139
$\hat{\sigma}_\epsilon$	0.027	0.005	0.019 - 0.038

Abbreviations: SE standard error, CI confidence interval

FIGURE 4-1

Within subject fit of the multilevel model: The data are log transformed ADC measurements and relative inflation volumes in 9 subjects (distinguished by colour and point symbols) with measurement pairs or triplets taken at one occasion (constant FRC) using different bolus volumes. The lines represent the expected change in ADC with inflation volume according to the fitted multilevel model (slope = β). The model has a random intercept for each subject. The intercepts of the lines shown are chosen so that the lines cross the subject specific means of $\ln(\text{ADC})$.



4.3.4. TESTING NULL HYPOTHESIS USING ADC

In the multilevel regression model above, the null hypothesis that lung grows only by expansion of pre-existing alveoli is satisfied if $\beta = \theta$ (or alternately $\gamma = 0$ (see section 4.3.3.2)). The equivalence of β and θ was tested by the Wald test (using the command ‘test’ in Stata). The Chi-squared statistic of this test was 22.26 which corresponds to a p-value = 2.4×10^{-6} with one degree of freedom. This implies that the chance that $\beta = \theta$ given our experimental data is 2.4×10^{-6} . This provides strong evidence against the null hypothesis that growth in lung volume within this age group is accomplished without neo-alveolarization. In summary, the concentration-corrected ADC (ADC_0) increased by 0.19% (95% Confidence Interval (CI): 0.13 -0.25%) for every 1% increment in FRC in the study

population but by 0.41% (95%CI: 0.31-0.52) for every 1% inflation in the bolus-effect study.

FIGURE 4-2

Scatterplot of ADC against functional residual capacity. Values are weighted mean ADC from 3 measurements in each individual child and corrected to zero ^3He concentration (ADC_0) and FRC. The solid red lines indicate back-transformed linear fits of $\log(\text{ADC})$ on $\log(\text{FRC})$ and the areas between the dotted red lines are 95% simultaneous confidence bands (Scheffe's method). The green curve shows the estimated change in ADC for a child with an initial FRC of 1 L assuming that the growth of the lung occurs only by proportional enlargement of pre-existing alveoli without new alveolarization. This curve was estimated from the multilevel model

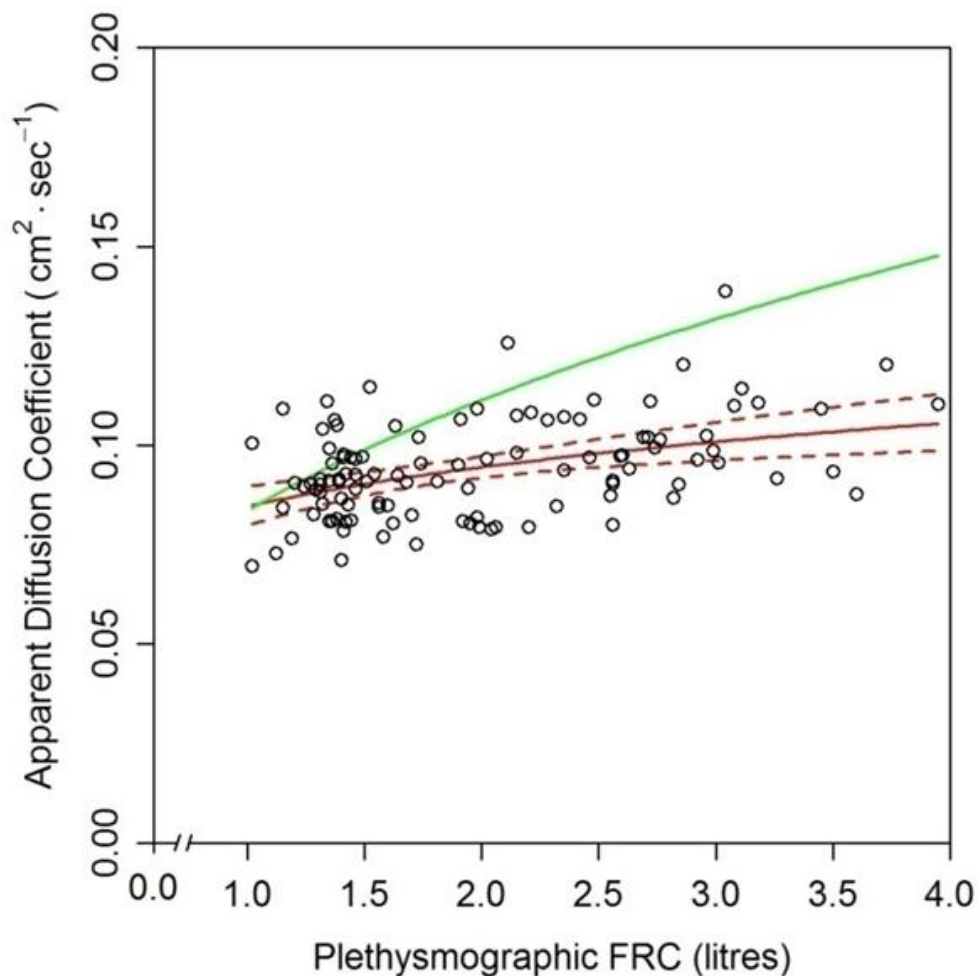


Figure 4-2 summarises the model fit in our subjects. ADC values are volume corrected to represent measurements taken at FRC (section 4.4.3 below). This correction eliminates the effect of varying relative inflation volumes because constant bolus volumes (0.6 L) were used. The figure does not contain any repeated measurements within the same subject and the red line represents a linear regression of $\log \text{ADC}$ on $\log \text{FRC}$ using these data only. The slope of this regression, $\theta = 0.160$, corresponds to θ from the

multilevel model. However, the two slopes are not exactly identical because the data used to fit multilevel model additionally included the multiple measurements within subjects. The green line is a representation of how log ADC would increase with lung volume if we apply the slope β instead of θ . This represents a scenario in which $\gamma = 0$, i.e. there is no neo-alveolarization

4.3.4.1. CONTRIBUTION OF ALVEOLARIZATION TO LUNG GROWTH

Using the 'nlcom' command in Stata, the value of γ is estimated to be 0.54 (95%CI: 0.41 – 0.68). This suggests as the lung grows throughout mid and late childhood, a growth in lung volume (FRC) by 1% is associated with an increase in number of alveoli by 0.54% (95% CI: 0.41% to 0.68%, from equation 4-4).

Using linear regression of FRC on age on our data, we obtained a 3.4-fold increase in mean estimated FRC from 7 (1.02 L) to 21 years (3.50 L). If s_0 , n_0 and x_0 are the initial mean size of alveoli, number of alveoli and FRC respectively (at age =7) and s_1 , n_1 and x_1 represent the final values (at age =21) then we have

$$\frac{n_1}{n_0} = \left(\frac{x_1}{x_0}\right)^{\gamma} \text{ from equation (4-4) and}$$

$$\frac{s_1}{s_0} = \left(\frac{x_1}{x_0}\right)^{(1-\gamma)} \text{ from equations (4-2) and (4-4)}$$

Because γ has been estimated as above, we can estimate that increase in FRC by about 3.4 fold between 7 and 21 years of age is accompanied by an increase in alveolar number by about $3.4^{0.54}$ (= 1.94) fold (95% CI: 1.64 -2.30 fold) and an increase in alveolar volume by about $3.4^{0.46}$ (=1.75) fold (95%CI: 1.48 – 2.07 fold).

4.3.4.2. VOLUME AND CONCENTRATION CORRECTED ADC

The final volume and concentration corrected values were estimated for each subject by mathematical means. From equation (4-5) (section 4.3.3.2), we have

$$\ln y = \alpha + \beta \ln w + \beta(1 - \gamma) \ln x$$

where y is the concentration corrected ADC (ADC_0) at relative inflation w ($w = (FRC + bolus)/FRC$). Let y_{corr} be the concentration corrected ADC at FRC (i.e., ADC_{corr}). Then,

$$\ln y_{corr} = \alpha + \beta(1 - \gamma) \ln x \quad \text{EQUATION 4-8}$$

because $w = 1$ when bolus =0.

From (4-5) and (4-8), $\ln y - \ln y_{corr} = \beta \ln w$ or

$$\frac{y}{y_{corr}} = w^{\beta} = \left(\frac{FRC+Bolus}{FRC}\right)^{\beta}$$

Therefore,

$$ADC_{corr} = ADC_0 \left(\frac{FRC + Bolus}{FRC} \right)^{-0.415} \quad \text{EQUATION 4-9}$$

because β has been estimated at 0.415 (see table 4-3).

4.3.5. TESTING NULL HYPOTHESIS USING X_{RMS}

Because X_{RMS} represents a linear measure, we can assume that it is proportional to the cubic root of mean alveolar size which is a measure of volume (section 3.4.5.2). In this case, the null hypothesis of growth by expansion of pre-existing alveoli is satisfied if alveolar dimensions represented by X_{RMS} increase proportional to the cubic root of resting lung volume as measured by FRC.

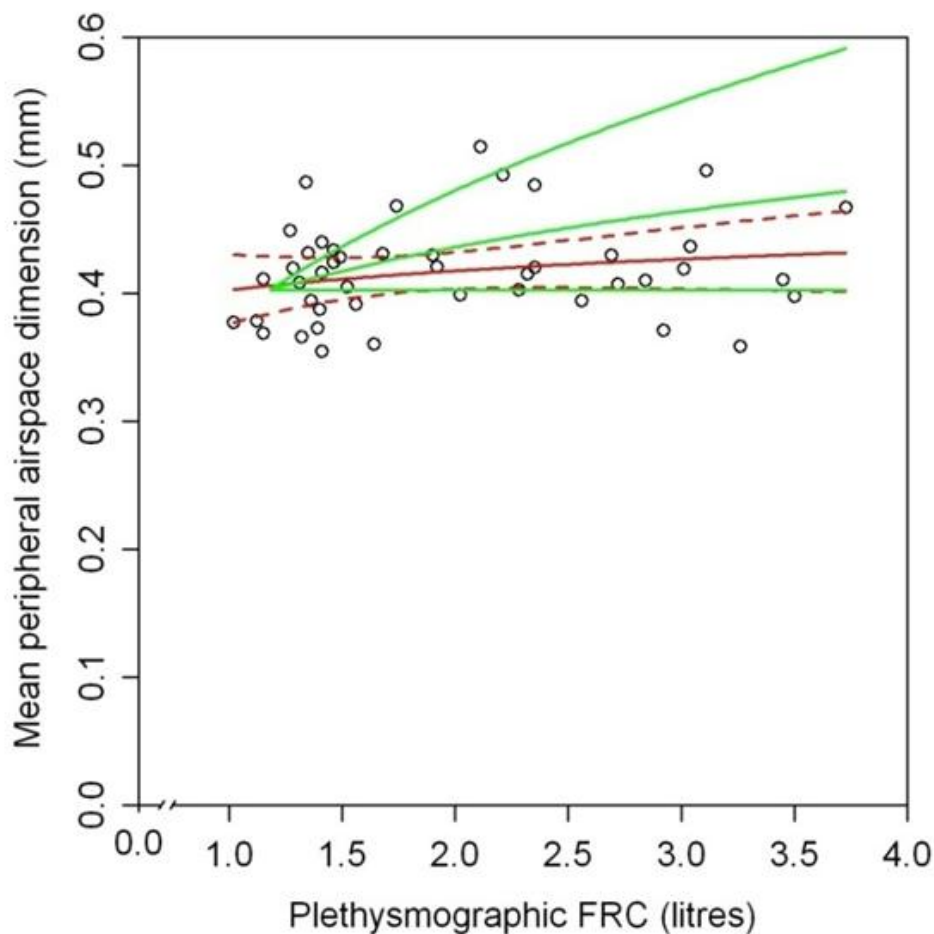
If z represents X_{RMS} , and s represents mean alveolar size, then equation (4-3) becomes $z \propto s^{0.33}$. Therefore, we can substitute β in equation (4-5) with 0.33 in the case of X_{RMS} to give:

$$\ln z = \alpha + 0.33 \ln w + 0.33(1 - \gamma) \ln x \quad \text{EQUATION 4-10}$$

In the 46 subjects where X_{RMS} was done, the coefficient of $\ln x$ in the above expression was 0.053 (95% confidence interval = -0.02 to 0.127). This value is equivalent to θ in equation 4-6 ($\theta_{X_{RMS}}$). In this case, the null hypothesis is satisfied if $\theta_{X_{RMS}} = 0.33$. This was tested by using the command `lincom` in Stata. The p value for this is <0.001 (95% confidence interval for difference between $\theta_{X_{RMS}}$ and 0.33 = -0.35 to -0.20). Using $0.33(1 - \gamma) = 0.053$, we obtain a rate of neo-alveolarization, γ , = $1 - (0.053/0.33) = 0.84$ (95 % CI = 0.615 to 1.06) which is higher than estimated using ADC. It is notable that the confidence interval of γ includes 1, i.e., the growth of the lung entirely by neo-alveolarization. The figure below (Fig 4-3) illustrates the relationship between X_{RMS} and FRC.

FIGURE 4-3

Scatterplot of mean peripheral airspace dimension (X_{RMS}) against FRC. The red lines indicate back transformed log-log fit. Dotted red lines indicate 95% confidence intervals. Green lines indicate the following in a child with an initial FRC of 1 L : top line – predicted change in X_{RMS} with FRC if lung growth was accomplished only by expansion of pre-existing alveoli (scenario of no alveolarization); middle line - predicted change if rate of neo-alveolarization was 0.54 (predicted from ADC vs. FRC multi-level model) ; lower line – predicted change if all growth of lung was by neo-alveolarization. The data suggest that the rate of neo-alveolarization may be even higher than predicted from the ADC vs. FRC model (figure 4-2).



4.3.6. APPARENT DIFFUSION COEFFICIENT

The within subject values of the raw ADC were internally consistent (mean coefficient of variation 3.1 %). Mean (SD) ADC_{corr} by ^3HeMR was 0.094 (0.012) cm^2s^{-1} (table 4-4). ADC_{corr} increased significantly with age (slope of $\log(ADC_{corr})$ vs. age = 0.013, 95%CI 0.006-0.021, $r^2=0.10$, $p<0.001$, Figure 4-4).

TABLE 4-4Parameters derived from $^3\text{HeMR}$

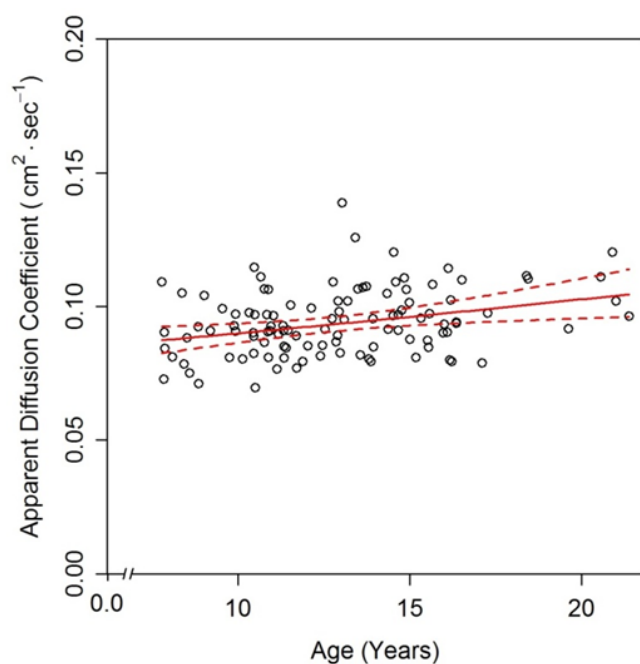
Measurement	n*	Mean (SD)	Range
$^3\text{HeMR}$ data			
$\text{ADC}_{\text{corr}}\ddagger, \text{cm}^2\text{sec}^{-1}$	109	0.094 (0.012)	0.070 – 0.139
$X_{\text{RMS}} \ddagger, \text{mm}$	45†	0.418 (0.039)	0.354 – 0.515
Alveolar duct radius including alveolar sleeve (R)§, mm	46†	0.426 (0.040)	0.318 – 0.503
Alveolar sleeve height (h)§, mm	46†	0.244 (0.043)	0.164 – 0.345

* - number of technically acceptable observations.

† Measurements were done only in a subset of the volunteers

‡ - ADC – apparent diffusion coefficient of ^3He , X_{RMS} - mean peripheral airspace diameter.§ - Alveolar duct radius (R) and alveolar sleeve height (h) derived from application of Yablonskiy's model of alveolar duct (124).**FIGURE 4-4**

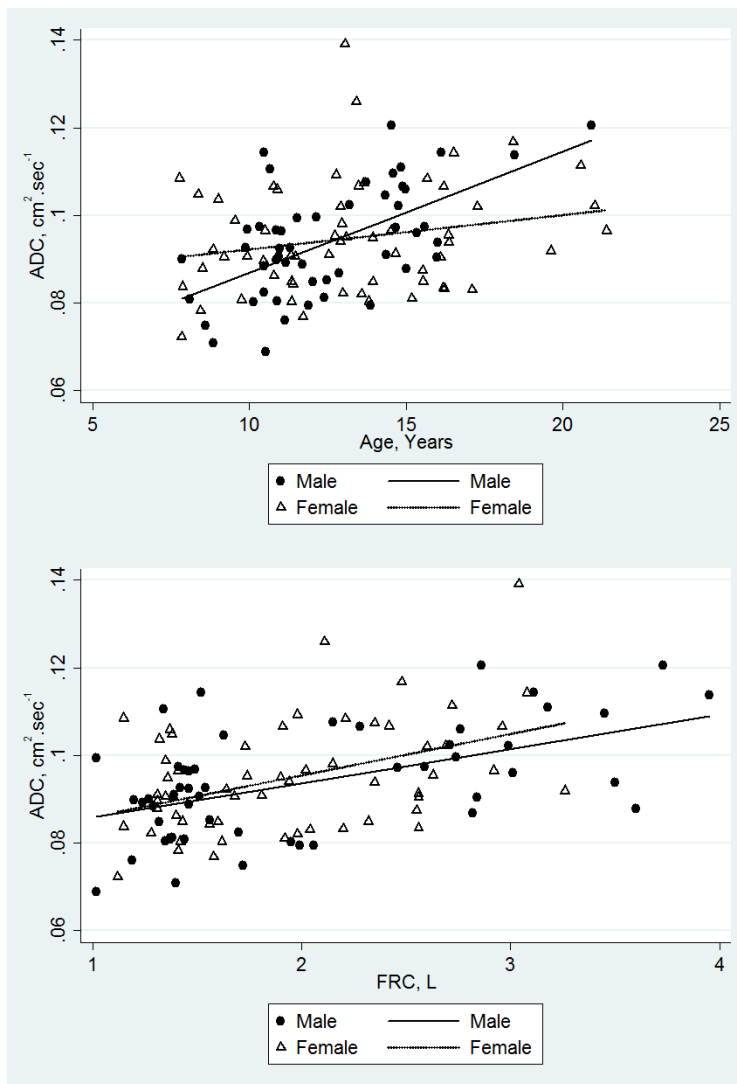
Scatterplot of ADC_{corr} against age. Values are weighted mean ADC from 3 measurements in each individual child and corrected to zero He concentration (ADC_0) and FRC. The solid red lines indicate back-transformed linear fits of $\log(\text{ADC})$ on age and the areas between the dotted red lines are 95% simultaneous confidence bands (Scheffe's method).



ADC_{corr} was similar in males and females ($p = 0.90$). There are differences in ADC distribution with age between males and females (younger boys have relatively lower ADC when compared to girls, whereas above age of about 13, boys have higher average ADC compared to girls, Figure 4-5 top panel). However, average ADC is almost identical in males and females of similar FRC (Fig 4-5 bottom panel).

FIGURE 4-5

Scatterplot of ADC against age (Top panel) and FRC (Bottom panel) by sex.



ADC_{corr} increased with FRC (slope of $\log(ADC_{corr})$ vs. $\log(FRC) = 0.16$, 95%CI 0.09-0.23, $r^2=0.18$, $p<0.001$, Figure 4-2). The change of ADC_{corr} with FRC remained less than in the predicted scenario of 'no alveolarization' after adjusting for confounders ($p=0.004$). Adjustment for potential confounders had negligible effects on the measured relationships of ADC with age or FRC (Table 4-5). History of smoking during pregnancy was the only

confounding variable which showed statistical significance (associated with increase in ADC). This is explored further in chapter 6.

TABLE 4-5

Multivariate regression model with confounders for the relationship between ADC_{corr} with age and FRC. Values given are coefficients of the regression model with 95% confidence interval in parenthesis. *- $p < 0.05$. ** - $p < 0.01$

ADC vs.	Age	FRC
Age	0.01 (0.002, 0.018)*	-
FRC	-	0.13 (0.04, 0.21)**
Height z-score	0.03(-0.01, 0.06)	0.008 (-0.03, 0.04)
Weight z-score	-0.005 (-0.04, 0.03)	-0.003 (-0.04, 0.03)
FEV ₁ z-score	0.04 (-0.01, 0.09)	0.04 (-0.01, 0.09)
FVC z-score	-0.02 (-0.07, 0.04)	-0.02 (-0.07, 0.04)
FRC z-score	0.008 (-0.02, 0.04)	-0.01 (-0.05, 0.02)
Sex (male =1)	0.02 (-0.03, 0.07)	-0.003 (-0.06, 0.05)
Mother ethnicity (Asian = 1)	-0.01 (-0.06, 0.04)	-0.01 (-0.06, 0.04)
Smoke during pregnancy (exposed =1)	0.11 (0.023, 0.19)*	0.09 (0.009, 0.18)*
ETS ever (exposed =1)	0.04 (-0.008, 0.094)	0.04 (-0.009, 0.18)
Steroid ever (exposed =1)	-0.002 (-0.07, 0.06)	- 0.000 (-0.06, 0.06)

4.3.7. Q-SPACE PARAMETERS (X_{RMS} , R AND H)

The mean peripheral airspace dimension (X_{RMS} ; range and mean reported in table 4-4) did not change with helium concentration. There was no significant increase with age (slope of $\log(X_{RMS})$ vs. age = 0.005, 95% CI -0.003 to 0.012, $r^2=0.04$, $p=0.2$, Figure 4-6) or with FRC (slope of $\log(X_{RMS})$ vs. $\log(FRC)= 0.053$, 95% CI -0.021 to 0.128, $r^2=0.05$, $p=0.16$, Figure 4-3). X_{RMS} data are compatible with an even higher rate of alveolarization than estimated from ADC data (see section 4.3.5).

Adjustment for potential confounders did not have any effect on the relationship of X_{RMS} with age and FRC (table 4-6). Z-score of FRC was significantly negatively associated with X_{RMS} . In other words, smaller FRC for a given height is associated with larger X_{RMS} (and therefore mean alveolar dimension).

The parameters derived from Yablonskiy's acinar model, R and h , changed significantly less with increasing FRC (slope of $\log(R)$ vs. $\log(\text{FRC}) = 0.105$, 95% CI -0.03 to 0.18, $r^2=0.159$, $p=0.006$, slope of $\log(h)$ vs. $\log(\text{FRC}) = 0.16$, 95% CI -0.013 to 0.30, $r^2=0.09$, $p=0.03$) than expected from the scenario of no new alveolarization. These results also did not alter after adjusting for potential confounders.

FIGURE 4-6

Scatterplot of mean peripheral airspace dimension (X_{RMS}) against age. The red line indicates back transformed log-linear fit.

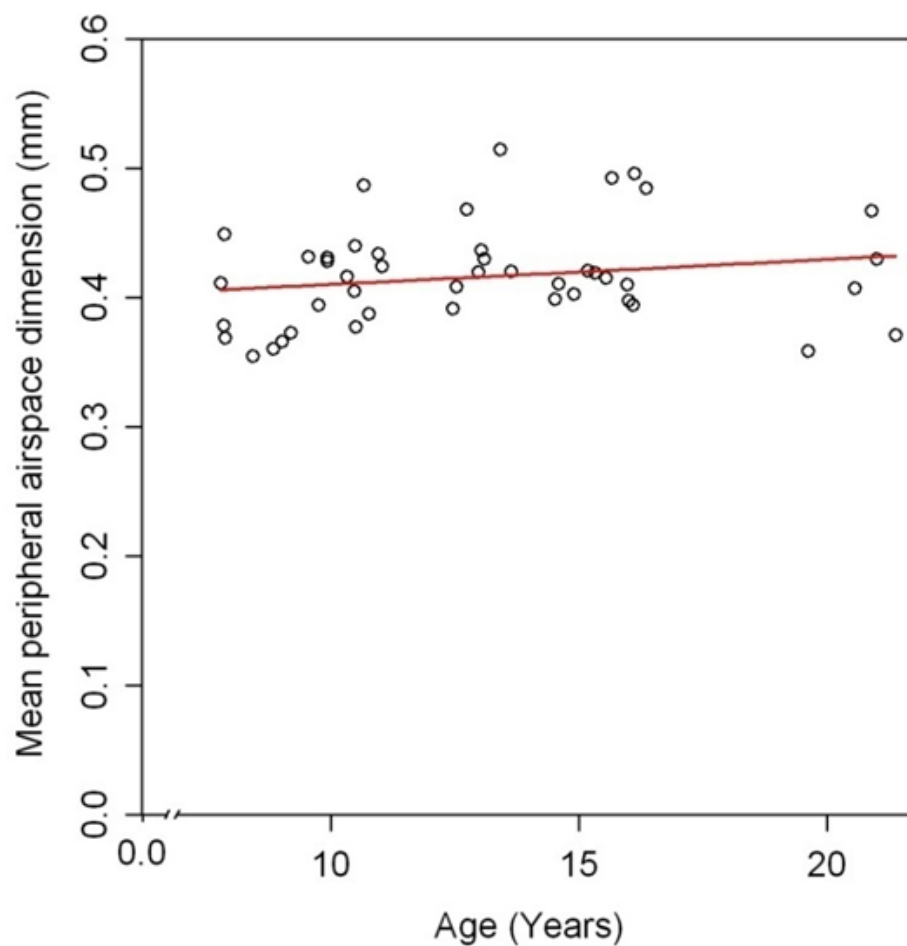


TABLE 4-6

Multivariate regression model with confounders for the relationship between X_{RMS} with age and FRC. Values given are coefficients of the regression model with 95% confidence interval in parenthesis. *- $p < 0.05$.

XRMS vs.	Age	FRC
Age	0.000(-0.003, 0.003)	-
FRC	-	0.006 (-0.01,0.02)
Height z-score	-0.006 (-0.03, 0.01)	-0.009 (-0.03, 0.01)
Weight z-score	0.01 (-0.006, 0.03)	0.01 (-0.005, 0.03)
FEV ₁ z-score	0.01 (-0.02, 0.04)	0.01 (-0.02, 0.04)
FVC z-score	0.002 (-0.03, 0.03)	0.005 (-0.03, 0.04)
FRC z-score	-0.02 (-0.03, -0.001)*	-0.02 (-0.03, -0.002)*
Sex (male =1)	0.01 (-0.01, 0.04)	0.01(-0.02, 0.04)
Mother ethnicity (Asian = 1)	0.005 (-0.01, 0.02)	0.006 (-0.01, 0.03)
Smoke during pregnancy (exposed =1)	0.02 (-0.01, 0.05)	0.02 (-0.02, 0.05)
ETS ever (exposed =1)	0.01 (-0.01, 0.04)	0.01 (-0.01, 0.04)
Steroid ever (exposed =1)	0.02 (-0.01, 0.05)	0.02 (-0.01, 0.05)

4.4.INTERPRETATION

We have shown that dimensions of alveoli determined by $^3\text{HeMR}$ increase with age and lung size during childhood and adolescence at a rate much less than would be expected if lung growth occurred only by expansion of the pre-existing airspaces. This is best explained by postulating that lung grows largely by neo-alveolarization through childhood and adolescence. This contradicts the prevailing hypothesis that alveolarization is restricted to fetal life and early childhood. A full discussion is given in chapter 7.

5. INFLUENCE OF PRETERM BIRTH ON ALVEOLAR DEVELOPMENT

5.1.BACKGROUND

Infants born very preterm are known to have arrested peripheral lung development, manifesting as fewer and larger alveoli(48,49). Most evidence regarding structure and development of periphery of the lung in preterm infants comes from histological studies, either from animal models (83) or from autopsies of severely affected infants (48,49) (see section 1.5.2.1). These studies are limited to infancy/early childhood, either because of constraints of keeping preterm animals alive for long periods or because majority of children who die following preterm birth die before school age.

Functionally many infants who are born very preterm need long-term oxygen therapy to maintain adequate oxygenation. This serves as the basis for the definition of chronic lung disease of prematurity (CLD, section 1.5.1.4). With advances in the neonatal care of preterm infants, most ex-preterm infants who need oxygen therapy can be weaned out of oxygen before 1 year of age (154,155). There is some evidence for persisting lung damage in school-age and adult survivors of preterm birth based on traditional lung function tests (decreased forced expiratory volumes and increased residual lung volumes (156,157). Poorer functional outcomes correlate with lower gestational age at birth and the presence of CLD (157,158) . However, these functional indices are overall estimates of function and do not necessarily correlate with peripheral lung structure.

Histological data regarding peripheral lung structure in preterm born children do not extend to children surviving beyond 3 years of age (50,51,159), with the sole exception of an 8 year old described by Husain et al (49). The histological data are necessarily from fatal cases. Animal studies have not assessed preterm survivors beyond a human equivalent of 3 years of age(83). Because human alveolarization was thought to be complete by 3 years of age (160,161), the histological data were extrapolated to postulate persistence of acinar damage in preterm and CLD survivors(76,83).

However, many animal studies done with modern techniques support ongoing alveolarization throughout the entire period of lung growth in mammals (111-113). The work described in chapter 4 suggests that alveolarization continues beyond early childhood in humans. This raises the possibility of recovery from acinar damage in CLD survivors. The near impossibility of obtaining appropriate histological specimens for morphometric analysis means that non-invasive techniques such as ³HeMR are essential to determine whether alveolarization recovers in CLD survivors.

5.1.1. AIMS AND HYPOTHESIS

The aim of this study was to investigate the possibility of alveolar catch up following birth <32 weeks of gestation and in survivors of CLD. We hypothesised that children born preterm, in particular those who had CLD in infancy, would have fewer and therefore larger alveoli than term-born children. This was tested using $^3\text{HeMR}$.

5.2.METHODS

5.2.1. SUBJECTS

Recruitment of the subjects is given in detail in chapter 3, section 3.1. In brief children were selected from 3 sources : Leicestershire Respiratory Cohorts (LRC), Leicestershire Specialist Community Child Health Services (LSCCHS) database and Trent Neonatal Survey (TNS). A majority of the very preterm born children were recruited from the TNS. For the purposes of this study, we invited 411 children, of whom 150 initially agreed to take part and 122 eventually participated. For children recruited via TNS and the LSCCHS, we ascertained likely ability to perform routine lung function measurements from the child's family doctor before sending the invitations. Written informed consent was obtained from all subjects and from their parents/legal guardians.

5.2.2. INCLUSION AND EXCLUSION CRITERIA

Only those children who were aged between 10 and 14 at the time of measurements were included in this analysis (influence of preterm birth on alveolar development). Children with any other co-existing chronic respiratory disorder (e.g. cystic fibrosis, diaphragmatic hernia, severe asthma needing repeated courses of oral corticosteroids) were excluded from the analysis leaving 119 children.

5.2.3. QUESTIONNAIRES, MEASUREMENTS AND VARIABLES

Complete details of the visit of the subject including the questionnaires and physiological measurements are given in chapter 3, section 3.2-3.3. Hyperpolarised 3-Helium(^3He) magnetic resonance(MR) was used to measure the apparent diffusion coefficient(ADC) of ^3He in the lungs of these subjects (section 3.4).

5.2.3.1. RISK FACTORS ASSOCIATED WITH CLD

For the purposes of this analysis, very preterm birth (birth <32 weeks of gestation) and chronic lung disease of prematurity (see section 5.2.4 for definition) were

analysed as primary risk factors. Risk factors associated with neonatal care, including duration of oxygen therapy and duration of mechanical ventilation which are potentially associated with lung damage were also assessed as secondary risk factors. This information was available from the TNS dataset. Only one child who was born very preterm did not belong to the TNS dataset. In this case, these risk factors were assessed from questionnaire and corroborated from pre-existing data available from the LRC.

5.2.3.2. CONFOUNDERS

The analysis controlled for age, sex, height and ethnicity. The main two ethnic groups in our study were white and south Asian. Children with mixed white/ south Asian ethnicity were classified as south Asian if the mother was south Asian. Apart from these variables, the following were analysed as confounding factors:

- Small for gestational age: defined as birthweight less than 10th centile for gestation from data of Wilcox et al (144).
- Exposure to environmental tobacco smoke (ETS): extensive data regarding dose and duration of exposure to ETS were available from the questionnaires. The data from the questionnaires was collated to two variables (early ETS exposure and late ETS exposure, based on exposure to ETS before or after 3 years of age respectively). Early ETS exposure variable also included exposure during fetal life.
- Exposure to corticosteroids (inhaled or oral): This data was also available from the questionnaires. Similar to the ETS variable, data regarding exposure to steroids was collated into 2 variables Early exposure to corticosteroids (exposure before 3 years of life including antenatal administration of corticosteroids) and Late exposure to corticosteroids (exposure after 3 years of life). The type of exposure (inhaled vs. oral) was not differentiated for the purposes of this study. Only one child had multiple courses of oral corticosteroids for asthma. This person was excluded from analysis because he was an extreme outlier in this regard.
- Crowding index: this was used as a surrogate marker for socio-economic background. It is calculated as ratio between number of individuals (≥ 2 years) in the household and the number of rooms (excluding kitchens, bathrooms and toilets). The information was available from the questionnaires.

5.2.3.3. OUTCOME VARIABLES

The outcome variables included apparent diffusion coefficient (ADC), a marker for alveolar dimensions measured by $^3\text{HeMR}$ and intrasubject standard deviation of ADC (SD_{ADC}) (see section 3.4.3.1). The reported values of ADC and SD_{ADC} were derived after

correction of the raw values for concentration of helium and relative size of bolus (section 4.3.4.2). We did not have enough measurements of mean peripheral airspace dimension (X_{RMS}) in very preterm subjects and therefore this parameter was not used in this part of the study.

Other outcome measures comprised traditional lung function measures including forced expiratory volume in one second (FEV₁), forced vital capacity (FVC) and functional residual capacity (FRC). These measurements are explained in detail in chapter 3, section 3.2.5.

5.2.4. DEFINITION OF GROUPS

For the purpose of analysis, the children included in the study were stratified into four groups by gestational age (GA, expressed in weeks) and presence of absence of chronic lung disease of prematurity (CLD).

The current definition of CLD (section 1.5.1.4) is based on the classification developed at a joint workshop conducted in Bethesda, Maryland in June 2000 by the National Institute of Child Health and Human Development (NICHD) with the National Heart Lung and Blood Institute (NHLBI) and the Offices of Rare Diseases (ORD) (70,162). According to this classification, mild BPD is defined in those babies born at less than 32 weeks gestation as treatment with oxygen for more than 28 days of age but breathing room air at 36 weeks post-menstrual age (PMA). Moderate BPD and severe BPD are defined in those babies born at less than 32 weeks gestation who need supplemental oxygen beyond 36 weeks PMA (moderate <30% oxygen requirement and severe ≥30% oxygen or positive pressure ventilation).

Two different definitions of CLD were used for stratification of subjects into groups as follows:

5.2.4.1.1. Definition of groups: analysis 1

- 1) *term-born* (GA 37-42, no neonatal respiratory support (n=61; of these 46 children were from the LRC and 15 from LSCCHS);
- 2) *mild preterm* (GA 32-36, neonatal respiratory support for <4 weeks, n=21; of these, 20 were from the LRC and 1 from LSCCHS);
- 3) *very preterm* (GA <32, neonatal respiratory support for <4 weeks, n=19; of these, 18 were from the TNS and 1 from LRC); and
- 4) *very preterm with CLD* (GA <32, neonatal respiratory support for >4 weeks, n=18; all children belonging to this group were recruited from the TNS).

5.2.4.1.2. Definition of groups: Analysis 2

In this case, the definition of the first 2 groups remained the same, while the definitions of group 3 and 4 were as follows:

3) *very preterm* (GA <32, neonatal respiratory support until less than 36 weeks post-menstrual age (PMA: gestational age at birth + age after birth, expressed in weeks, n=28; of these, 27 were from the TNS and 1 from LRC); and

4) *very preterm with chronic lung disease* (CLD; born <32 weeks GA, who needed neonatal respiratory support for at least 36 weeks PMA, n=9; all children belonging to this group were recruited from the TNS).

Thus, the key difference between Analysis 1 and Analysis 2 lies in the definition of CLD. In analysis 1 there are 18 subjects deemed to have CLD, whereas in Analysis 2, only 9 subjects were considered to have CLD. The CLD group in analysis 1 contains mild, moderate and severe BPD from the NICHD classification while in analysis 2, it contained only the moderate and severe BPD from NICHD classification and the mild BPD was incorporated into the very preterm without CLD group (group 3). Further analysis was carried out in exactly the same way for both classifications.

5.2.5. STATISTICS

5.2.5.1. ANALYSIS

The outcome variables, ADC and SD_{ADC} , were compared between the four groups of children in the entire sample using linear regression after first controlling for age, sex, height and ethnicity (basic model). Then, the analysis was repeated after adjusting for potential confounders (adjusted model): SGA, exposure to ETS, exposure to corticosteroids and crowding. For 20 children, information was missing on one or more potential confounders. The whole analysis was repeated using multiple imputation (see below).

In a subsample of children born very preterm, the association of ADC and SD_{ADC} with risk factors associated with neonatal care (*SGA, number of days with oxygen therapy, on ventilator and on CPAP*) was analysed by linear regression in a stepwise fashion. Administration of antenatal corticosteroids was not included in this model because of the high uptake (>94%), which would decrease statistical power. First, the subsample was adjusted for age, sex, height and ethnicity (basic model). Then, the risk factors were analysed separately, along with the factors in the basic model (i.e. 4 separate analyses taking SGA, number of days with oxygen therapy, on ventilator and CPAP respectively along with the parameters of the basic model - age, height, sex and ethnicity). Then, the

risk factors were taken together along with factors in the basic model. This was done to determine if any of the risk factors become significant when any effect of collinearity of these confounders is excluded.

Though ADC is known to increase slightly with increasing FRC (i.e. with lung growth - see chapter 4), the models were not adjusted for FRC because the CLD group had higher probability of air trapping, which can be associated with higher FRC. The analysis was, however, repeated including FRC in the models to check for unexpected results. The analysis was performed using each of the 2 definitions of CLD (Analysis 1 and Analysis 2, see section 5.2.4). We used Stata 11.2 for analysis (Stata Corporation, Austin Texas).

5.2.5.2. MULTIPLE IMPUTATION

The multiple imputation procedure involves two steps. First a number of datasets are created, in which the variables have their missing values imputed based on a regression model with all other variables as predictors. The process is iterative, adding an “uncertainty” factor at each iteration, so that the imputed values in the datasets created will vary slightly. Second, the study analysis is performed separately within each dataset and the estimates are pooled. In our case, 30 multiple-imputed datasets were created. Numeric variables (lung function measurements, age, height, weight, birth weight, gestational age and crowding) were imputed using linear regression and categorical variables (sex, ethnicity, family education, small for gestational age, ETS exposure, corticosteroid exposure, smoking during pregnancy) using logistic regression.

5.2.5.3. CALCULATION OF STATISTICAL POWER

Statistical power was calculated based on the parameters calculated in the previous analysis (chapter 4). The mean (SD) of ADC in this study of normal volunteers was 0.094(0.012) cm².sec⁻¹ in this study. Using the correlation between volume and ADC calculated as described in chapter 4 (ADC ratio = volume ratio^{0.415}), statistical power was calculated for hypothetical increases in alveolar volume in the preterm group using the number of children included in the study.

For example, this study had a 90.5% statistical power of picking up a 20% increase in alveolar volume (i.e., a 7.9% increase in ADC) between the 2 very preterm groups (37 subjects) and the term group (61 subjects) with an alpha of 95% (this was same for analysis 1 and analysis 2 because the difference is only in the subdivision of the very preterm groups). Analysis 2 (using the alternate definition of CLD) had 89.7% statistical power of picking up a 35% increase in alveolar dimensions between the CLD group and the term groups (i.e., a 13.3% increase in ADC) with an alpha of 95%. Another

way of looking into the statistical power was to determine the minimum increase in alveolar dimensions in the preterm groups that could be determined by this study with a power of at least 80% and alpha of 95% (table 5-1)

TABLE 5-1

Percentage differences in alveolar dimensions that can be picked up by this study with a statistical power of at least 80% and alpha of 95%

Comparison between	Analysis 1 -% differences in			Analysis 2 - %differences in		
	Volume	Linear	ADC	Volume	Linear	ADC
Term vs. very preterm (with or without CLD)	16.9	5.3	6.6	16.9	5.3	6.6
Term vs. CLD	21.7	6.7	8.5	29.5	9	11.3

5.3. RESULTS

5.3.1. DEMOGRAPHICS AND CONFOUNDERS

The study participants had a mean (SD) age of 11.85 (1.01) years and 60 (50.4%) were boys. Ninety two (77.3%) were of white Caucasian and 25 (21.1%) of south Asian ethnicity. Two children belonged to other ethnic groups. The results did not change when these two children were excluded from the analysis.

5.3.1.1. Analysis 1

Compared to mild preterm and term-born children, the very preterm group (with or without CLD) were marginally younger and shorter. They were more likely to have received corticosteroid therapy (inhaled or systemic) before age 3 (Table 5-2). The CLD group had also received more corticosteroids after age 3 and were less likely to have a south-Asian mother (Table 5-2). The very preterm groups were more likely to be small for gestational age. Thirty two % of children were exposed to environmental tobacco smoke (ETS) at some point (antenatally or postnatally). ETS exposure, crowding index and wheeze were similar between the groups (Table 5-2).

TABLE 5-2

Characteristics of the study participants, by gestational age and presence of CLD (n =119) (where CLD is defined as oxygen requirement at 28 days of life - Analysis 1)

	Term-born n=61	Mild preterm n= 21	Very preterm without CLD, n=19	Very preterm with CLD, n=18	<i>p</i>
Age (years)*	12.0 (1.2)	12.2 (0.9)	11.3 (0.6)	11.6 (0.6)	0.016
Height (cm)*	151.7 (10.5)	154.6 (7.0)	148.2 (5.8)	148.2 (7.6)	0.068
Weight (kg)*	45.2 (10.5)	47.4 (15.4)	42.3 (9.7)	43.1 (12.1)	0.49
South-Asian†	15 (24.6)	4 (16.0)	5 (26.3)	1 (5.9)	0.36
Gestational age (weeks)*	39.6(1.4)	35.2(1.2)	29.7 (1.3)	28.0 (2.0)	<0.001
SGA†	1 (1.7)	0 (0)	3 (16.7)	4 (23.5)	0.004
Crowding (persons/room) *‡	0.79 (0.50)	0.81 (0.25)	0.91 (0.38)	0.76 (0.30)	0.67
ETS during pregnancy†	6 (9.8)	1 (7.7)	4 (20.0)	2 (11.8)	0.48
ETS exposure at 0-3 years†	14 (23.0)	6 (17.7)	9 (45.0)	5 (29.4)	0.313
ETS exposure after 3 years†	16 (26.2)	6 (17.7)	6 (30.0)	6 (35.3)	0.88
Steroids: 0-3 years†,§	3 (4.9)	0 (0)	4 (21.1)	6 (37.5)	0.001
Steroids: after 3 years†,§	6 (9.8)	2 (11.8)	2 (10.0)	7 (43.8)	0.012
Steroids: current†,§	4 (6.9)	2 (18.2)	2 (10.0)	3 (18.7)	0.53
Current wheeze†	7 (11.5)	1 (8.3)	2 (10.5)	2 (11.8)	0.841

Abbreviations: CLD - chronic lung disease of prematurity; ETS=environmental tobacco smoke;

SGA=small for gestational age.

*Mean (SD), †N (%)

‡ Crowding Index – Calculated as mean number of inhabitants (>= 2 years) per room in the residence of the subject (excluding kitchen, bathroom and toilets)

§Steroids – inhaled or oral corticosteroids for any disease (given mainly for reactive lung disease (asthma) in this group). One child who had multiple doses of oral corticosteroids for severe asthma was excluded from analysis.

5.3.1.2. Analysis 2

TABLE 5-3

Characteristics of the study participants, by gestational age and presence of CLD (where CLD is defined as oxygen requirement at 36 weeks of postmenstrual age- Analysis 2)

	Term-born n=61	Mild preterm n= 21	Very preterm without CLD, n=28	Very preterm with CLD, n=9	<i>p</i>
Age (years)*	12.0 (1.2)	12.2 (0.9)	11.4 (0.6)	11.5 (0.5)	0.022
Height (cm)*	151.7 (10.5)	154.6 (7.0)	148.2 (7.0)	148.3 (5.3)	0.067
Weight (kg)*	45.2 (10.5)	47.4 (15.4)	43.3 (11.4)	40.8 (8.5)	0.445
South-Asian†	15 (24.6)	4 (16.0)	6 (22.2)	0 (0)	0.47
Gestational age (weeks)*	39.6(1.4)	35.2(1.2)	29.1 (1.56)	28.1 (2.57)	<0.001
SGA†	1 (1.7)	0 (0)	4(15.3)	3 (33.3)	0.001
Crowding (persons/room) *‡	0.79 (0.50)	0.81 (0.25)	0.91 (0.36)	0.62 (0.19)	0.357
ETS during pregnancy†	6 (9.8)	1 (7.7)	5 (17.9)	1 (11.1)	0.498
ETS exposure at 0-3 years†	14 (23.0)	6 (17.7)	11 (39.3)	3(33.3)	0.436
ETS exposure after 3 years†	16 (26.2)	6 (17.7)	10(35.7)	2 (22.2)	0.83
Steroids: 0-3 years†,§	3 (4.9)	0 (0)	6(22.2)	4 (50.0)	<0.001
Steroids: after 3 years†,§	6 (9.8)	2 (11.8)	6 (21.4)	3(37.5)	0.104
Steroids: current†,§	4 (6.9)	2 (18.2)	3 (10.7)	2 (25.0)	0.357
Current wheeze†	7 (11.5)	1 (8.3)	2 (7.41)	2 (22.2)	0.486

Abbreviations: CLD - chronic lung disease of prematurity; ETS=environmental tobacco smoke; SGA=small for gestational age.

*Mean (SD), †N (%), ‡ Crowding Index – Calculated as mean number of inhabitants (>= 2 years) per room in the residence of the subject (excluding kitchen, bathroom and toilets), §Steroids – inhaled or oral corticosteroids for any disease (given mainly for reactive lung disease (asthma) in this group). One child who had multiple doses of oral corticosteroids for severe asthma was excluded from analysis, Bold: statistical significance different from analysis 1.

The demographic variables and distribution of confounders in analysis 2 (definition of CLD by oxygen requirement at 36 weeks of postmenstrual age) are given in table 5-3. There is no change in statistical significance between the 2 analysis, except that distribution of corticosteroid exposure after 3 years of age between the groups became non-significant in analysis 2.

5.3.2. CHARACTERISTICS OF THE VERY PRETERM GROUPS

TABLE 5-4

Clinical characteristics of children born very preterm, by presence of CLD (Analysis 1 -where CLD is defined as oxygen requirement at 28 days of life)

	Without CLD N=19*	With CLD N=18*	p
Antenatal corticosteroids ¹	17 [94.4]	16 [94.1]	1.0
Spontaneous pre-labour rupture of membranes ¹	2 [11.8]	4 [23.5]	0.66
Fetal distress ¹	8 [47.1]	5 [29.4]	0.48
Birthweight centile ² †	63.4 [34-78]	36.0 [20-56]	0.038
Apgar score at 5 mins ² †	9 [8-10]	9 [8-10]	0.86
Surfactant administered ¹	8[47.1]	14 [82.4]	0.07
Mechanical ventilation, days ³ †	0.0 [0-2], 0-8	7.0 [5-19], 0-44	<0.001
CPAP, days ³ †	1.0 [0-2], 0-12	7.0 [0-21], 0-53	0.49
O ₂ therapy alone, days ³ †	2.0 [0-4], 0-14	30.0 [7-46], 0-115	0.015
Duration of any respiratory support ††, days ³ †	4.0 [2-8], 1-24	62 [38-73], 29-172	<0.001
Total length of stay, days ³ †	41.0 [28-62], 20-92	83.0 [69-112], 45-249	0.005

Abbreviations - CLD- chronic lung disease of prematurity; IQR - interquartile range; CPAP- continuous positive airway pressure.

*- some of the neonatal data were not available for all the children.

¹- values depicted as n(%), ² - values depicted as - median (IQR), ³- values depicted as median (IQR), range. †- N= 17 for both groups. ††- total duration of Ventilation+ CPAP+ oxygen

Within the very preterm subjects, CLD survivors had lower birth weight, received more surfactant, needed longer respiratory support (including more ventilation days, more days on continuous positive airway pressure (CPAP) and more days on supplemental

oxygen) and a longer duration of hospitalisation compared to the non-CLD group (Table 5-4). A high proportion of children were exposed antenatally to corticosteroids in both the very preterm subgroups. Of children belonging to the CLD group, 82.4 % received surfactant. Among children who were born at less than 30 weeks gestation (both CLD and non- CLD groups), 80% received surfactant.

TABLE 5-5

Clinical characteristics of children born very preterm, by presence of CLD (Analysis 2- where CLD is defined as oxygen requirement at 36 weeks of postmenstrual age)

	Without CLD N=28*	With CLD N=9*	p
Antenatal corticosteroids ¹	25 [96.1]	8 [88.9]	0.41
Spontaneous pre-labour rupture of membranes ¹	5[20]	1[11.1]	0.55
Fetal distress ¹	11[44]	2[22.2]	0.25
Birthweight centile ² †	48.0 [34-69]	20.2 [7.6-55.9]	0.08
Apgar score at 5 mins ² †	9 [8-10]	8[8-10]	0.70
Surfactant administered ¹	14[56]	8[88.9]	0.08
Mechanical ventilation, days ³ †	1.0 [0-5], 0-37	15.0 [7-39], 5-44	<0.001
CPAP, days ³ †	1.0 [0-7], 0-51	7.0 [1-17], 0-53	0.15
O ₂ therapy alone, days ³ †	3.0 [0-13], 0-62	43.0 [43-69], 1-115	0.001
Duration of any respiratory support † †, days ³ †	8.0 [3-29], 1-67	73 [66-86], 42-172	<0.001
Total length of stay, days ³ †	60.0[36-69], 20-104	112.0 [83-142], 62-249	<0.001

Abbreviations - CLD- chronic lung disease of prematurity; IQR - interquartile range; CPAP- continuous positive airway pressure.

*- some of the neonatal data were not available for all the children.

¹- values depicted as n(%), ² - values depicted as - median (IQR), ³- values depicted as median (IQR), range

†- N= 17 for both groups, ††- total duration of Ventilation+ CPAP+ oxygen, Bold: statistical significance different from analysis 1

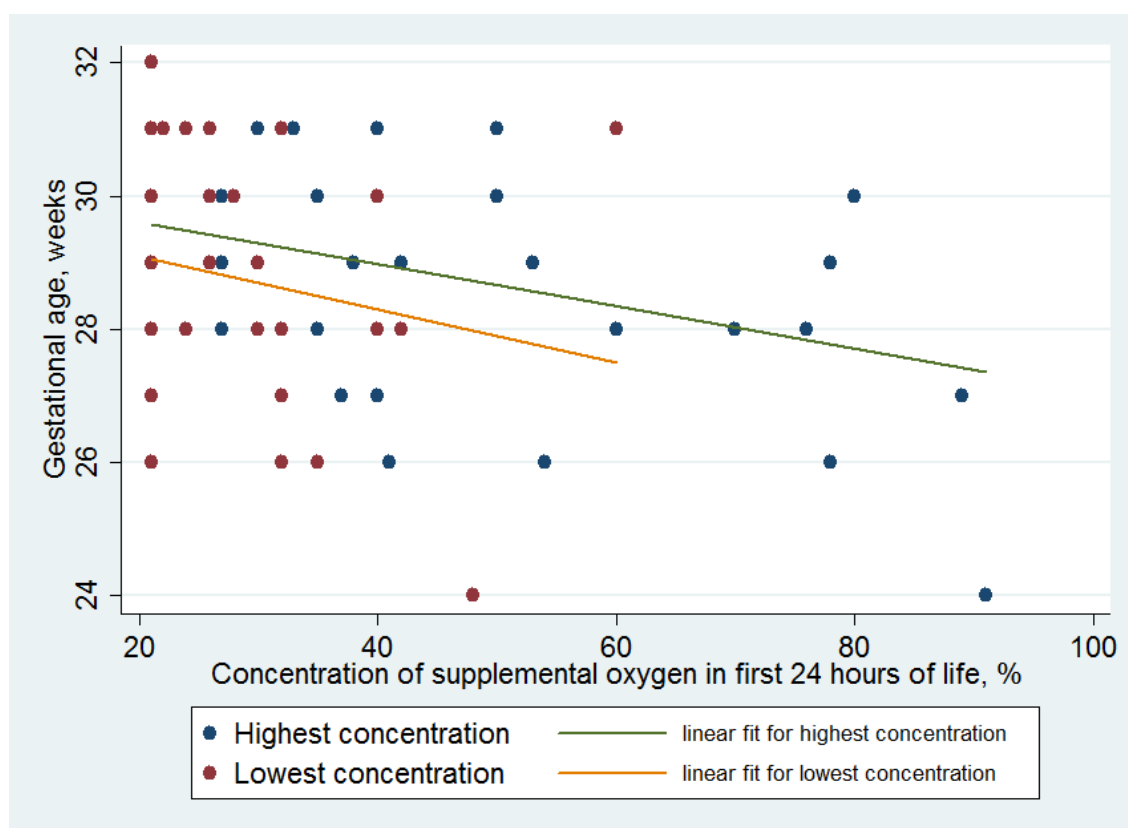
Using the rigorous definition of CLD (Analysis 2), the differences are essentially the same, except that the difference in birthweight centile between CLD and non-CLD groups becomes non-significant (Table 5-5). The number of days where the child needed

ventilation, CPAP and oxygen therapy and total length of stay were very high in the CLD group (either definition), illustrating the severe lung damage sustained in the neonatal period in this group.

Concentration of supplemental oxygen in first 24 hours of life was inversely related to gestation at birth (Figure 5-1). Only 3 children (9% of the 33 where this data was available) in the preterm group were exposed to oxygen concentrations above 80% in the first 24 hours of life. In contrast, 22 children (66%) had weaned down to 30% FiO₂ or below by 24 hours of life (of who 13 (39.3%) were weaned down to air.

FIGURE 5-1

Highest and lowest concentration of fractional inspired oxygen in first 24 hours of life by gestation.



5.3.3. LUNG FUNCTIONS

The very preterm born children had lower one second forced expiratory volume (FEV₁) z-scores compared to the term and mild preterm born children in both analysis 1 and analysis 2 (Table 5-6 & 5-7). Z-scores of forced vital capacity (FVC) were similar between the groups. In analysis 2, the CLD group had significantly higher residual volume (RV) z-scores than the other groups (Table 5-7).

TABLE 5-6

Lung function parameters in the study population by gestational age and presence of CLD (n =119)
(analysis 1: where CLD is defined as oxygen requirement at 28 days of life)

	Term-born n=61	Mild preterm n=21	Very preterm without CLD n=19	Very preterm with CLD n=18	<i>p</i>
FEV ₁ * (z-score)	0.15 (0.91)	0.31 (0.87)	-0.37(1.23)	-0.51(0.98)	0.017
FVC* (z-score)	0.04 (1.02)	0.25 (0.81)	-0.16 (1.07)	-0.28 (1.09)	0.39
FRC* (z-score)	-0.04 (1.20)	0.05 (0.74)	-0.08 (0.67)	0.17 (0.88)	0.75
RV* (z-score)	-0.15 (1.00)	0.22 (0.88)	-0.03 (0.85)	0.24 (1.29)	0.35
TLC* (z-score)	- 0.02 (1.12)	0.28 (0.78)	-0.11 (0.87)	-0.15 (0.98)	0.39

Abbreviations - FEV₁-Forced expiratory volume in one second, FRC-functional residual capacity, FVC-forced vital capacity, RV-residual volume, TLC-total lung capacity.

*z-scores were calculated within sample, adjusting for age, sex, height and ethnicity. Numbers in parenthesis represent standard deviation.

TABLE 5-7

Lung function parameters in the study population by gestational age and presence of CLD (n =119)
(analysis 2: where CLD is defined as oxygen requirement at 36 weeks of postmenstrual age)

	Term-born n=61	Mild preterm n=21	Very preterm without CLD n=28	Very preterm with CLD n=9	<i>p</i>
FEV ₁ * (z-score)	0.15 (0.91)	0.31 (0.87)	-0.26(1.15)	-1.03(0.75)	0.003
FVC* (z-score)	0.04 (1.02)	0.25 (0.81)	-0.10 (1.10)	-0.59 (0.90)	0.22
FRC* (z-score)	-0.04 (1.20)	0.05 (0.74)	-0.10 (0.77)	0.27 (0.80)	0.25
RV* (z-score)	-0.15 (1.00)	0.22 (0.88)	-0.15 (0.97)	0.95 (0.91)	0.01
TLC* (z-score)	- 0.02 (1.12)	0.28 (0.78)	-0.14 (0.96)	-0.08 (0.78)	0.52

Abbreviations - FEV₁-Forced expiratory volume in one second, FRC-functional residual capacity, FVC-forced vital capacity, RV-residual volume, TLC-total lung capacity.

*z-scores were calculated within sample, adjusting for age, sex, height and ethnicity. Numbers in parenthesis represent standard deviation.

Bold: statistical significance different from analysis 1

5.3.4. ALVEOLAR DIMENSIONS

5.3.4.1. ANALYSIS 1: CLD DEFINED BY OXYGEN REQUIREMENT FOR 28 DAYS

Apparent diffusion coefficient: The ADC of ^3He was very similar across the 4 groups. There was no evidence for an association between ADC and either preterm birth or presence of CLD after controlling for age, sex, height and ethnicity (basic model, table 5-8, figure 5-2). There was no relation between ADC and gestational age ($p=0.3$, figure 5-3). The results remained similar in the model additionally adjusted for pre-and postnatal ETS exposure, treatment with corticosteroids, crowding, and small for gestational age (adjusted model, Table 5-8). Sex and height were the only confounders showing a significant association with ADC in the adjusted model (Table 5-9). ADC was lower in males and increased with height (as expected from previous chapter). Inclusion of FRC in the model did not change the results: there was no statistically significant difference between the ADCs of the 4 groups. There was no trend for ADC across the groups with either basic model or adjusted model (p value for trend - 0.312 and 0.359 respectively). The model using multiple imputation gave similar results (Table 5-10).

Within subject standard deviation of the apparent diffusion coefficient. The within subject standard deviation of the apparent diffusion coefficient (SD_{ADC}) gives an idea about the uniformity of ADC within the lung (section 3.4.3.1). In the basic model there was evidence for an association between preterm type and SD_{ADC} , which remained on adjustment with confounding factors (basic and adjusted model, table 5-8). Compared to term-born children, SD_{ADC} was $0.003 \text{ cm}^2/\text{sec}$ (95% CI= -0.006, 0.000, $p=0.025$) lower in very preterm without CLD group, but $0.003 \text{ cm}^2/\text{sec}$ (95% CI=0.000,0.006, $p=0.048$) higher in CLD group. On multiple imputation, these associations remained (Table 5-10), but the difference between of SD_{ADC} of CLD and term group was not statistically significant anymore. The SD_{ADC} of very preterm without CLD group was significantly lower than that of the term group even after employing multiple imputation (Table 5-10).

TABLE 5-8

Alveolar dimensions and intra-subject spread of alveolar dimensions(ADC and SD_{ADC}) estimated by ^3He magnetic resonance (linear regression adjusted for anthropometric data (basic model) and additional confounders (adjusted model)

	ADC, $cm^2 \cdot sec^{-1}$			SD_{ADC} $cm^2 \cdot sec^{-1}$		
Basic model*						
	mean (CI)	difference (CI)	p	mean (CI)	difference (CI)	p
<i>Term</i>	0.092 (0.089,0.095)	~~	~~	0.007 (0.005,0.008)	~~	~~
<i>Mild</i>	0.096 (0.091,0.101)	0.004 (- 0.002,0.010)	0.160	0.007 (0.005,0.009)	0.001 (-0.002,0.003)	0.664
<i>Very Preterm</i>	0.090 (0.085,0.095)	-0.002 (- 0.008,0.004)	0.514	0.003 (0.001,0.006)	-0.003 (-0.006,- 0.001)	0.018
<i>CLD</i>	0.089 (0.083,0.094)	-0.003 (- 0.010,0.003)	0.301	0.010 (0.008,0.013)	0.003 (0.000,0.006)	0.022
Adjusted model†						
	mean (CI)	difference (CI)	p	mean (CI)	difference (CI)	p
<i>Term</i>	0.093 (0.089,0.096)	~~	~~	0.007 (0.005,0.008)	~~	~~
<i>Mild</i>	0.093 (0.088,0.099)	0.001 (- 0.005,0.007)	0.795	0.007 (0.004,0.010)	0.000 (-0.003,0.003)	0.865
<i>Very Preterm</i>	0.091 (0.085,0.097)	-0.001 (- 0.008,0.005)	0.668	0.003 (0.000,0.006)	-0.004 (-0.007,- 0.000)	0.036
<i>CLD</i>	0.089 (0.083,0.095)	-0.003 (- 0.010,0.004)	0.328	0.010 (0.007,0.014)	0.004 (0.000,0.007)	0.038

Abbreviations: CI= 95% confidence interval. CLD- chronic lung disease of prematurity.

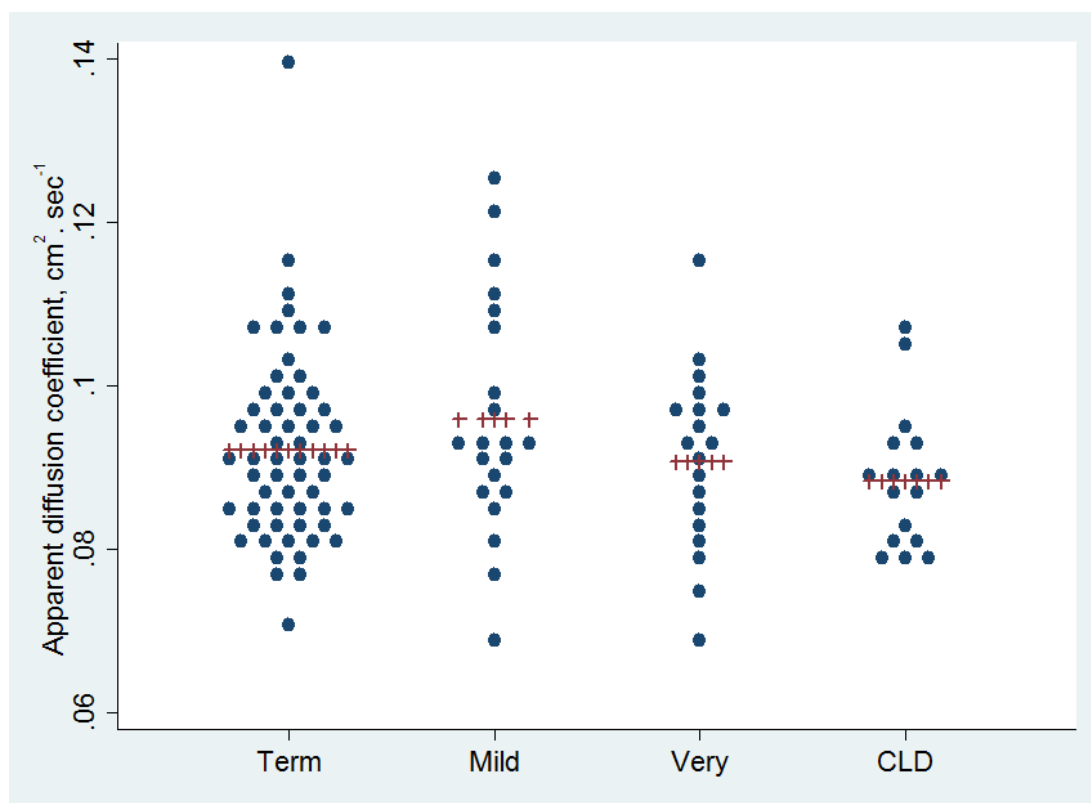
Group names - Term = term born, Mild = mild preterm, Very preterm = very preterm without CLD, CLD= very preterm with CLD.

*Basic model: adjusted for age, sex, height and ethnicity (n=119)

†Adjusted model: adjusted, in addition, for ETS exposure (before and after 3 years), corticosteroid treatment (inhaled or oral, before or after 3 years) and crowding (number of persons/room, indicator of socio-economic status)(n=98)

FIGURE 5-2

Apparent diffusion coefficient, by degree of prematurity and presence of chronic lung disease CLD



Term= Term born, Mild = mild preterm, Very = Very preterm, CLD = Very preterm with CLD.

FIGURE 5-3

Apparent diffusion coefficient against gestational age.

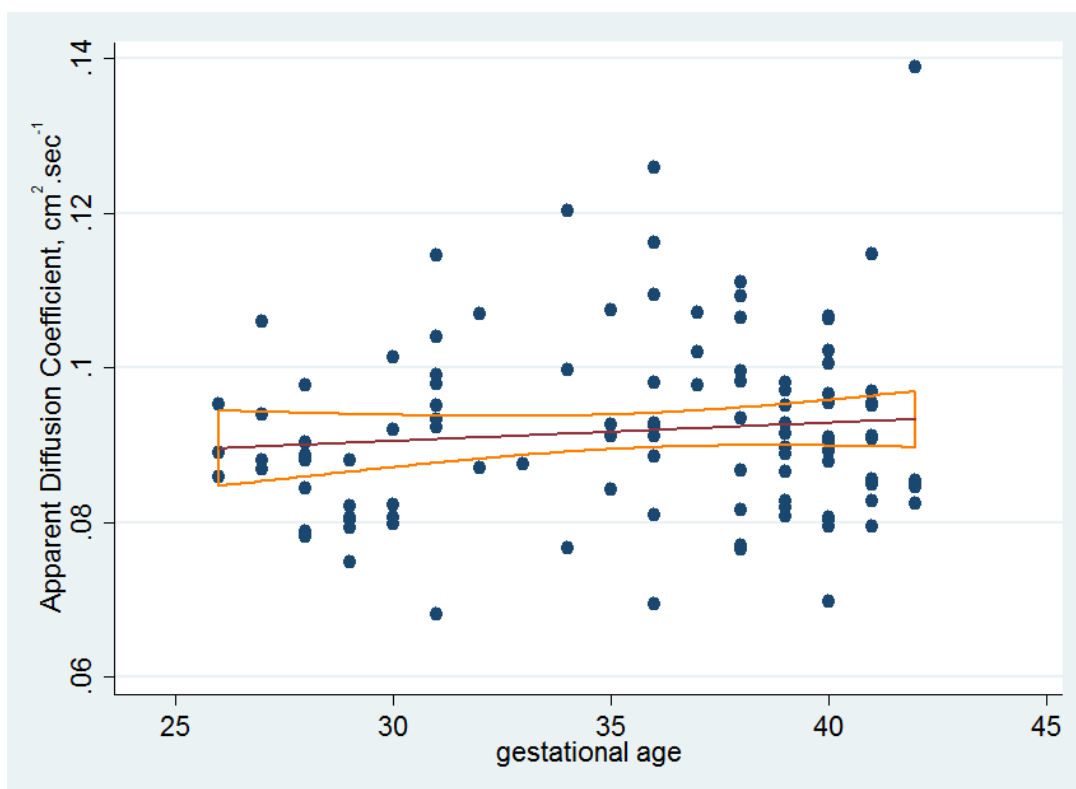


TABLE 5-9

Factors associated with apparent diffusion coefficient (ADC) from a multivariate linear analysis within the study population – not imputed model (n=99)

	Coefficient	Beta	SE	95% CI	p
Term- born	Reference	~~	~~	~~	~~
Mild preterm	0.001	0.026	0.003	-0.005,0.007	0.795
Very preterm without CLD	-0.001	-0.046	0.003	-0.008,0.005	0.668
Very preterm with CLD	-0.003	-0.102	0.004	-0.010,0.004	0.326
Male sex	-0.007 [†]	-0.279 [†]	0.002	-0.012,-0.002	0.009
Age	-0.003	-0.220	0.002	-0.006,0.001	0.119
Height	<0.001*	0.311*	0.000	0.000,0.001	0.032
White ethnicity	0.004	0.119	0.003	-0.002,0.009	0.236
ETS exposure at 0-3 years	0.006	0.237	0.004	-0.003,0.015	0.162
ETS exposure after 3 years	0.002	0.093	0.005	-0.007,0.011	0.584
Steroids [†] : 0-3 years	-0.005	-0.135	0.004	-0.014,0.003	0.241
Steroids [†] : after 3 years	0.003	0.077	0.005	-0.006,0.013	0.472
Small for gest. age	-0.002	-0.058	0.004	-0.010,0.006	0.589
Crowding	<0.001	-0.011	0.003	-0.006,0.005	0.911
Constant	0.093	~	0.002	0.089,0.096	~

Abbreviations: Coefficient - regression coefficient; Beta- standardized regression coefficient; SE - standard error; CI = confidence interval; CLD- chronic lung disease of prematurity; ETS = environmental tobacco smoke; coefficient= regression coefficient; beta =standardized regression coefficient (standard deviations units),

[†]steroid - inhaled or oral corticosteroids

* $p < 0.05$; [†] $p < 0.01$.

TABLE 5-10

Alveolar dimensions and intra-subject spread of alveolar dimensions(ADC and SD_{ADC}) estimated by ^3He magnetic resonance (linear regression adjusted for anthropometric data (basic model) and additional confounders (adjusted model) - results following multiple imputation.

	ADC cm².sec⁻¹			SD_{ADC} cm².sec⁻¹		
Basic model*						
	mean (CI)	difference (CI)	p	mean (CI)	difference (CI)	p
<i>Term</i>	0.092(0.089,0.095)	~~	~~	0.007 (0.005,0.008)	~~	~~
<i>Mild</i>	0.096(0.091,0.101)	0.004 (-0.002,0.010)	0.186	0.007 (0.005,0.010)	0.001 (-0.002,0.003)	0.713
<i>Very preterm</i>	0.091(0.085,0.096)	-0.001(-0.008,0.005)	0.679	0.004 (0.001,0.006)	-0.003 (-0.006,-0.000)	0.036
<i>CLD</i>	0.088(0.082,0.094)	-0.004(-0.010,0.003)	0.231	0.010 (0.007,0.012)	0.003 (-0.000,0.006)	0.068
Adjusted model†						
<i>Term</i>	0.092(0.089,0.095)	~~	~~	0.007 (0.005,0.008)	~~	~~
<i>Mild</i>	0.096(0.090,0.101)	0.004 (-0.002,0.010)	0.217	0.007 (0.005,0.010)	0.000 (-0.003,0.003)	0.780
<i>Very Preterm</i>	0.090(0.085,0.096)	-0.001(-0.008,0.005)	0.669	0.003 (0.001,0.006)	-0.003 (-0.006,-0.000)	0.047
<i>CLD</i>	0.089(0.083,0.095)	-0.003(-0.010,0.004)	0.430	0.010 (0.007,0.012)	0.003 (-0.000,0.006)	0.082

Abbreviations:

ADC= Apparent diffusion coefficient.

SD_{ADC} = Intra-subject standard deviation of ADC, a marker of uniformity of alveolar dimensions within subject.

CI= 95% confidence interval. CLD- chronic lung disease of prematurity. Group names - Term = term born, Mild = mild preterm, Very Preterm = Very preterm without CLD, CLD= very preterm with CLD

*Basic model: adjusted for age, sex, height, , ethnicity (n=119)

†Adjusted model: adjusted, in addition, for ETS exposure (before and after 3 years), corticosteroids treatment (inhaled or oral, before or after 3 years), and crowding (number of persons/room, indicator of socio-economic status): Note analysis done using multiple imputation (n=119)

5.3.4.2. ANALYSIS2: CLD DEFINED BY OXYGEN REQUIREMENT AT 36 WEEKS POSTMENSTRUAL AGE

TABLE 5-11

Results from analysis using alternative definition of CLD. Alveolar dimensions and intra-subject spread of alveolar dimensions(ADC and SD_{ADC}) estimated by ^3He magnetic resonance. Estimated by linear regression adjusted for anthropometric data (basic model) and additional confounders (adjusted model).

	ADC $cm^2 \cdot sec^{-1}$			SD_{ADC} $cm^2 \cdot sec^{-1}$		
Basic model*						
	mean (CI)	difference (CI)	p	mean (CI)	difference (CI)	p
<i>Term</i>	0.092 (0.089,0.095)	~~	~~	0.007 (0.005,0.008)	~~	~~
<i>Mild</i>	0.096 (0.091,0.101)	0.004 (- 0.002,0.010)	0.135	0.007 (0.005,0.01)	0.000 (- 0.002,0.003)	0.732
<i>Very Preterm</i>	0.089 (0.085,0.094)	-0.003 (- 0.008,0.003)	0.308	0.005 (0.003,0.007)	-0.001 (- 0.004,0.001)	0.327
<i>CLD</i>	0.090 (0.082,0.098)	-0.002 (- 0.011,0.006)	0.626	0.010 (0.006,0.013)	0.003 (- 0.001,0.007)	0.165
Adjusted model†						
	mean (CI)	difference (CI)	p	mean (CI)	difference (CI)	p
<i>Term</i>	0.093 (0.089,0.096)	~~	~~	0.007 (0.005,0.008)	~~	~~
<i>Mild</i>	0.093 (0.088,0.099)	0.001 (- 0.005,0.007)	0.805	0.007 (0.004,0.010)	0.000 (- 0.003,0.003)	0.938
<i>Very Preterm</i>	0.090 (0.085,0.095)	-0.003(- 0.008,0.003)	0.359	0.006 (0.003,0.008)	-0.001(- 0.004,0.002)	0.441
<i>CLD</i>	0.091 (0.082,0.101)	-0.001 (- 0.011,0.009)	0.823	0.011(0.006,0.016)	0.004 (- 0.001,0.009)	0.187

This table corresponds to table 5-8 but uses the alternative definition of CLD

Abbreviations: CI= 95% confidence interval. CLD- chronic lung disease of prematurity, defined here as children born at less than 32 weeks of gestation and oxygen dependent up to at least 36 weeks post menstrual age. Group names - Term = term born, Mild = mild preterm, Very Preterm= Very preterm without CLD, CLD Very preterm with CLD.

*Basic model: adjusted for age, sex, height, , ethnicity.

†Adjusted model: adjusted, in addition, for ETS exposure (before and after 3 years), corticosteroids treatment (inhaled or oral, before or after 3 years), and crowding (number of persons/room, indicator of socio-economic status)

With the rigorous definition of CLD (oxygen dependency at 36 weeks PMA), the pattern of results were very similar. Once again the ADC of ^3He was very similar across the 4 groups, both in the basic model and the adjusted model (Table 5-11). ADC was lower in males and increased with height (not shown). There was no trend for ADC across the groups with either basic model or adjusted model (p value for trend - 0.439 and 0.483 respectively). The results did not change after multiple imputation (not shown).

The differences in SD_{ADC} between the groups were not statistically significant in analysis 2 (table 5-11). Compared to term-born children, SD_{ADC} was lower in the very preterm without CLD group, and higher in the very preterm with CLD group but the differences were not statistically significant ($p=0.441$ and 0.187 in the adjusted model).

5.3.4.3. FACTORS ASSOCIATED WITH PRETERM BIRTH AND NICU CARE

In a subsample of children born very preterm, the association between ADC and factors associated with preterm birth and to neonatal intensive care was determined (Table 5-12). This analysis is independent of the definition of neonatal chronic lung disease. The basic model showed negative association between male sex and ADC and a positive association between white ethnicity and ADC. There was no association between ADC and the following risk factors: ventilation days, CPAP days, days on oxygen therapy and being born small for gestational age, whether taken individually or collectively (Table 5-12, figure 5-4 and 5-5). In the adjusted model, the only factor that remained statistically significant was the association between ADC and sex of the child. These results did not change on multiple imputation (not shown).

TABLE 5-12

Neonatal/ Perinatal factors associated with ADC from a multiple linear regression within the very preterm subgroups

a. Basic model (N=35)					
	coefficient	Beta	SE	CI	p
<i>Male sex</i>	-0.00981†	-0.49133†	0.00318	-0.01631,-0.00332	0.00435
<i>Age</i>	0.00199	0.11998	0.00290	-0.00392,0.00791	0.49625
<i>Height</i>	-0.00028	-0.17320	0.00029	-0.00088,0.00032	0.34703
<i>White ethnicity</i>	0.00904*	0.34243*	0.00417	0.00053,0.01755	0.03808
<i>Constant</i>	0.09000	~	0.002	0.087,0.093	~

b. Individual risk factors analysed along with factors in basic model

	coefficient	Beta	SE	CI	p
<i>Small for gest. age‡</i>	0.00012	0.00460	0.00446	-0.00904,0.00928	0.97850
<i>Number of days ventilated‡</i>	-0.00012	-0.13261	0.00018	-0.00048,0.00024	0.50953
<i>Number of days on CPAP‡</i>	0.00004	0.08150	0.00009	-0.00014,0.00022	0.64663
<i>Number of days of O₂ therapy‡</i>	0.00000	-0.00555	0.00013	-0.00028,0.00027	0.97396

c. Fully adjusted model

	coefficient	Beta	SE	CI	p
<i>Male sex</i>	-0.01226†	-0.60525†	0.00400	-0.02053,-0.00399	0.00546
<i>Age</i>	0.00213	0.11629	0.00403	-0.00621,0.01048	0.60220
<i>Height</i>	-0.00035	-0.20661	0.00040	-0.00118,0.00048	0.39461
<i>White ethnicity</i>	0.00805	0.31067	0.00479	-0.00187,0.01796	0.10679
<i>Number of days ventilated</i>	-0.00020	-0.22535	0.00022	-0.00066,0.00026	0.38210
<i>Number of days on CPAP</i>	0.00005	0.06421	0.00015	-0.00027,0.00037	0.74363
<i>Number of days of O₂ therapy</i>	0.00006	0.11269	0.00010	-0.00014,0.00025	0.56425
<i>Small for gest. Age</i>	0.00193	0.07458	0.00540	-0.00925,0.01311	0.72400
<i>Constant</i>	0.08949	~	0.00176	0.08585,0.09312	~

Abbreviations: Coefficient - regression coefficient; Beta- standardized regression coefficient; SE - standard error; CI = confidence interval; CLD- chronic lung disease of prematurity; CPAP - continuous positive airway pressure.

* $p < 0.05$; † $p < 0.01$. ‡-Values given are for 4 separate multivariate regressions of ADC against these four risk factors taken separately along with factors in the basic model (Sex, Age, Height and ethnicity). Values for the parameters of the basic model are not given for these 4 regressions

FIGURE 5-4

ADC against duration of oxygen therapy (very preterm groups only)

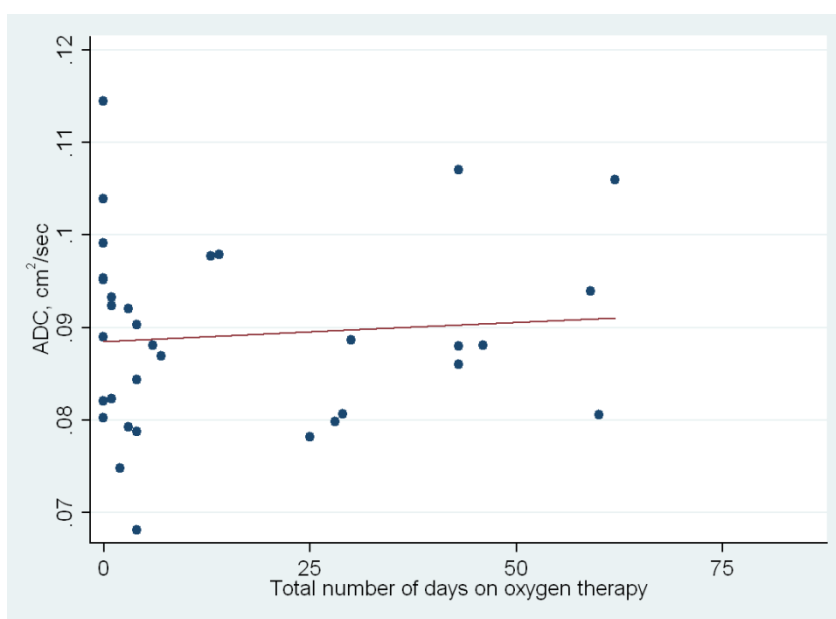
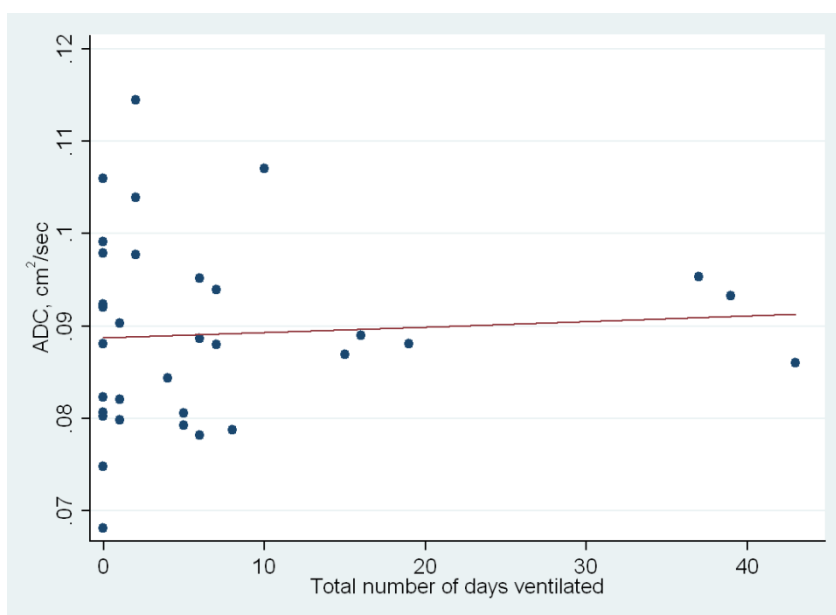


FIGURE 5-5

ADC against duration of mechanical ventilation (very preterm groups only)



5.4.INTERPRETATION

With regard to traditional lung function tests, this analysis showed evidence for abnormal airway function and air trapping (lower FEV₁ and higher RV) in children who were born very preterm. However, alveolar dimensions at later childhood, as measured by ADC in this study were essentially similar between all four groups. There was no relationship between ADC and gestational age or presence of neonatal chronic lung disease. Moreover, alveolar dimensions were not related to risk factors for CLD such as duration of ventilation and oxygen therapy. This is inspite of the fact that our group of children born very preterm (especially the CLD group) were severely affected in the neonatal period (Table 5-4 and 5-5).

This indicates that in survivors of birth at very preterm gestation, deranged alveolar development found in early childhood may be compensated within the first decade of life in survivors, probably via later alveolarization. A complete discussion is given in chapter 7.

6. OTHER FACTORS AFFECTING ALVEOLAR DEVELOPMENT

6.1.BACKGROUND

There has been little research into factors affecting human alveolar development beyond early life, apart from preterm birth and the factors involved with the care of the preterm infant. A multitude of animal studies have contributed to our understanding of effect of some factors on human lung development (summarised in section 1.5.2). These studies have mainly focused on the early neonatal period because of the consensus that human alveolar development was confined to the first 3 years of life.

We have evidence supporting continuing alveolar development through childhood and early adolescence in humans (chapter 4). Our study has also demonstrated the possibility of catch-up alveolarization in later childhood following preterm birth, (chapter 5).

The risk factors operating in early infancy (apart from preterm birth) which may be associated with deranged peripheral lung development are considered in section 1.5.2. They include: 1. maternal smoking during pregnancy and early life exposure to environmental tobacco smoke, 2. antenatal and early life exposure to corticosteroids., 3. intra-uterine growth retardation, leading to being born small for gestational age., 4. exposure to either hyperoxia or hypoxia. Similar to alveolar catch-up following preterm birth, catch-up alveolarization may be possible following exposure to these risk factors as well. However, it is also possible that these risk factors work in a different way to preterm birth and lead to persisting damage to peripheral lung structures.

Some of these risk factors may operate potentially throughout childhood and early adolescence. Given the new evidence that supports alveolarization throughout childhood (Chapter 4), the following risk factors may also affect peripheral lung development: 1. continuing exposure to environmental tobacco smoke (after 3 years of age), 2. corticosteroid exposure beyond 3 years of age and 3. nutritional inadequacy.

³HeMR is an ideal tool to resolve this issue. This chapter deals with our study using ³HeMR to determine the impact of early and later life risk factors on peripheral lung development. Effect of preterm birth was not included in this analysis because it was explored fully in Chapter 5. Data regarding the risk factors were either available at the outset of the study or obtained from questionnaires (see section 3.3). As none of our cohort of children were born at high altitudes and only one child who was born at greater than 32 weeks of gestation received neonatal intensive care, we did not explore exposure to hyperoxia or hypoxia.

6.1.1. AIMS AND HYPOTHESIS

The main aim was to determine the impact of various risk factors on peripheral lung development using $^3\text{HeMR}$.

Hypotheses: Factors that affect alveolar development in pregnancy and early childhood

- maternal smoking during pregnancy and early life exposure to environmental tobacco smoke,
- antenatal and early life exposure to corticosteroids and
- intra-uterine growth retardation, leading to being born small for gestational age

can affect alveolar dimensions measured in childhood and early adolescence measured using $^3\text{HeMR}$.

Factors that could potentially have an ongoing influence on peripheral lung structure

- continuing exposure to environmental tobacco smoke
- corticosteroid exposure beyond early life and
- nutritional status

can also affect alveolar dimensions in childhood and early adolescence as measured by $^3\text{HeMR}$.

6.2. ANALYSIS

6.2.1. SUBJECTS

Details of subject recruitment are given in chapter 3. For the purpose of this study, we analysed the association between risk factors that could potentially affect alveolar development and alveolar dimensions as measured by $^3\text{HeMR}$ in children who were not born very preterm (ie. those belonging to the LRC or the LCCHS. Those belonging to the TNS cohort were excluded in this analysis).

The exclusion criteria included children who:

- were born at less than 32 weeks gestation.
- had neonatal ventilatory/CPAP support
- had surgical lesions in chest - e.g. congenital diaphragmatic hernia
- had a severe chronic respiratory disorder, e.g. cystic fibrosis

6.2.2. VARIABLES

6.2.2.1. OUTCOME VARIABLES

The outcome variables were measures of alveolar dimensions by $^3\text{HeMR}$ including apparent diffusion coefficient (ADC), intrasubject standard deviation of ADC (SD_{ADC}), mean peripheral airspace dimension (X_{RMS}), and traditional lung function measures including forced expiratory volume in one second (FEV_1), forced vital capacity (FVC) and functional residual capacity (FRC). Measurements from $^3\text{HeMR}$ were corrected for relative inhaled bolus size and concentration of Helium in the lung (section 3.4.4 and 4.3.4.2). The measurements are explained in detail in chapter 3.

6.2.2.2. RISK FACTORS

The risk factors considered in this part of the study were: exposure to environmental tobacco smoke (ETS), exposure to corticosteroids, being born small for gestational age (SGA), and nutritional status of the subject (BMI). The rationale for choosing these risk factors is explained in 6.1.1. and 1.5.2.

1. ETS exposure:

Detailed information about timing, period and degree of ETS exposure were available from the questionnaires (appendix 1). Information from the questionnaires was validated against pre-existing data regarding parental smoking available for the children belonging to LRC. Measurements of urinary cotinine were also used to cross check questionnaire data regarding current exposure. For the purpose of this study, we collated the information available into 4 binary variables and one ordinal variable:

- Early ETS exposure: binary variable describing exposure to ETS from mother, father or other member ordinarily resident with family either in antenatal period or between 0-3 years of age.
- Late ETS exposure: binary variable describing exposure to ETS from mother, father or other member ordinarily resident with family or past personal history of smoking after 3 years of age.
- Current ETS exposure: binary variable describing exposure to ETS from mother, father or other member ordinarily resident with family or current personal history of smoking.
- Any ETS exposure: binary variable describing any exposure to ETS (positive if any of the above 3 is positive).

- ETS_category: Ordinal variable, with three values : 0- no exposure to ETS., 1- some exposure (exposure to ETS after 3 years of age) and 2- strong exposure (exposure to ETS before 3 years of age with or without exposure after 3 years of age).

2. Exposure to corticosteroids:

Similar to the ETS exposure, corticosteroid exposure was also categorised on similar lines to 4 binary variables and one ordinal variable based on exposure to either systemic or inhaled corticosteroids as follows:

- Binary variables: Early steroid exposure (exposure to antenatal corticosteroids and/or oral or inhaled corticosteroid therapy before 3 years of life) , late steroid exposure (corticosteroid therapy after 3 years of life), current steroid exposure and any steroid exposure.
- Ordinal variable: Steroids_category was an ordinal variable with the following defined values : 0- no exposure to corticosteroids, 1- exposure to corticosteroids after 3 years of age and 2- exposure to corticosteroids before 3 years of age with or without exposure after 3 years of age.

3. Small for gestational age:

Children were classified as being born small for gestational age using data available from records (gestation at birth and birthweight). Small for gestational age was defined as birthweight less than 10th centile for gestation from data of Wilcox et al (144).

4. Current nutritional status:

Normalised body mass index was used as the marker for current nutritional status. Body mass index was calculated from anthropometric measures taken at the time of the subject visit ($BMI = \text{weight(kg)}/\text{height(m)}^2$). BMI was normalised for age by the LMS method (163) using standardised WHO reference values (148,149).

6.2.2.3. CONFOUNDERS

Age, height, sex, ethnicity, gestation at birth, childhood wheeze, childhood respiratory infections and parental asthma were analysed as confounding factors.

1. Childhood wheeze: Two variables were created for childhood wheeze - early childhood wheeze (0-3 years) and late childhood wheeze (3 years and above). In both cases, the variable was coded as '1' if either of the following questions were answered 'yes':

- Has the child ever had wheezing or whistling in the chest at (the appropriate age range)? (Questionnaire 1, q12 and Questionnaire 2, q1)
- Did he/she use a steroid (brown/purple/orange) inhaler at (the appropriate age range)? (Questionnaire 1, q13)

2. Childhood respiratory illness: The term 'childhood respiratory illness' is used here as an umbrella term to cover other respiratory problems (other than wheezy illness) that could occur in an otherwise well child. Similar to childhood wheeze above, we created two variables for childhood respiratory infections (early, 0-3 years; late, 3 years and above). The relevant question in the questionnaire was:

- Has the child ever suffered from any other chest problems (other than wheeze or asthma)? (Questionnaire 1, q15)

3. Parental asthma: This was a binary variable coded as '1' if either parent had ever suffered from asthma or wheezing disorders.

6.2.3. STATISTICAL MODELS

First, the influence of each risk factor on indices associated with alveolar dimensions was analysed separately by linear regression after controlling for age, height, sex and ethnicity (univariable model). Following this, multivariate regression models were created for each outcome including all the risk factors and the confounders together in the same model. Influence of the risk factors on lung function indices (FEV1, FVC and FRC) were analysed in the same way. We used Stata 11.2 for analysis (Stata Corporation, Austin Texas).

6.3. RESULTS

6.3.1. DESCRIPTIVE STATISTICS:

Of the 209 subjects who participated in our study, 168 were eligible for inclusion in this analysis. 37 subjects were excluded because they were born at less than 32 weeks gestation and four subjects were excluded due to the following reasons:

- (i) congenital diaphragmatic hernia,
- (ii) cystic fibrosis
- (iii) severe asthma, with multiple doses of oral corticosteroids
- (iv) neonatal mechanical ventilation (>32 week gestation)

Complete data (excluding X_{RMS}) was available in 119 subjects. Only a subset of 50 children had data on X_{RMS} . Demographic data are shown in Tables 6-1 and 6-2.

TABLE 6-1

Demographic data. (Note that BMI z-score (shaded) is one of the risk factors analysed in the study)

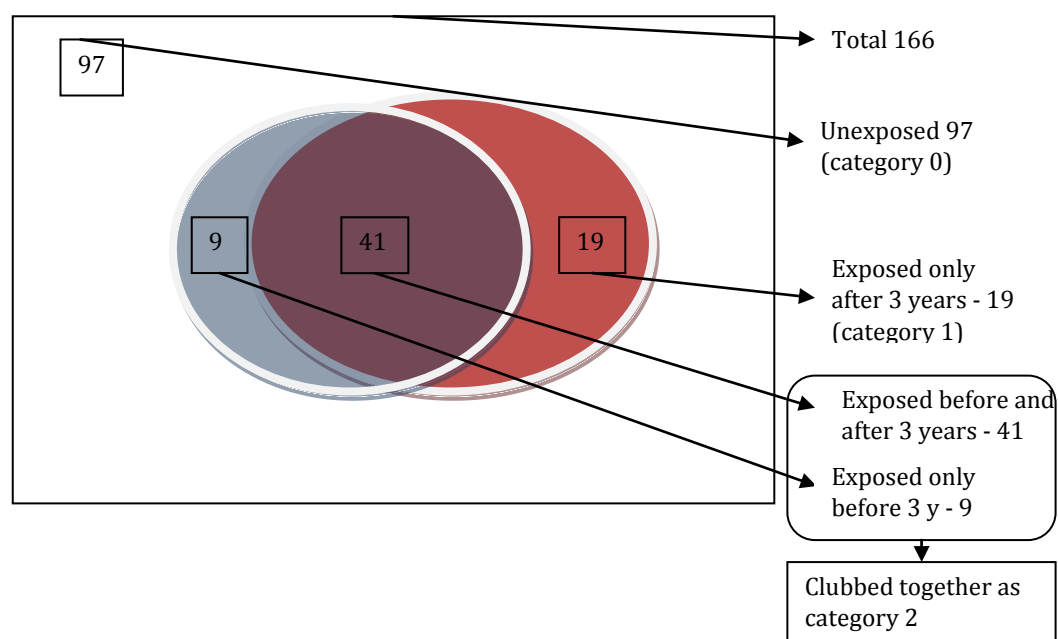
	<i>Mean</i>	<i>SD</i>	<i>Min</i>	<i>Max</i>	<i>N</i>
Age (years)	13.01	3.02	7.75	21	168
Height (m)	1.55	0.14	1.23	1.85	168
Weight (kg)	49.22	14.97	22.5	99.7	168
BMI	20.05	3.76	13.82	36.13	168
BMI z-score	0.42	1.09	-2.11	3.53	168
Gestation (weeks)	38.8	2.05	32	42	156

6.3.1.1. EXPOSURES

6.3.1.1.1. ETS exposure:

FIGURE 6-1

Venn Diagram describing ETS exposure status in the subjects



Of 166 subjects in whom exposure data to ETS was available, 97 had no history of exposure to tobacco smoke. Fifty subjects were exposed to ETS before 3 years of age (of

this, 41 were exposed before and after 3 years of age). Nineteen subjects were exposed only after 3 years of age. Therefore the ordinal variable ETS_category had :

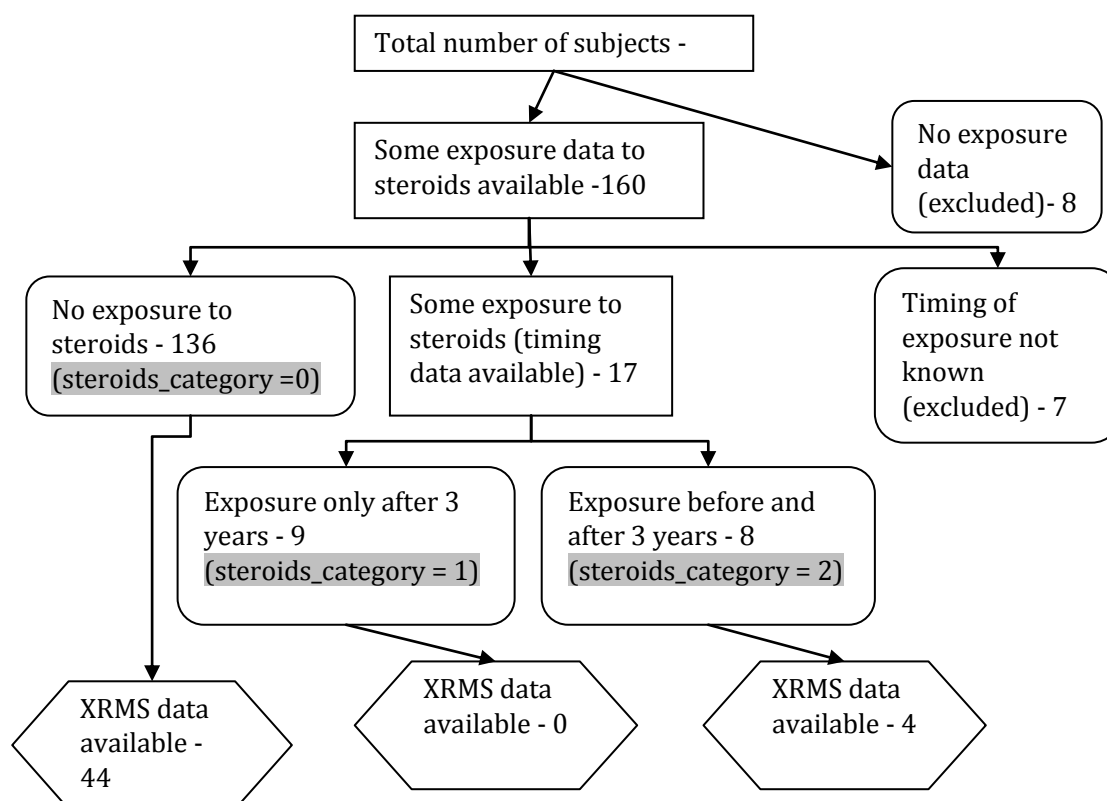
- 97 subjects with value 0- no exposure to ETS
- 19 subjects with value 1 - exposure to ETS only after 3 years of age
- 50 subjects with value 2 - exposure to ETS before and/or after 3 years of age (Table 6-2, Figure 6-1)

Of the 166 subjects with valid exposure data to ETS exposure, 49 had valid X_{RMS} measurements. Of these, 24 had ETS_category = 0, 7 had ETS_category = 1, and 18 had ETS_category = 2. In our sample, only 6 subjects admitted to personal history of smoking and of these, only one had smoked regularly (This was the only person who was also a current smoker). This subject belonged to a family with strong exposure to ETS. The other 5 were 'experimental' smoke exposures (3 who had smoked a single cigarette and other 2 for a period of less than one month). These were corroborated by the cotinine data.

6.3.1.1.2. Corticosteroid exposure:

FIGURE 6-2

Flow chart describing corticosteroid exposure status in the subjects



There were 160 subjects in whom data relating to oral and inhaled corticosteroids were available. Twenty four reported some exposure to corticosteroids. Complete data

regarding timing of exposure was available in only 153 (136 no exposure and 17 with some exposure to corticosteroids). Of these 17 subjects, 8 subjects reported exposure before (and/or after) 3 years of age and 9 reported exposure only after 3 years of age (Figure 6-2).

Therefore, the ordinal variable `steroids_category` had:

- 136 subjects with value 0 - no exposure to corticosteroids
- 9 subjects with value 1- exposure to corticosteroids only after 3 years of age
- 8 subjects with value 2- exposure before and after 3 years of age (Table 6-2)

TABLE 6-2

Risk factors (shaded boxes) and confounders (unshaded boxes)

Risk factors/ confounders	Number of subjects (number with available data)	Percent
Female sex	88 (168)	52.4
White ethnicity	129 (168)	76.8
Small for gestation	44 (152)	26.2
Never exposed to ETS	97 (166)	57.7
Only late exposure to ETS	19 (166)	11.3
Early and/or late exposure to ETS	50 (166)	29.8
Never exposed to corticosteroids†	136 (160)	80.9
Only late exposure to corticosteroids†	9 (155)	5.36
Early and/or exposure to corticosteroids†	8 (153)	4.8
Early wheeze	28 (159)	16.7
Late wheeze	38 (161)	22.6
Early chest problems	16 (155)	9.5
Late chest problems	14 (155)	8.3
Family History of asthma	38 (125)	30.4

†- inhaled or oral corticosteroids

Of the 153 subjects with valid exposure data to steroid exposure, 48 had valid X_{RMS} measurements. Of these, 44 had `steroids_category` = 0, 0 had `steroids_category` =1, and 4

had steroids_category = 2. Therefore, this variable was treated as a binary variable for the purpose of analysing influence of risk factors on X_{RMS} . Four subjects were exposed to corticosteroids antenatally - one was exposed to systemic corticosteroid given to mother for anticipated preterm birth and 3 were asthmatic mothers who took inhaled corticosteroids for asthma attacks during pregnancy

6.3.1.2. CONFOUNDERS AND LUNG FUNCTION:

Twenty eight subjects (16.7%) reported wheeze in early childhood (before 3 years of age) and 38 subjects (22.6%) reported wheeze after 3 years of age (table 6-2). Sixteen subjects (9.5%) had at least one chest infection before 3 years of age and 14 (8.3%) had at least one chest infection after 3 years of age. Family history of asthma was reported by 30.4 % of the subjects. Lung function measures from spirometry (FEV_1 and FVC) were appropriately distributed (mean z-scores around 0 and SD of z-scores around 1, Table 6-3). The standardised values are normally distributed. Results from plethysmography (FRC) were slightly lower than predicted (average z-score = -0.6, Table 6-3). This is likely due to the fact that the normalisation was based on school children on white ethnicity (164), whereas our data consists of children of both white and Asian ethnicities. However, the standardised values are normally distributed.

6.3.1.3. OUTCOME MEASURES:

TABLE 6-3

Summary of key results

	<i>Mean</i>	<i>SD</i>	<i>Min</i>	<i>Max</i>	<i>N</i>
FEV_1 (L)	2.74	0.86	1.18	5.16	166
FEV_1 z-score	0.04	0.98	-2.89	2.92	166
FVC (L)	3.16	1	1.3	6.28	165
FVC z-score	-0.04	1.00	-2.78	2.61	165
FRC (L)	1.98	0.7	0.9	4.25	168
FRC z-score	-0.6	0.95	-3.23	1.92	168
ADC	0.094	0.012	0.07	0.139	153
SD_{ADC}	5.9×10^{-3}	4.3×10^{-3}	9.9×10^{-4}	3.3×10^{-2}	146
X_{RMS}	0.42	0.04	0.35	0.51	49

Apparent diffusion coefficient (ADC) ranged from 0.0695 to 0.139 cm²/sec (mean 0.0943 cm²/sec, SD 0.0121 cm²/sec). Intra-subject spread of ADC (SD_{ADC}) ranged from 9.9x10⁻⁴ cm²/sec to 3.3x10⁻² cm²/sec (mean 5.9 x 10⁻³ cm²/sec , SD 4.3x10⁻³ cm²/sec). 49 subjects had valid X_{RMS} measurements. X_{RMS} ranged from 0.355 to 0.514 mm with a mean of 0.42 and SD of 0.037 mm.

6.3.2. UNIVARIATE ANALYSIS:

Univariate analysis was done by regression of various risk factors separately on the outcomes. The analysis was controlled for age, sex, height and ethnicity.

6.3.2.1. EFFECT OF RISK FACTORS ON LUNG FUNCTIONS

TABLE 6-4

Effect of risk factors on lung functions - univariate analysis (controlled for age, sex, height and ethnicity)

Risk Factor	FEV1, litres		FVC, litres		FRC, litres	
	Coefficient	95% Confidence interval	Coefficient	95% Confidence interval	Coefficient	95% Confidence interval
ETS - never	Reference		Reference		Reference	
ETS - after 3y	0.044	(-0.1426,0.2305)	0.081	(-0.1242,0.2863)	0.128	(-0.0532,0.3091)
ETS - before and/or after 3y	0.113	(-0.0164,0.2422)	0.147*	(0.0035,0.2906)	0.074	(-0.0509,0.1986)
Steroids - never†	Reference		Reference		Reference	
Steroids - after 3y†	-0.168	(-0.4305,0.0938)	-0.006	(-0.2980,0.2865)	-0.036	(-0.2910,0.2182)
Steroids - before and/or after 3y†	-0.024	(-0.3012,0.2534)	0.203	(-0.1060,0.5118)	0.3957**	(0.1266,0.6648)
Not small for gestation	Reference		Reference		Reference	
Small for gestation	0.154	(-0.0193,0.3279)	0.169	(-0.0256,0.3629)	0.2586**	(0.0961,0.4210)
BMI z-score	0.016	(-0.0385,0.0695)	0.053	(-0.0064,0.1128)	-0.0953**	(-0.1465,-0.0442)

*- p<0.05, ** - p<0.01, †- exposure to inhaled or oral corticosteroids.

None of the risk factors analysed had any effect on FEV1 or FVC in this study apart from a borderline positive association between strong ETS exposure (i.e. before and/or after 3 years of life) and FVC ($p=0.045$) (table 6-4). There was no association between ETS exposure and FRC. Strong corticosteroid exposure and SGA at birth were associated with higher FRC ($p=0.004$ and 0.002 respectively). On the other hand, higher BMI was associated with lower FRC ($p < 0.001$).

6.3.2.2. EFFECT OF RISK FACTORS ON ALVEOLAR DIMENSIONS

Exposure to environmental tobacco smoke was significantly associated with increased alveolar dimensions, measured by both ADC ($p= 0.007$) and X_{RMS} ($p=0.012$) (see table 6-5). There was greater impact on alveolar dimensions if smoke exposure started before 3 years of age. SD_{ADC} was not associated with ETS exposure.

TABLE 6-5

Effect of risk factors on alveolar dimensions- univariate analysis (controlled for age, sex, height and ethnicity)

Risk Factor	ADC, $\text{cm}^2.\text{sec}^{-1}$		X_{RMS} , mm		SD_{ADC} , $\text{cm}^2.\text{sec}^{-1}$	
	Coefficient	95% CI	Coefficient	95% CI	Coefficient	95% CI
ETS - never	Reference		Reference		Reference	
ETS - after 3y	0.004	(-0.0024,0.0097)	0.014	(-0.0166,0.0445)	0.001	(-0.0015,0.0032)
ETS - before and/or after 3y	0.0057**	(0.0016,0.0098)	0.0287*	(0.0067,0.0507)	-0.001	(-0.0025,0.0007)
Steroids - never†	Reference		Reference		Reference	
Steroids - after 3y†	0.000	(-0.0089,0.0082)	-		0.000	(-0.0035,0.0030)
Steroids - before and/or after 3y†	-0.003	(-0.0125,0.0057)	-0.009	(-0.0492,0.0315)	0.003	(-0.0005,0.0065)
Not small for gestation	Reference		Reference		Reference	
Small for gestation	0.003	(-0.0023,0.0088)	-0.014	(-0.0531,0.0248)	-0.0028**	(-0.0049,-0.0008)
BMI z-score	0.000	(-0.0019,0.0017)	0.010	(-0.0004,0.0203)	0.000	(-0.0006,0.0007)

*- $p < 0.05$, ** - $p < 0.01$, †- exposure to inhaled or oral corticosteroids.

There was no relationship between exposure to corticosteroid and alveolar dimensions in our study. However, the numbers of those exposed are low. We did not find

any association between SGA or BMI and alveolar dimensions. SGA was associated with slightly lower SD_{ADC} in our subjects ($p=0.007$, table 6-5).

6.3.3. MULTIVARIATE ANALYSIS

6.3.3.1. EFFECT OF RISK FACTORS ON LUNG FUNCTIONS

TABLE 6-6

Multivariate analysis of effect of various antenatal and postnatal risk factors on lung functions (controlled for age, sex, height, ethnicity, gestational age, wheeze in early life, wheeze in later life and family history of asthma)

Risk Factor/ confounder	FEV1, litres		FVC, litres		FRC, litres	
	Coefficient	95% Confidence interval	Coefficient	95% Confidence interval	Coefficient	95% Confidence interval
Constant	2.3186	(2.099,2.538)	2.5276	(2.259,2.796)	1.7196	(1.532,1.907)
Age, y	0.0803*	(0.012,0.149)	0.0948*	(0.022,0.168)	0.0718**	(0.025,0.118)
Height, m	4.7584***	(3.486,6.031)	5.0547***	(3.596,6.513)	3.0121***	(1.855,4.169)
White ethnicity	0.3387***	(0.150,0.527)	0.5162***	(0.299,0.733)	0.2330**	(0.073,0.393)
Sex (Male=1)	0.0515	(-0.123,0.226)	0.1867	(-0.016,0.389)	0.1219	(-0.027,0.271)
ETS - after 3y	-0.0458	(-0.279,0.187)	-0.0111	(-0.267,0.245)	-0.006	(-0.315,0.303)
ETS - before and/or after 3y	0.1332	(-0.049,0.315)	0.1097	(-0.097,0.316)	0.0077	(-0.144,0.160)
Steroids - after 3y†	-0.0153	(-0.431,0.401)	0.1405	(-0.407,0.688)	-0.1018	(-0.433,0.229)
Steroids - before and/or after 3y†	0.2019	(-0.211,0.615)	0.3785	(-0.129,0.886)	0.5289**	(0.173,0.885)
Small for gestation	0.3180*	(0.008,0.628)	0.1901	(-0.149,0.529)	0.1924	(-0.130,0.515)
BMI z-score	0.0059	(-0.064,0.076)	0.0896*	(0.005,0.175)	-0.1081*	(-0.195,-0.021)
Gestation, wks	-0.0141	(-0.053,0.024)	-0.008	(-0.053,0.037)	0.0097	(-0.029,0.048)
Early wheeze	0.0026	(-0.278,0.283)	-0.0027	(-0.332,0.326)	-0.0027	(-0.228,0.222)
Late wheeze	-0.2468	(-0.514,0.020)	-0.1702	(-0.480,0.139)	-0.0353	(-0.220,0.149)
Family history of asthma	0.0383	(-0.154,0.231)	0.0386	(-0.174,0.251)	0.0321	(-0.133,0.198)

*- $p<0.05$, ** - $p<0.01$, *** - $p<0.001$

†- exposure to inhaled or oral corticosteroids. Risk factors shaded in gray in above table.

FEV1, FVC and FRC showed a strong correlation with age, height and white ethnicity (as expected, see table 6-6). FEV1 did not show any statistically significant relation to risk factors, except for being higher in those born small for gestation ($p=0.044$). FVC showed a positive relation with BMI z-score ($p=0.039$: this relationship had not reached statistical significance in univariate analysis). FRC was higher in those with strong history of corticosteroid exposure ($p=0.004$) and lower with higher BMI z-score ($p=0.016$), confirming the trends seen in univariate analysis.

6.3.3.2. EFFECT OF RISK FACTORS ON ALVEOLAR DIMENSIONS

TABLE 6-7

Multivariate analysis of effect of various antenatal and postnatal risk factors on alveolar dimensions (controlled for age, sex, height, ethnicity, gestational age, wheeze in early life, wheeze in later life and family history of asthma)

Risk Factor / confounder	ADC, $\text{cm}^2 \cdot \text{sec}^{-1}$		XRMS, mm		SDADC, $\text{cm}^2 \cdot \text{sec}^{-1}$	
	Coefficient	95% Confidence interval	Coefficient	95% Confidence interval	Coefficient	95% Confidence interval
Constant	0.0880	(0.081,0.095)	0.4076	(0.375,0.441)	0.0045	(0.003,0.006)
Age, y	0.0001	(-0.002,0.002)	-0.0007	(-0.013,0.011)	0.0001	(-0.001,0.001)
Height, m	0.0472*	(0.011,0.084)	0.0392	(-0.146,0.224)	-0.0027	(-0.013,0.008)
White ethnicity	0.0034	(-0.002,0.009)	0.0081	(-0.017,0.033)	0.0005	(-0.001,0.002)
Sex (Male=1)	-0.0023	(-0.008,0.003)	0.0147	(-0.010,0.040)	-0.0006	(-0.002,0.001)
ETS - after 3y	0.0021	(-0.005,0.009)	-0.0029	(-0.032,0.027)	0.0032	(-0.001,0.008)
ETS - before and/or after 3y	0.0064*	(0.000,0.012)	0.0193	(-0.009,0.047)	0.0006	(-0.000,0.002)
Steroids - after 3y [†]	-0.0054	(-0.015,0.004)	dropped	-	-0.0007	(-0.002,0.001)
Steroids - before and/or after 3y [†]	-0.0044	(-0.015,0.006)	-0.0005	(-0.048,0.047)	0.0012	(-0.001,0.004)
Small for gestation	0.0062	(-0.002,0.015)	-0.0157	(-0.053,0.021)	-0.0011	(-0.004,0.002)
BMI z-score	0.0005	(-0.002,0.003)	0.0148**	(0.005,0.025)	0.0002	(-0.000,0.001)
Gestation, wks	-0.0011	(-0.003,0.000)	-0.0025	(-0.009,0.004)	-0.0001	(-0.000,0.000)
Early wheeze	-0.0026	(-0.008,0.003)	-0.0263	(-0.063,0.011)	0	(-0.002,0.002)
Late wheeze	0.0004	(-0.006,0.007)	0.0153	(-0.054,0.085)	0.0007	(-0.001,0.002)
Family history of	0.0014	(-0.004,0.007)	-0.0031	(-0.030,0.024)	-0.0002	(-0.001,0.001)

asthma						
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*- $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$, †- exposure to inhaled or oral corticosteroids.

Risk factors shaded in gray in above table.

ADC increased with height ($p=0.011$, table 6-7). ETS exposure was associated with higher ADC ($p=0.036$), confirming the findings of univariate analysis. The effect of ETS exposure on X_{RMS} was not significant on multivariate analysis. Higher BMI z-score is associated with larger X_{RMS} in this study.

6.4.SUMMARY AND INTERPRETATION

TABLE 6-8

Summary of significant findings in the analysis.

Risk Factor	Univariate analysis		Multivariate analysis	
	Lung functions	Alveolar dimensions	Lung functions	Alveolar dimensions
Environmental tobacco smoke - after 3 years				
Environmental tobacco smoke- before and after 3 years	↑FVC	↑ADC, ↑ X_{RMS}		↑ADC
Exposure to corticosteroids - after 3 years				
Exposure to corticosteroids - before and after 3 years	↑FRC		↑FRC	
Small for gestation	↑FRC	↓ SD_{ADC}	↑ FEV_1	
BMI z-score	↓FRC		↑FVC , ↓FRC	↑ X_{RMS}

With regard to traditional lung function tests, the consistent findings in our study (both univariate and multivariate analysis) are that functional residual capacity is higher in children who were exposed to corticosteroids before and after 3 years of age. Given that these children are most likely to have asthma (and associated inhaled corticosteroid

therapy), air trapping and associated increase in FRC is expected. The other finding was that higher BMI z-score was associated with lower FRC.

With regard to parameters from $^3\text{HeMR}$, strong exposure to tobacco smoke (both before and after 3 years) is associated with increased ADC in both univariate and multivariate analysis (and increased X_{RMS} in univariate analysis only). Therefore, there is a strong association of larger alveolar dimensions with increased exposure to tobacco smoke. Since this is not associated with changes in lung volume (FRC), it follows that children exposed to ETS have fewer, larger alveolar structures. A complete discussion is given in chapter 7.

7. DISCUSSION

7.1. SUMMARY

In this chapter, I summarise the results of the 3 studies (chapters 4, 5 and 6) included in this thesis and provide a detailed discussion of the results. I have opted to discuss all the results together because there are several common threads in the discussion, which becomes repetitive and disjointed if dealt with separately in the individual chapters.

In essence, measurements from $^3\text{HeMR}$ include the apparent diffusion coefficient (ADC) and the mean peripheral airspace dimension (X_{RMS}). Both these measurement provide surrogate measurements of alveolar dimensions. While ADC is a measure of degree of limitation to the self diffusion of helium (units $\text{cm}^2 \cdot \text{sec}^{-1}$), X_{RMS} is a measure of length (chapter 2) which can be described as a measure of mean peripheral airspace dimensions (2.2.3.1).

The findings of this work can be summarised as below:

1. Dimensions of alveoli determined by $^3\text{HeMR}$ increase with age and lung size during childhood and adolescence at a rate much less than would be expected if lung growth occurred only by expansion of the pre-existing airspaces. This can only be explained by postulating that new alveoli continue to form through childhood and adolescence
2. Alveolar dimensions determined by $^3\text{HeMR}$ in very preterm born children and CLD survivors (aged 10-14 years) were similar to that in term-born and mild preterm children. Moreover, alveolar dimensions were not related to risk factors for CLD. Therefore deranged alveolar development secondary to prematurity may be compensated within the first decade of life in survivors, probably via later alveolarization.
3. On analysis of various risk factors, exposure to environmental tobacco smoke was associated with larger alveolar dimensions as determined by $^3\text{HeMR}$. Environmental tobacco smoke may have a detrimental effect on normal alveolar development.

7.2. VALIDITY OF $^3\text{HEMR}$ AS A SURROGATE FOR ALVEOLAR DIMENSIONS

This interpretation of our results depends on the premise that measurements from $^3\text{HeMR}$ are valid proxies for alveolar dimensions. Many studies have validated measurements from $^3\text{HeMR}$ (including ADC and the q-space measures) against histological measures of alveolar dimensions in both animal and human lungs. Many other studies

have used other indirect techniques to demonstrate that $^3\text{HeMR}$ is a good measure of peripheral lung structure.

7.2.1. DIRECT EVIDENCE: HISTOLOGICAL STUDIES WITH ANIMAL LUNGS

Chen et al (126) demonstrated that ADC measured by $^3\text{HeMR}$ was higher in elastase-treated rat lungs. These lungs were then histologically demonstrated to have larger alveoli. However, the study did not show a direct correlation between ADC and histology. Peces-Barba et al (127) demonstrated similar findings in elastase-treated rat lungs. In addition, they demonstrated direct correlation between the regional ADC and histological measures such as mean linear intercept and alveolar internal area. Mata et al (129) used $^3\text{HeMR}$ to evaluate the progression of emphysema in elastase-treated rabbits. At euthanasia, regional and global histological measurements were found to correlate with respective measurements from $^3\text{HeMR}$. This study demonstrated the utility of $^3\text{HeMR}$ as a non-invasive tool to assess peripheral airspace dimensions. Using an elastase-treated rat model, Jacob et al (132) correlated parameters derived from applying Yablonskiy's acinar model (124) to histological measures. Further details of these studies are given in section 2.3.1 and 2.3.4.

7.2.2. DIRECT EVIDENCE: HISTOLOGICAL STUDIES WITH HUMAN LUNGS

Woods et al (165) compared histology and ADC in explanted human lungs (both diseased and normal). There was strong correlation between the global ADC and morphometric measurements. Regional correlations were also significant, with higher regional ADC corresponding to higher regional mean linear intercept and lower regional surface area to volume ratio. Yablonskiy et al (125) compared histological measures with parameters derived from application of his acinar model on $^3\text{HeMR}$ measurements in 6 human lung specimen. His group showed that histological measures correlated very closely with $^3\text{HeMR}$ measurements. Further details are given in section 2.3.1 and 2.3.4.

7.2.3. INDIRECT EVIDENCE

7.2.3.1. CHANGE WITH INFLATION

The fact that measures derived from $^3\text{HeMR}$ change with inflation of the lung provides indirect evidence that it measures dimensions of peripheral airspaces. Waters et al (131) reported the existence of a tight linear relation between fractional change of volume and fractional change of ADC. Halloran et al (133) showed that XRMS increases with inflation of the lung. In our study, we show that the change of ADC with lung inflation

can be modelled by a power function with the exponent 0.41 (Section 4). This is discussed further in 7.3.5.1.

7.2.3.2. POSTURAL VARIATIONS

Fichele (166) conducted regional measurements of ADC at four separate postures in 6 volunteers and concluded that alveolar dimensions were highest in the uppermost regions of the lung and lowest in the dependant areas for all postures. This gradient of relative alveolar dimensions has been previously demonstrated in histological studies (167) and using modern techniques such as positron emission tomography (168).

7.2.3.3. MEASUREMENTS IN DISEASE

In adults with COPD, where there is damage to alveoli, ADC from $^3\text{HeMR}$ has been consistently shown to be higher. In adults with emphysema, Saam et al (169) showed that mean ADC values were nearly 2.5 times higher than that of healthy volunteers. In a study group consisting of 16 healthy volunteers and 11 patients with COPD, Salerno (170) demonstrated that ADC showed a strong negative correlation with FEV₁ percent predicted ($r = -0.797$) and FEV₁/FVC percent predicted ($r = -0.930$).

7.2.3.4. COMPARISON WITH OTHER IMAGING TECHNIQUES

Fain et al (171) determined lung function parameters such as FEV₁, FVC and DLCO (Diffusing capacity of carbon monoxide) and also measured ADC by $^3\text{HeMR}$ and performed CT scans of the chest in 19 healthy subjects (of who 11 were smokers). There was a strong association between number of pack years and mean ADC, after adjustment for age. DLCO was negatively related to pack years. They also found that ADC was strongly negatively associated with DLCO and FEV₁/FVC. The authors found that $^3\text{HeMR}$ was sensitive to picking up pre-clinical changes in lung functions in these subjects, and was better than CT in this regard.

7.2.4. REPEATABILITY AND SENSITIVITY

The intra-subject coefficient of variation of ADC in our case was 3.1% despite the fact that we measured children and adolescents from 7 years of age onwards. Our X_{RMS} measurement had a coefficient of variation of 1.6%. These values compares favourably with values from Morbach et al (134) (5.1-6.1%) and Mata et al (129) (1.7%).

In the current study on normal alveolar development in children and adolescents, the difference in slopes between ADC against lung volumes over the period of lung growth and the ADC against various degrees of lung inflation in the bolus effect study was

measured by the Wald test. This test estimated the p-value for equivalence of the 2 slopes to be 2.4×10^{-6} .

The preterm study also had ample statistical power (a 90.5% statistical power of detecting a 20% increase in alveolar volume between the very preterm and the term groups with alpha of 95% - see section 5.2.6). Therefore our technique should reliably detect structurally significant differences in alveolar dimensions between the groups if they existed (also see section 7.4.4.3).

7.2.5. VALIDATION - SUMMARY

In summary, the evidence given above shows that $^3\text{HeMR}$ technique we use to measure alveolar dimensions is a sufficiently sensitive, repeatable and accurate measure. Furthermore, there are several direct histological comparisons and indirect evidence to demonstrate that they are valid measures of alveolar dimensions.

7.3. LUNG GROWTH BY CONTINUOUS ALVEOLARIZATION

7.3.1. PREVIOUS THEORY AND DRAWBACKS

At the time this study started, the widely prevalent view regarding human alveolarization was that it was complete by about three years of age. Further growth of the lung was postulated to occur by expansion of pre-existing alveoli. This was based on studies done using older techniques of morphometry, whose drawbacks have been discussed (section 1.3.4.2). The most influential study in this regard was Thurlbeck's study (32) which postulated that alveolarization was completed by 2-3 years in humans. This contributed to the prevailing paradigm (section 1.4.4) that neo-alveolarization was not possible after the stage of micro-vascular maturation, which occurs around 2-3 years in human lungs (35,160).

There have been newer developments in the field of morphometry, where some of those drawbacks mentioned in 1.3.4.2 have been addressed (1.2.1.3). These new techniques however, have not been applied in studies of human alveolar development, largely due to problems with availability of suitable specimens.

7.3.2. EVIDENCE FOR CONTINUOUS ALVEOLARIZATION IN ANIMAL STUDIES

Recent studies in animals using both old and new morphometric techniques have showed that new alveolar development is possible throughout the period of lung growth and even in adulthood.

Hsia et al (114) demonstrated that alveolar surface area tends to normalise by about 16 months following right pneumonectomy in adult dogs. They examined lungs using Weibel's morphometric techniques 5 months (in case of 2 dogs) and 16 months (in case of 3 dogs) following right pneumonectomy. Alveolar surface density was found to progressively increase between 5 to 16 months postpneumonectomy to reach values approximating the surface density in control dogs. This adaptive response was postulated to be due to initial expansion of alveolar airspaces to fill the thoracic cavity following pneumonectomy followed by septation of enlarged airspaces.

Kovar et al (112) determined alveolar number by Weibel's morphometric methods in rabbits at various ages from birth to 36 weeks of life (adulthood). They found that alveolar number increases progressively from birth to adulthood, though the rate of new alveolarization decreases with age. They concluded that rabbits were good models for onset of alveolarization but were doubtful about its role as a model for cessation of alveolarization.

Hyde et al (111) examined the lungs of 26 rhesus monkeys at various ages from 4 days of life to 2675 days of life (i.e. neonatal period to adulthood: somatic growth complete by 2160 days). The microscopic evaluation to determine alveolar development was based on the new technique of design based stereology. The number of alveoli showed a significant increase with age, throughout the period of somatic growth. Rhesus monkeys are more plausible as models for cessation of alveolarization in humans than rabbits. The authors called for reconsideration of previous reports of postnatal alveolar development in humans because the previous reports were based on bias-prone techniques.

Massaro et al (60) showed that calorie restriction in adult mice resulted in alveolar loss, manifested by decreased alveolar number and increased alveolar volume. Within 72 hours of refeeding, the alveoli showed complete regeneration, and the alveolar number and size reverted to values prior to calorie restriction. The degree of plasticity of adult alveoli was revealed in this study. There have been reports of emphysema in human lungs associated with anorexia nervosa (172) and it is suggested that this is an adaptive response conserved during evolution (173).

Schittny et al (113) determined the progress of alveolarization in Sprague-Dawley rats from 4 days of life to 60 days (adulthood in these rats) using design based stereology techniques. They found that new alveolar septae were being formed well into adulthood. Using 3D synchrotron radiation X-ray tomography, they showed local duplication of single capillary layers in areas of postmaturity septal growth which indicated a potential mechanism for post-mature alveolarization. This dispelled the notion that the immature double capillary layer in alveolar walls is a pre-requisite for new septation.

Taken together, all this information from mammals supports alveolarization beyond early childhood. There is no reason why this could not happen in humans.

7.3.3. EVIDENCE FOR CONTINUOUS ALVEOLARIZATION IN HUMAN STUDIES

Prior to this study, there was some indirect evidence indicating that alveolarization continues beyond early childhood in humans too. Brown et al (46) used electrical impedance tomography to determine an average alveolar number of 90 million at age 2-3 years compared to 300 million in adults, implying that alveolarization continues to take place after 3 years of age.

Using design based stereology in adult human lungs, Ochs et al (30) showed that alveolar number was closely related to adult lung volume and that mean alveolar size was almost constant between subjects. If alveolarization were completed by 2-3 years, the final number of alveoli, and by extrapolation, the final size of the lung would have to be set by then, which is implausible. This provides more indirect evidence in favour of human alveolarization beyond early childhood.

Following the completion of this study, neo-alveolarization has been reported following pneumonectomy in an adult human using $^3\text{HeMR}$ (174). This again provides evidence that alveolarization need not be confined to early childhood in humans.

7.3.4. OTHER STUDIES OF ALVEOLAR DIMENSION IN CHILDREN BY $^3\text{HeMR}$

The only previous study to look at lung alveolar development in humans using $^3\text{HeMR}$ did not conclude that there was a possibility of continuous alveolarization in children and young adults. In this report, Altes et al (175) measured ADC in 29 healthy subjects ranging from 4 to 30 years. They reported an increase of ADC with age. However, they did not measure lung size by independent means and there was no attempt to determine the expected increase of ADC with age. Our study also showed that ADC

increased with age, but we were able to show that this increase is much less than expected for the scenario of no new alveolarization.

Using $^3\text{HeMR}$, Shanbhag et al (121) measured X_{RMS} in 5 children aged 6 years and in adults and found that X_{RMS} was lower in children than adults. Again, there was no independent assessment of lung volume nor attempt to determine the expected change in X_{RMS} with age in this study. The mean age of adult subjects was 49 years. Previous human studies have shown evidence of increase in alveolar size with age in adulthood (176), which is thought to be part of the ageing process. Similarly ADC has also been shown to increase with age in adulthood (131). Therefore the difference between X_{RMS} in children and adults may be due to this effect. In support of this theory, X_{RMS} reported for the 5 children was very close to the values in the 2 youngest adults (both 28 years) in their study.

7.3.5. DISCUSSION OF OUR STUDY RESULTS

7.3.5.1. INHALATION AS A MODEL FOR GROWTH WITHOUT NEW ALVEOLARIZATION

In our study, we use $^3\text{HeMR}$ to compare the change of dimensions of alveoli with age and lung size during childhood to change of dimensions with expansion due to inhalation. In other words, inhalation is used as a model for lung enlargement without alveolarization. This assumes that inhalation does not distort the anatomy of the peripheral lung structures. The bolus of gas used in the bolus - effect study did not inflate the lung by more than 64% of FRC (except in one case, where we inflated by 86% of FRC). This is well below the limits of expansion of the lung, as total lung capacity is about twice to three times FRC (177,178).

O'Halloran et al (133) argued that lung inflation could be by recruitment of previously closed alveoli. This is likely only towards maximal inhalation (179). Also, if this were the case, inflation would be an underestimate of the degree of alveolar enlargement expected if lung growth were by expansion of pre-existing alveoli. Therefore, the true difference between the observed and expected change of ADC with growth would be greater than calculated in section 4.3.4 (and the green line representing expected increase in ADC only by expansion of pre-existing alveoli in figure 4-2 would be steeper) strengthening the case for lung growth by alveolarization even further.

7.3.5.2. ABSENCE OF GEOMETRICAL ASSUMPTIONS

Our results were not based on any geometrical model of the peripheral airspaces. In fact, the only assumption is that expansion of the lung during moderate degrees of

inhalation is an acceptable model for lung growth without new alveolarization. Given this assumption, subsequent analysis is based on statistical approaches. The change of ADC with inflation is modelled by a power function ($y \propto s^\beta$: section 4.3.3.1), where the exponent β is allowed to vary between subjects (implying that we do not assume any particular geometry holds between subjects). It is worth noting that the variability of β between subjects is negligible ($\sigma_\beta = 1.38 \times 10^{-8}$: section 4.3.3.4), implying that there might indeed be similar geometrical determinants of ADC in our subjects.

7.3.5.3. CONSIDERATION OF OTHER EXPLANATIONS FOR OUR RESULTS

We considered the possibility that the relative lack of increase in ADC and X_{RMS} with growth (than expected from the prevailing paradigm) could be explained by changes in geometry of lung acinus with growth rather than neo-alveolarization. However, there is morphometric data to suggest that the relative dimensions of alveoli and alveolar ducts remain constant during lung growth (32). Also, our q-space data, analysed using the acinar model of Yablonskiy et al (124) show that both alveolar sleeve diameter and alveolar duct diameter increased less than expected with lung growth. In addition, because ADC and the parameters derived from q-space MR are measured with diffusion times of 7 and 5 milliseconds respectively, they measure different geometric aspects of the peripheral airspaces and changes in relative geometry should be reflected in different relationships of these parameters with growth. However both increase with FRC at a rate considerably slower than would be expected in the absence of neo-alveolarization. Therefore, it is unlikely that the results can be explained by changes in geometry.

7.3.6. STRENGTHS

The strengths of the study include the large number of volunteers from a wide age range spanning most of the period of lung growth. Prospectively collected data on early life exposures and pre-existing lung disease were available in a large number of volunteers (135), which allowed selection of volunteers known to be healthy. Repeated MR measurements at varying inflation volumes were performed in some subjects, which facilitated the statistical test of the hypothesis of no new alveolarization. Independent measurement of lung size by plethysmography, enabled the calculation of expected slope in the models. Finally, the application of two different techniques of MR permitted evaluation of lung geometry.

7.3.7. LIMITATIONS

Studies of alveolar size and number in subjects at different ages, including this study, share the common assumption that the cross-sectional data are representative of longitudinal changes during the period of growth. We found wide inter-individual variability in ADC, but our large number of volunteers and the repeatability of ADC measurements (within subjects) minimises the effect of this problem. Also, preliminary longitudinal data (part of a separate study) corroborates the evidence from this study. Further longitudinal measurements of ADC will help refine the results of this study and begin to explain the wide population scatter of ADC values.

7.4.NEOALVEOLARIZATION IN PRETERM SURVIVORS

7.4.1. HISTOLOGICAL STUDIES OF PRETERM SURVIVORS

Birth at very preterm gestations (<32 weeks) and neonatal chronic lung disease (CLD) are important factors that affect alveolar development (section 1.5.2.1). Many studies (48-50,81) have used histological techniques to determine peripheral lung structure on lung specimen of children who have died of CLD. However, there are few human studies looking at alveolar structure in longer-term survivors of preterm birth and CLD. This is partly because such studies depend on morphometric histological techniques which can only be carried out on autopsy specimens.

In the 1970s, Bonikos et al (81) examined the lungs of 21 infants who were born between 28 weeks gestation to term, who required ventilatory support. The preterm survivor who survived longest was born at 32 weeks gestation and was 217 days of life at death. Multiple abnormalities were found at pathological examination and damage to alveoli and the gas exchanging zone was a feature of the infants who survived more than 33 days.

Sobonya et al (50) described the morphometric analysis of the lungs of a male infant born at 31 week gestation and died at 33 months of age. The authors report greatly reduced alveolar internal surface area and number of alveoli.

Stocker(51) reported his microscopic analysis of the lungs of 28 infants who died between 3 months of age to 40 months of age after preterm birth (born between 1974-1984). The gestation at birth was not reported. The infant who survived longest (40 months) weighed 740 g at birth. Alveolar septal fibrosis was the predominant finding in the lung periphery, but morphometric measurements of alveolar size were not reported.

Erickson et al (180) examined autopsied lung specimen of 46 subjects who died following preterm birth. They identified 3 morphological patterns. Pattern 1 was seen in infants who died early following preterm birth (mean 39 days, range 5-150 days), where lungs showed marked interstitial fibrosis. Pattern 3 was seen in infants who survived the longest (mean 277 days, range 35- 790 days) and lungs showed marked enlargement of airspaces and apparent reduction in alveolar numbers. Pattern 2 was the intermediate pattern in intermediate survivors (mean 142 days, range 21- 425 days) and showed features of both pattern 1 and 3. The oldest survivor analysed in this study is 790 days (2.16 years). These studies were done in the pre-surfactant era and therefore the findings are characteristic of classical or 'Old BPD'.

In more recent studies, derangement of alveolar development was the major feature seen following preterm birth. Hislop (48) compared histological and morphometric appearance of the lungs in 3 groups of preterm born infants (non ventilated 31-36 week gestation, low pressure ventilated 26-38 week gestation and high pressure ventilated 25-33 week gestation infants) with term born infants who died of non-respiratory causes. The ventilated group had the lowest alveolar number, highest mean linear intercept (Lm) and lowest total surface area (TSA) of alveoli. Slope of change of alveolar number with age was reduced in the ventilated infants. The age of death of these infants were from 1 day of life to 14 months of life.

Margraf (58) compared 8 children born at 24-32 weeks who were ventilator dependent for 2-12 months (BPD group) with 6 children who were term born but died due to non-respiratory causes (Control group). Lm was increased and alveolar number was reduced in the ventilated group. The age of histological analysis in case of the BPD group was from 2 months to 28 months.

Husain et al (49) compared peripheral lung histology of 14 infants who had surfactant treated BPD (S-BPD) with 8 infants who had non-surfactant treated BPD (NS-BPD) and 15 age matched controls using standard histology and morphometric methods. They showed that S-BPD had little alveolar septal fibrosis, but almost complete arrest of acinar development (resulting in larger, simpler and fewer alveoli). Of the 22 subjects who had BPD, only one was older than 2 years of age at the time of autopsy (7.75 years).

In summary, apart from the child who survived up to 7.75 years in Husain's series, all other human histological data regarding the lung structure in preterm survivors are from infants who died before 40 months of age. Overall, these studies suggest that there is delay in acinar and alveolar development in these infants up to 3 years of age. When it was taken together with the existing theory that human alveolarization is complete at 3 years,

it was assumed that deranged alveolar structure would be a lifelong feature in preterm survivors (76,83).

7.4.2. DRAWBACKS OF EXISTING THEORY

This theory could be challenged for 2 reasons. First, histological data are necessarily limited to the fatal, severe cases of preterm CLD and therefore cannot be generalized to survivors. The second challenge is to the assumption that human alveolarization is complete at 3 years.

7.4.2.1. ANIMAL STUDIES

As described above, human histological studies preferentially illustrate the severe end of the spectrum of CLD. Data from animal studies were designed to resolve this conundrum by determining alveolar structure in animals who survived birth at very preterm gestations and did not die due to its complications. Bland and coworkers (181,182) have developed the preterm lamb model of CLD, by delivering lamb fetuses at 120-130 days of life (term = 145 days) and ventilating them. The longest survival reported is 33 days.

However, the most well known of the animal models was the preterm baboon model developed by Coalson's team (see detailed description in 1.5.2.1.3). The animal models confirmed that alveolar hypoplasia was the predominant feature of 'new CLD' (seen in ex-preterm infants following modern neonatal care, characterised by administration of corticosteroids antenatally to mother and postnatal administration of surfactant combined with "gentle ventilation"). Despite this pioneering work, there were no data on long term survivors, since the maximum survival reported in the preterm baboons was 8 months – a human developmental equivalent of 3 years.

7.4.2.2. ASSUMPTION REGARDING ENDPOINT OF HUMAN ALVEOLAR DEVELOPMENT

The second drawback to the existing theory is to the assumption that human alveolarization is complete at 3 years. New evidence from animal models (section 7.3.2), indirect human data (section 7.3.3) and evidence from this study (chapter 4 and section 7.3.5) show that this assumption may not be valid.

Animal models of survivors of preterm birth and neonatal CLD into adulthood may be developed in the future to answer the question regarding alveolar structure in human long-term survivors. However, this remains an expensive undertaking. The only realistic method to resolve this question is the use of non-invasive techniques of measuring alveolar structure.

Prior to the currently reported study, only one previous study has looked at alveolar structure in preterm survivors using $^3\text{HeMR}$, with results being reported only in abstract form (183-185). They reported a higher ADC in survivors of CLD compared to term born children. They included a wide age range (5 -18 years) but did not have independent measures of lung volumes. There was no attempt to adjust the results for confounding factors. Furthermore, there was a trend towards normalisation of ADC in preterm survivors from age 5 to 15 years.

7.4.3. DISCUSSION OF OUR STUDY RESULTS

7.4.3.1. STUDY POPULATION

The children who were recruited to our study of preterm alveolarization were born between 1995 and 1998. Overall, only 9% of the children born very preterm received greater than 80% oxygen in the first 24 hours of life and 66% had weaned down to FiO_2 of 30% or below by first 24 hours of life (Section 5.3.2). Therefore it is more likely that the modern neonatal policies of gentler ventilation were implemented during the care of these infants. This observation is corroborated by the fact that nearly all of these infants were exposed to antenatal corticosteroids and a high proportion had surfactant therapy (Table 5-4, 5-5). Therefore, it is likely that the pathological picture of lungs in these infants who met the diagnostic criteria for CLD was 'new CLD'(70).

Conversely, it is also to be noted that the CLD group had length of stay between 45 to 249 days and median respiratory support for 62 days (73 days in case of analysis 2). The duration of oxygen dependency in this cohort of subjects is similar to that seen in previous studies of chronic lung disease of prematurity (86). It is therefore unlikely that our population was skewed to the milder end of the spectrum of disease. The fact that the main results of the analysis did not change on using the stricter definition of CLD substantiates the robustness of the results.

With regard to the term group, there are recent reports regarding increased respiratory morbidity faced by children born at 37-38 weeks gestation compared to children born after 39 weeks of gestation (186). However, this study looked at the short-term risks and not at long term outcomes. In any case the effect is very minor and became significant only on a population based study of 33,488 subjects. Also, we excluded term infants who needed respiratory support from our study. Therefore we do not think that this would impact on the results.

Birthweight centile is lower in the CLD group compared to very preterm without CLD group. This is consistent with previous findings that IUGR is one of the risk factors for CLD (187).

7.4.3.2. CLASSICAL LUNG FUNCTION TESTS

Classical lung function tests show that the 2 groups of very preterm born children had findings suggestive of obstructive airway disease (lower FEV₁ z-score and higher RV z-score). This finding has been replicated in other studies. Narang et al (187) reviewed studies of lung function in survivors of preterm birth and CLD. Most studies report lower FEV₁ and other features of airway obstruction in ex-preterms. However, these studies were from the pre-surfactant era. Fawke et al (157) compared spirometric lung function in groups of extreme preterm children born below 25 weeks of gestation (with or without CLD) with controls. FEV₁ and FEV₁/FVC were significantly lower in both preterm groups (with CLD group being more severely affected) compared to controls. These children were born in 1995 and were treated with surfactant and antenatal corticosteroids (consistent with new CLD). Assessment of lung volumes by plethysmography was not done in that study.

Vrijlandt et al (188) performed lung function tests in a group of 42 young adults (approx 19 years old) who were born at less than 32 weeks of gestation and against 48 age matched term born controls. The preterm group had lower FEV₁, FVC, FEV₁/FVC, and higher RV %TLC suggesting airway obstruction. Again, this cohort was from pre-surfactant era.

7.4.3.3. ADC - SENSITIVITY TO DETECT DIFFERENCES IN ALVEOLAR SIZE

We showed that ADC in children born very preterm and in survivors of neonatal CLD were similar to that in term-born and mild preterm children. Also, ADC was not related to risk factors for CLD. Given that ADC is a reliable and valid measure of alveolar dimensions (7.2), we can surmise from these results that any derangement in alveolar development due to preterm birth/CLD may be compensated within the first decade of life in survivors. The validity of this statement depends on the ability of our technique to discriminate between expected differences in alveolar dimensions between the groups.

Our study could pick up differences of 6.6% in ADC between the term group and the very preterm groups with a statistical power of 80% and alpha of 95% (Section 5.2.5.3). This equates to a 16.9% difference in volume or 5% difference in linear dimensions. Similarly, the technique can distinguish differences of 8.5 % in ADC

(equivalent to 21.7% difference in volume or 6.7% in linear dimensions) between the term group and CLD group.

The difference in linear dimensions of alveoli between term and CLD groups in histological studies is far higher than this. For example in Husain's study (49), term born controls had average mean linear intercept (Lm) of 0.1222, while the mean Lm for surfactant-BPD group was 0.21 (71.8 % average difference in linear dimensions) and non-surfactant-BPD group was 0.172 (40.7% average difference in linear dimensions). If alveolar catch-up does not occur, then this difference would be maintained as these infants grow up. Even allowing for the fact that Husain's study was looking at post-mortem specimen (and therefore the severe end of the spectrum), the expected differences are so high that we are confident that our study could identify differences in alveolar dimensions between the term and BPD groups. Thus the results are likely to have clinical significance.

7.4.3.4. INTRASUBJECT STANDARD DEVIATION OF ADC

The intrasubject standard deviation of ADC (SD_{ADC}) has been used by other authors to determine the degree of homogeneity of alveolar size within the lung. There is evidence to show that SD_{ADC} is higher in disease. Swift (188) compared SD_{ADC} between healthy non smokers, healthy smokers and COPD patients. While COPD patients had higher mean and standard deviation of ADC, healthy smokers had higher SD_{ADC} but similar mean ADC when compared to the non-smoker group. The implication is that less homogenous ADC values indicate early damage to the gas exchange area of the lung. Similar results were shown by Fain et al (171). It is important to note that these authors used techniques which provided more 'resolution' to regional ADC. Both Swift and Fain (171,189) used techniques that provided in excess of 10000 regional ADC values (pixels), whereas our technique only provides 64 regional ADC values from 64 parasagittal sections of the thorax.

In our study, SD_{ADC} was higher in the CLD group and lower in the very preterm without CLD group compared to the term group (in analysis 1). While it was predictable that the CLD group has higher SD_{ADC} than the term group (because of multiple neonatal insults including longer duration of positive pressure ventilation leading to patchy lung disease), it was unexpected to find that SD_{ADC} was lower in very preterm without CLD group (compared to term group). There are two plausible explanations for these results. It is conceivable that extreme preterm born infants who do not develop CLD have genetic factors that confer advantage and this may manifest as relative homogeneity of alveolar dimensions. Also, because this group (by definition) did not need supplemental oxygen

after the first 4 weeks of life, it is plausible that more new alveoli formed in this group over a shorter timescale than the CLD or term born groups. If many alveoli are formed at a similar age, I suggest that they are more likely to be uniform in size when the lung grows and therefore, SD_{ADC} is likely to be lower.

The differences in SD_{ADC} remained substantially the same when the analysis was done using the alternative definition of CLD (analysis 2). However, the difference was no longer statistically significant, perhaps because the number of CLD subjects in the latter analysis was only 9. The relationship of SD_{ADC} with very preterm birth and CLD, therefore, requires validation in larger studies.

7.4.4. STUDIES USING OTHER TECHNIQUES

Numerous studies applied high resolution computed tomography (HRCT) to CLD survivors, though only a few have studied 'new CLD' survivors. Aukland et al (190) studied two cohorts of preterm survivors born between 1982-85 and 1991-92. They performed HRCT in both inspiration and expiration and found a decrease in hypo-attenuated areas from expiratory CT (26%) to inspiratory CT (14%), compatible with air trapping (these numbers represent combined values from both birth cohorts). However, the emphysema score in these subjects was 0% (189). Therefore, it seems likely that hypo-attenuation seen in HRCT images of preterm survivors is not due to deranged alveolar development but due to air trapping resulting from airways disease.

In any case, information from studies using HRCT should not be interpreted in the same way as $^3\text{HeMR}$ studies. HRCT attenuation is affected not just by alveolar air, but also by parenchyma (including blood vessels). Also, the resolution of HRCT is in the order of mm (for example, in Aukland's study, it is 1.25 mm). Van Beek et al(191), in an adult study of COPD, suggested that HRCT gives different information from $^3\text{HeMR}$ and that $^3\text{HeMR}$ correlates better to measures of alveolar function such as diffusing capacity of carbon monoxide (DLCO).

Many studies looking at DLCO in preterm survivors have showed decreased DLCO in preterm survivors compared to controls (73,188). However, DLCO is a test of alveolo-capillary function and is influenced by the permeability of the alveolo-capillary barrier and ventilation-perfusion mismatch, apart from the alveolar surface area. Therefore, DLCO can be abnormal even if alveolar structure has normalised. Such a dichotomy between alveolar structure and function may be more likely to happen in preterm survivors, where the damage occurs during alveologenesi and capillary maturation, than in COPD, where damage occurs in mature alveolar-capillary units. Narang et al (192) have

shown that though DLCO is decreased at rest in ex-preterm subjects studied at 21 years of age, it normalises with exercise. Such an improvement with dynamic testing is not plausible with persistent structural damage to alveoli.

7.4.5. STRENGTHS OF THIS STUDY

Our inclusion of a control group of children who had similar characteristics to the study groups was one of the strengths. These groups were recruited from subjects randomly selected from population-based databases. The 2 very preterm groups were selected from the Trent Neonatal Survey database which is a database of all children born less than 32 weeks gestation in the Trent region. Details of important antenatal and neonatal risk factors (e.g. antenatal corticosteroids, surfactant administration) were collected prospectively in the database and made available to us. All subjects performed lung function tests including plethysmography, which enabled us to correct ADC measurements for relative difference in the helium bolus size and helium concentration.

7.4.6. LIMITATIONS

With regard to the preterm study, our data were necessarily limited to the group of children who were able to perform lung function tests adequately. This might have excluded children at the most severe end of the spectrum of CLD. Another limitation of this study is that we assumed that our very preterm born children had deranged alveolar structure in infancy. In the absence of non-invasive techniques applicable to infancy and in the presence of good histological evidence that infants born at this gestation have deranged alveolar structure, this was a reasonable assumption. In this study, we did not do measurements of peripheral airspace dimensions, with the second technique of MR (q-space technique) on these subjects. Finally, we did not assess alveolar function using techniques such as DLCO, because this would have increased the burden of the demanding protocol to an unacceptable extent.

7.5. FACTORS AFFECTING ALVEOLAR DEVELOPMENT

7.5.1. IMPACT OF ETS EXPOSURE

7.5.1.1. LUNG FUNCTION AND ETS EXPOSURE

Smoking is a well known causative factor of chronic obstructive pulmonary disease (COPD), which is characterised by decrease in spirometric indices and damage to

the peripheral architecture of the lung (emphysema) (98). Exposure to environmental tobacco smoke (ETS) in childhood and young adulthood is linked to decreased lung function in adulthood (193). Exposure to ETS during antenatal period is also associated with decreased childhood lung function (194, 195). These effects of ETS on lung function has been documented to be one of the childhood risk factors associated with development of COPD in later life(7). In our study, however, we did not find a detrimental association between ETS exposure and lung function. It has to be noted, however, that the studies that linked ETS exposure with decreased lung function had far more subjects than our study. For example, in Moshammer's study 626 children had complete lung function and questionnaire data while Gilliland had 3357 subjects with complete data(194,195). Similarly data was available for 15901 subjects in Svanes' study(7) and 2195 subjects in Upton's study(193). The aim of our study was not to detect differences in lung function due to exposure to ETS and it is likely that this study may have been underpowered to detect any difference.

7.5.1.2. HISTOLOGICAL EVIDENCE

The association between tobacco smoking and damage to the structure of lung periphery has been well documented in adults with COPD (196,197). There are no direct histological studies in humans linking second hand smoke exposure to deranged lung structure. Elliot et al histologically examined airways and parenchymal tissues in 32 infants who died of sudden infant death syndrome(101). The mean distance between alveolar attachment points on intraparenchymal airways was significantly higher in infants who were exposed to tobacco smoke in utero compared to those who were not exposed. Postnatal exposure did not have a similar effect. Thus there is some indirect human histological evidence linking ETS exposure and deranged lung structure.

However, there is abundant evidence from animal models. Section 1.5.2.4 explores the association between ETS exposure to alveolar development in animal models. In summary, it is known from experimental animal studies that exposure to tobacco smoke (antenatal or postnatal) is associated with decreased alveolar number and increased alveolar volume in early childhood (64). We can conclude from experimental studies on pregnant rhesus monkeys and their offspring that both antenatal and postnatal exposure is associated with derangement in alveolar development (64,100). In fact, antenatal exposure may be more important in this regard (64).

7.5.1.3. PERSISTENCE OF LUNG DAMAGE FOLLOWING ETS EXPOSURE

Maritz et al exposed pregnant and lactating rats to nicotine (injected subcutaneously) and compared alveolar volume and number in their offspring with control rat offspring who were not exposed(63). Morphometry was carried out on the sacrificed rat pups at day 14, 21, 35 and 42 respectively. They found that alveolar volume was significantly higher and alveolar number was lower in the nicotine exposed group than the controls at day 35 and 42 (after nicotine exposure had ceased), but not at day 14 and 21. There was no difference in the gestation duration and birthweight between the nicotine exposed group and the control group. The authors hypothesize that nicotine exposure could have resulted in some fundamental alteration in genes controlling lung development and integrity of peripheral lung structures.

Lovasi et al (102) assessed measures of early emphysema in CT scans of 1781 healthy non-smokers, done as a part of the multi-ethnic study of atherosclerosis (MESA study). They correlated the findings with self reported childhood ETS exposure and lung function. The group that had higher ETS exposure in childhood were found to have more severe and earlier onset of emphysema than the other group. The authors conclude that there is evidence of long term persistence of damage to peripheral lung structure as a result of exposure to tobacco smoke in childhood. It was noted previously (section 7.4.5) that CT findings should not be interpreted in the same way as $^3\text{HeMR}$ results. However, the findings of our study corroborate the conclusion from their study.

7.5.1.4. DISCUSSION OF OUR STUDY RESULTS

In our study, exposure to environmental tobacco smoke (ETS) was consistently associated with increased alveolar size (both increase ADC and increase X_{RMS}). ETS exposure was associated with increased ADC after controlling for confounding factors including gestational age and IUGR. Interestingly, presence of exposure to tobacco smoke before 3 years of life (ETS_category = 2) was more closely associated with increased alveolar dimensions in later childhood and adolescence than exposure after 3 years of life (ETS_category =1).

Our findings corroborate the animal studies mentioned above that ETS can cause persistent damage to lung alveolar structure and are consistent with the finding by Maritz et al (63) mentioned above. It is interesting that whereas damage to alveolar structure due to preterm birth has a potential to recover (chapter 5, section 7.4), damage due to ETS tends to be more persistent. This is compatible with influence of ETS being mediated by genetic or epigenetic factors while influence of very preterm birth/CLD is caused by direct structural damage (and hence recoverable after the damaging influence ceases).

While we did not find any detrimental effects on lung function parameters due to tobacco smoke exposure, we did indeed find evidence of structural damage. This is similar to the findings from Lovasi et al (102). Techniques which determine lung structure may be more sensitive in assessing lung damage than traditional lung function tests.

7.5.1.5. IMPLICATIONS - EARLY LIFE ONSET OF COPD

The implications of these results are potentially far-reaching. This is the first direct evidence for damage to alveolar structure during childhood as a result of exposure to ETS (especially in-utero ETS exposure). This may play a part in increasing the risk of COPD in later life (as surmised by Svanes et al (7)).

7.5.2. IMPACT OF EXPOSURE TO CORTICOSTEROIDS

In our study, we found an association between corticosteroid exposure and higher FRC. There were no other significant differences in lung function between the two groups. Higher FRC in the corticosteroid treated group is likely to be because they are commonly prescribed for children who have asthma and these children are likely to have a higher than average FRC because of air trapping (i.e. it is an association rather than causally related).

In our study, exposure to inhaled or oral corticosteroids was not associated with changes in alveolar dimensions. From animal models, exposure to corticosteroids is associated with deranged alveolar development (section 1.5.2.6). These studies involved administration of systemic corticosteroids. In our analysis, there were only 17 subjects who had been exposed to corticosteroids. Those who were exposed typically had exposure only to low doses of inhaled corticosteroids and/or occasional oral steroid courses (the one subject who had multiple doses of oral corticosteroids was excluded from analysis). Therefore, this study may be underpowered to detect the effect of corticosteroids on alveolar development. Effect of corticosteroids needs further evaluation in future studies.

7.5.3. IMPACT OF NUTRITION

7.5.3.1. CURRENT KNOWLEDGE REGARDING IMPACT OF NUTRITION ON ALVEOLAR STRUCTURE

As explained in section 1.5.2.3, poor nutrition has been shown to affect peripheral lung structure in adult humans and lead to emphysema-like changes in lung. Coxson(97) compared CT images of 21 young adults with anorexia nervosa with that of 16 age matched controls (all females). The mean BMI of the anorexic subjects was 18 kg/m²

whereas that of the control subjects was 27 kg/m². There were no differences in spirometric or plethysmographic indices between the groups. CT measures of attenuation were lower for the anorexic group. Therefore, they concluded that adults with anorexia nervosa had changes similar to emphysematous lungs. This is in spite of the fact that the control subjects smoked significantly more tobacco (in terms of pack-years) than the anorexic group. Cook et al(172) reported the presence of significant emphysema and bullae on quantitative analysis of CT scan in the case of a woman with severe anorexia (BMI =10 kg/m²).

The above evidence from human studies is also corroborated by animal studies. Massaro's group conducted several studies on impact of calorie restriction and refeeding on adult mice. First (198) they compared alveolar surface area and number of alveoli between calorie restricted (CR) mice and controls. The CR group was restricted to an intake of roughly 1/3 their requirement for 15 days and the controls were allowed to feed ad libitum. The CR group had 55% fewer alveoli and 25% lower surface area of alveoli at the end of 15 days. Another group of mice were restricted to 1/3 of their requirement for 15 days, and then allowed to feed ad libitum for 21 days (CR-Adlib group). This group did not show any difference in the alveolar number and surface area compared to the controls. They showed that calorie restriction can cause loss of alveoli, which can recover after refeeding.

Massaro's group also showed that calorie restriction can cause rapid loss of alveoli and that it may be related to activation of apoptosis genes (60). Three groups of adult male mice were calorie restricted to 1/3 their daily requirement, the first group for 3days (CR-3), the second for 15 days (CR-15) and 3rd one for 15 days but allowed to feed ad libitum for next 3 days (CR-Adlib). The lungs were morphometrically compared to controls which were allowed to feed ad libitum for the equivalent length of time. The mice lost 25% of their weight in 3 days and 33% in 15days. The volume of alveoli increased by 44% from baseline by 3 days and 58% from baseline by 15 days. Just 3 days of Adlib feeding restored weight and alveolar volume back to control values. Analysis of gene expression profiling showed induction of Caspase, TNF and Granzymes A & B in the calorie restricted mice (indicating apoptosis).

Similar findings of enlarged alveolar dimensions and reduced number of alveoli in calorie restricted animals were also reported in rats (199-201) and hamsters (202).

7.5.3.2. DISCUSSION OF OUR STUDY RESULTS

In our study, we analysed BMI z-score rather than BMI value as an indicator for current nutritional status. This is because the normal values for BMI vary with age in

children. We did not find any relationship between ADC and BMI z-score. BMI z-score was positively correlated with X_{RMS} only in the multivariate analysis. Therefore there was no consistent link between alveolar dimensions and nutritional status in our study. The only positive result was in the opposite direction to expected (from the animal studies it is expected that lower BMI z-score should be associated with alveolar enlargement (higher X_{RMS} and ADC)).

However, there were no obviously malnourished subjects in our group (only 2 subjects had BMI z-score < -2 (-2.1 and -2.07 respectively)). The subjects in the studies mentioned in the previous section had evidence for severe nutritional impairment. In Coxson's study, 7 out of 21 in the anorexia group had BMI below the WHO cutoff for starvation (97). The BMI of the person reported by Cook (172) was 10 kg/m². In Massaro's studies (60), there was a weight loss of between 25 to 33% in the calorie restricted mice.

In fact, more subjects were on the overweight side of the spectrum in our study (17 subjects had BMI z-score >2). The link between alveolar dimensions and obesity has not yet been explored. There is a potential for further studies using ³HeMR to determine the link between alveolar dimensions and nutrition.

7.5.4. IMPACT OF LOW BIRTH WEIGHT

7.5.4.1. EVIDENCE FOR IMPACT ON LUNG FUNCTION

Many important studies have looked at the impact of birthweight on adult lung function. The most prominent was the study by Barker et al (203) which was one of the cornerstones of his 'fetal-origins hypothesis'. In this study they administered questionnaires and measured lung functions on 825 adults (aged 59-70 years) who were born in Hertfordshire between 1920-1930. Linking the birth records to this data, they were able to show an association between low birth weight and poorer lung function in adulthood. They showed that each pound (450 g) increase in birthweight was associated with a 0.06 litre increase in FEV₁, after adjusting for smoking status. Similar results were found in a study by Boezen et al (204) from Netherlands (597 subjects performed lung function tests: a reduction of 0.013L in FEV₁ was demonstrated for every 100g reduction in birthweight). However, a smaller study from Scotland by Shaheen et al (205) on 239 subjects failed to show a relationship between birthweight and lung function in adulthood.

7.5.4.2. EVIDENCE FOR IMPACT ON LUNG STRUCTURE

In very premature infants, being born small for gestational age is known to increase the risk of bronchopulmonary dysplasia (89,90) (therefore, this was examined as a confounder in the preterm study - chapter 5). Intra-uterine growth restriction in term babies has not been directly linked to alveolar structure at birth. However, intra-uterine nutritional restriction has been shown to be associated with reduction in number of alveoli in near term rabbits (92) and sheep (93). There are no human or animal studies looking at whether any damage due to IUGR on alveolar structure persists in later life.

7.5.4.3. DISCUSSION OF OUR STUDY RESULTS

We had 152 subjects (born > 32 weeks gestation) for whom birthweight and lung function data were available simultaneously, of whom 26% were born small for gestation (defined as birthweight being below the 10th centile for gestation). The results with respect to lung function were not consistent, which may have been due to the low numbers. Both Barker's and Boezen's study mentioned above (203, 204) had in excess of 500 subjects, whereas Shaheen's study (where no link was found between lung function and IUGR) had only 239 subjects(205).

Being born IUGR did not have an effect on alveolar dimensions in our study population (aged 7-21 years). It is conceivable that even if there was a detectable effect of IUGR on lung development, alveolar catch up growth may have occurred after birth (similar to very preterm birth). Any future study looking at influence of birth weight on alveolar dimensions must consider the effect of possible alveolar catch-up growth.

7.5.5. STRENGTHS

A large number of subjects took part in the study, in particular considering the cost and complexity of the ³HeMR technique. Prospectively collected data on early life exposures and pre-existing lung disease were available in many of these subjects(135). The questionnaires explored all aspects of exposure to environmental tobacco smoke and corticosteroids in detail, including detailed exploration of the timing, duration and dosage of exposure. All subjects performed lung function tests including plethysmography, which enabled us to correct ADC measurements for relative difference in the helium bolus size and helium concentration.

7.5.6. LIMITATIONS

Because of the exploratory nature of this study, we could not do a precise power calculation. Retrospectively, data regarding two of the risk factors: corticosteroid exposure and current nutrition may have been underpowered. However, as explained in

7.5.5 above, the cost and complexity of the $^3\text{HeMR}$ technique precluded selection of more subjects. In addition, the questionnaire data regarding exact timing of corticosteroid exposure was not available in 7 out of 24 children who were exposed to corticosteroids. Further future studies may be needed to explore the role of nutrition and corticosteroid exposure on alveolar structure.

7.6.CONCLUSIONS AND SUMMARY

In summary, the overwhelming belief regarding human alveolarization at the time of commencing this study was that it would be limited to early childhood. However, new evidence from other animal studies and indirect evidence from human studies had already begun to question the current paradigm. We have demonstrated using $^3\text{HeMR}$ that alveolarization can continue through childhood into adolescence (chapter 4, section 7.3).

The prevailing thought regarding alveolar structure in children and young adults born very preterm was that any damage sustained due to preterm birth and chronic lung disease of prematurity could not catch up in these survivors. Using $^3\text{HeMR}$, we have shown that alveolar catch up is possible following damage due to very preterm birth (chapter 5, section 7.4).

We have also explored the impact of 4 risk factors on alveolar structure in childhood and adolescence. Of these, we have shown that exposure to environmental tobacco smoke (especially in early life) can be associated with deranged alveolar structure in later childhood and adolescence (chapter 6, section 7.5). Further studies may be needed to explore the impact of some of the other risk factors (exposure to corticosteroids, current nutritional status, low birth weight).

8. IMPLICATIONS, CONTROVERSIES, FUTURE DIRECTIONS

8.1.IMPLICATIONS

There are important implications to our observation that alveolarization is not confined to early life. This suggests that there is potential for repair of any damage to peripheral lung structures sustained during early life.

This suggests a potential for catch-up alveolarization following very preterm birth, and indeed, our results suggest that this takes place. These results have a significant impact on our understanding of human lung development. They alter our understanding of the long term outcome of perinatal lung injury (206). The results may help in assigning prognosis for survivors of very preterm birth. They support research into new therapies to ameliorate CLD, such as vitamin A therapy (207). While our study does not show precisely when catch-up occurred, it suggests that the window for therapeutic strategies is wider than previously thought.

Other areas where alveolar catch-up may be possible is from processes associated with diminished alveolar number in early life (e.g. following pulmonary hypoplasia due to severe oligohydramnios or congenital diaphragmatic hernia) or later in life (e.g. surgical lung resection). However, it must be noted that congenital diaphragmatic hernia is a field defect that starts much earlier in the fetal life when compared to preterm birth (the fetal lung is in canalicular stage at limits of viability whereas it is at pseudoglandular stage when the defect due to CDH becomes obvious (208)). Therefore, it may not be appropriate to extrapolate the results of catch-up growth following preterm birth on other fetal pathologies. Conversely, there is a report of alveolar catch up following surgical lung resection in an adult female (174). This corroborates evidence from our study that new alveoli can form after early childhood.

We have shown evidence that early life environmental exposures such as exposure to environmental tobacco smoke (ETS) can cause long term effects on alveolar structure. Impaired alveolarization may be a potential mechanism linking passive smoking during childhood to increased lung senescence and emphysema, and therefore COPD. While other studies have shown the link between early life risk factors and poor lung function, ours is the first study to show a direct link between ETS exposure in early life and deranged alveolar structure. While some of this, undoubtedly, is due to persistence of exposure to the above risk factors (it is likely that mothers who smoked during pregnancy continue to smoke during childhood and adolescence of the offspring), it may also be associated with fundamental epigenetic changes triggered by ETS, which precludes catch-up alveolarization.

The safety, efficacy and repeatability of $^3\text{HeMR}$ measurements in children has been demonstrated in this study. The advent of potential 'alveolar therapy' to restore damaged alveolar structure (207,209,210) requires safe, non-invasive repeatable measurements to study the outcome of future therapeutic trials. $^3\text{HeMR}$ provides such a method.

8.2.CONTROVERSIES

The conclusions we have drawn from our experimental findings have very recently been challenged by Parra-Robles and Wild (211). The crux of the criticism was based on the concept of diffusion time (t), the time that is available for the ^3He atoms to diffuse between adjacent MR signals (in case of the RARE sequence, half the interval between two echo pulses). In free space, the ^3He atoms undergo diffusion with a root mean square displacement, s , which is related to t as

$$S = \sqrt{2Dt}, \quad \text{EQUATION 8-1}$$

where D = the free diffusion coefficient of ^3He .

In restricted space, the mean displacement in time ' t ' is correspondingly reduced due to the barriers and the measured diffusion coefficient (apparent diffusion coefficient, ADC) is a measure of the restriction due to the barriers.

The contention by Parra-Robles et al was that the diffusion time employed in our study (7ms for the RARE sequence and 5ms for the Q-space sequence) result in average displacement that could 'sample' structures beyond the confines of the individual alveoli. They have attempted to model this using a mathematical model with a hypothetical geometrically constructed alveolar duct (212).

Diffusion weighted $^3\text{HeMR}$ uses the degree of restriction to diffusion of ^3He as a proxy for dimensions of the enclosing structure. It follows that the diffusion displacement, s , should be of a similar order of magnitude to the distance between the barriers. If ' s ' is too small, the ^3He molecules are not restricted by the barriers and ADC approximates free diffusion coefficient, D . If ' s ' is too large, it is affected by the structures outside the barriers. Parra-Robles et al contended that the diffusion time employed in this study would result in ' s ' that would be sensitive to structures outside the alveoli. However, ' s ' in our case is only 1.58 times larger than they suggested (because of the square root relationship). Also, while it was true that some of the ^3He atoms in our study do sample

the space outside an individual alveolus and may move to the alveolar duct space, we hold that the measurements still reflect alveolar dimensions, as the alveolar duct does not have an independent wall. The ultrastructure of the periphery of the lung is made up of alveolar septae. As long as the alveolar duct dimension does not increase or decrease independent of alveolar dimensions, our conclusions remain valid. Alveolar duct dimensions increase along with alveolar dimensions from 7 to 14 years (32). This is explored in further detail in our reply to Parra-Robles et al (213).

Other technical questions posed by the authors have been answered in detail in our response (213). In addition, the mathematical model (212,214) based on which Parra-Robles et al base their criticism has several shortcomings (213). We have suggested that a similar study to ours should be done with shorter diffusion times as mathematical modelling alone is insufficient to resolve the issue.

8.3.FUTURE RESEARCH DIRECTIONS

This study has treated cross-sectional data on children and young adults from different age groups as equivalent to longitudinal data. Fortunately, we were able to study large enough number of subjects to overcome the effect of inter-individual variations in alveolar dimensions. Still, it would be worthwhile to confirm these results on a longitudinal sample of subjects. As mentioned above, it would be useful to do the diffusion MR measurements with a shorter diffusion timescale as well as the timescale employed in this study.

Similarly, in the part of this study looking at catch up alveolarization following preterm birth, we did not have q-space data on the very premature subjects. This would be something that a future study could attempt. Because the technique requires some degree of co-operation, it is not possible to currently study children much younger than 7 years of age (we have demonstrated the feasibility for 7 years and above in this study). In our preterm study, we had subjects older than 10 years of age. Repeating this study with younger participants may be able to give a clue regarding the age of alveolar catch-up in very premature subjects.

The result of our study corroborates the findings in animal studies of Massaro et al (209) that alveoli in mature animals may be more plastic than previously thought. The risk factor study was probably underpowered to detect any effect of corticosteroids and

undernutrition. Future studies may be required to delineate effects of these risk factors. We did not look at factors such as air pollution, hypoxia (eg. effects of living at a higher altitude) and hyperoxia (oxygen therapy) on alveolar development. These may be the subject of future studies.

There are exciting new studies suggesting therapeutic measures to promote alveolarization (207). In the past, animal models and in-vitro studies were the only ways to determine efficacy of these measures. $^3\text{HeMR}$ provides an ideal and safe technique for determining efficacy on any future studies of these therapeutic measures.

In summary, $^3\text{HeMR}$ has been shown to be a safe and reliable technique to determine alveolar structure in children and adolescence. This study has answered several unanswered questions on human alveolar development. I believe that, despite the expense and complexity of the technique, $^3\text{HeMR}$ has the potential to answer many more unanswered questions regarding structure, function and development of alveoli and other structures in the periphery of the lung.

APPENDICES

APPENDIX 1 - QUESTIONNAIRES

Questionnaire 1:

Helium³ magnetic resonance Questionnaire - Birth and early childhood

First Name: _____ No: _____ 2008_He_Birth

Please complete by either ticking the appropriate box: ☒ or filling in a number 93

⇒ Persons completing questionnaire: Mother ☐ Father ☐ Other ☐
if other who
⇒ Date questionnaire completed: day _____ month _____ year _____ (please fill in today's date)

When the child was born

1. Immediately after the child was born, did he/she need to be admitted to a special care unit (the neonatal unit)? yes ☐ no ☐

⇒ If you answered "no" to this question please skip to question **8**

2. Was the child admitted because he/she was premature? yes ☐ no ☐

If yes:

- (a) How many **weeks early (before the due date)** was the baby born? weeks (please fill in number)
(b) Many maternity units give steroids to the mother before the birth in order to make baby's lung mature. Was the child's mother **given steroids** (such as dexamethasone, betamethasone)?

yes ☐ no ☐ don't know ☐

If no, what was the **reason for admission** to the neonatal unit?

- Lung problems yes ☐ no ☐ don't know ☐
- Feeding problems yes ☐ no ☐ don't know ☐
- Infection yes ☐ no ☐ don't know ☐
- Jaundice yes ☐ no ☐ don't know ☐
- Heart problem yes ☐ no ☐ don't know ☐
- Fits or other brain problems yes ☐ no ☐ don't know ☐

3. How long did your child stay in the neonatal unit? days (please fill in number) don't know ☐

4. Did the child need any **support with breathing in the neonatal unit**? yes ☐ no ☐ don't know ☐

If yes: Was the breathing **supported by**

- Breathing machine (ventilator) yes ☐ no ☐ don't know ☐

If yes: for how long? days (please fill in number)

- CPAP* by nasal prong or mask yes ☐ no ☐ don't know ☐

If yes: for how long? days (please fill in number)

- Extra oxygen yes ☐ no ☐ don't know ☐

If yes: for how long? days or weeks (please fill in number)

(*CPAP – continuous positive airway pressure – device to assist breathing by providing continuous pressure to keep the breathing passage open)

5. In the neonatal unit, did the child have **any tube inserted in the chest** (for treating air leak into the chest)?

yes ☐ no ☐ don't know ☐

6. During the period after the child's birth, did the child have **any operation on chest or heart**?

yes ☐ no ☐ don't know ☐

If yes: please describe (what operation, when and how many days after birth):

7. Were there **any other problems** with the stay in the neonatal unit that have **NOT** been covered above?

yes ☐ no ☐ don't know ☐

If yes: please specify:

- | | | | |
|--|------------------------------|-----------------------------|-------------------------------------|
| • Bleeding into brain (intraventricular haemorrhage) | yes ☐ | no ☐ | don't know ☐ |
| • PDA (Patent Ductus Arteriosus- a heart problem) | yes ☐ | no ☐ | don't know ☐ |
| • Retinopathy of prematurity (an eye problem) | yes ☐ | no ☐ | don't know ☐ |
| • NEC (Necrotising enterocolitis- a bowel problem) | yes ☐ | no ☐ | don't know ☐ |
| • Severe lung infections | yes ☐ | no ☐ | don't know ☐ |
| • Other | yes ☐ | no ☐ | don't know ☐ |

if other, please specify _____

8. Was the child **breastfed**? yes ☐ no ☐ don't know ☐

If yes:

(a) How **many months**? months (please fill in number)

(b) How **many months** was he/she **exclusively breast fed**? (meaning that the child received only breast milk without solids, formula milk or other drinks except water)? months (please fill in number)

9. During the first year of life, in what **position** was the child usually put **to sleep**?

on the tummy ☐ on the back ☐ on the side ☐ no specific position ☐ cannot remember ☐

10. Did the child's mother have **asthma or wheeze** when she was pregnant with her child?

yes ☐ no ☐ don't know ☐

11. Did the child's mother receive **treatment for asthma** when she was pregnant with her child?

yes ☐ no ☐ don't know ☐

If yes: did the child's mother use **steroid inhaler** (brown, purple or orange inhaler) or **steroid tablets** (eg. prednisolone) when she was pregnant with her child? yes ☐ no ☐ don't know ☐

Symptom history

12. Has the child ever had **wheezing or whistling in the chest** at any time in the past? yes ☐ no ☐

(By "wheeze" we mean breathing that makes a high-pitched whistling or squeaking sound from the chest, not the throat)

If yes, during **which periods**?

- | | | | |
|---|------------------------------|-----------------------------|-------------------------------------|
| • When he/she was <u>0-3 years old</u> | yes ☐ | no ☐ | don't know ☐ |
| • When he/she was <u>3-10 years old</u> | yes ☐ | no ☐ | don't know ☐ |

13. Has the child ever **used any type of inhaler**? yes ☐ no ☐ don't know ☐

⇒ If you answered "**no**" to this question please skip to question **15**

14. Did he/she use a steroid (brown/purple/orange) inhaler? yes ☐ no ☐ don't know ☐

If yes, during **which period** did he/she use the **steroid** (brown/purple/orange) inhaler?

- | | | | |
|--|------------------------------|-----------------------------|-------------------------------------|
| • When he/she was <u>0-3 years old</u> | yes ☐ | no ☐ | don't know ☐ |
|--|------------------------------|-----------------------------|-------------------------------------|

- When he/she was 3-10 years old yes ☐ no ☐ don't know ☐

15 Has the child ever suffered from any other chest problems (other than wheeze or asthma)?

yes ☐ no ☐ don't know ☐

⇒ If you answered "no" to this question please skip to question 17

16. During **which period** did he/she suffer from **the chest problems** (other than wheeze or asthma)?

- When he/she was 0-3 years old yes ☐ no ☐ don't know ☐

If yes:

(I) please describe the chest problem(s) _____

(II) was he/she **admitted to the hospital** for these problems? yes ☐ no ☐ don't know ☐

If yes: (i) How **many times** during this period? (please fill in number)

(ii) Was he/she admitted to **intensive care** for these problems?

yes ☐ no ☐ don't know ☐

(iii) If admitted to intensive care, was the child connected to a breathing machine (ventilator)

yes ☐ no ☐ don't know ☐

- When he/she was 3-10 years old yes ☐ no ☐ don't know ☐

If yes:

(I) please describe the chest problem(s) _____

(II) was he/she **admitted to the hospital** for these problems? yes ☐ no ☐ don't know ☐

If yes: (i) How **many times** during this period? (please fill in number)

(ii) Was he/she admitted to **intensive care** for these problems?

yes ☐ no ☐ don't know ☐

(iii) If admitted to intensive care, was the child connected to a breathing machine (ventilator)

? yes ☐ no ☐ don't know ☐

17. Did the child ever have **any other diseases** for which he/she has been taking **regular** (for a period of at least 2-3 months) **treatment**? yes ☐ no ☐

⇒ If you answered "no" to this question please skip to question 19

18. During **which period** did he/she suffer from **other diseases** for which he/she has been taking **regular treatment**?

- When he/she was 0-3 years old yes ☐ no ☐ don't know ☐

If yes: please specify

(I) the disease _____ (please discuss with the study team if you are unsure)

(II) and the treatment _____

- When he/she was 3-10 years old yes ☐ no ☐ don't know ☐

If yes: please specify

(I) the disease _____ (please discuss with the study team if you are unsure)

(II) and the treatment _____

19. Did the child ever take **oral steroid tablets** (prednisolone) for any disease (e.g. renal disease, rheumatoid arthritis, tuberculosis, asthma)? yes ☐ no ☐

If yes: at what age? _____ years

for which problem? _____ (please discuss with the study team if you are unsure)

for how long? weeks or months (please fill in number)

Smoking

20. Did the child's **mother** ever **smoke cigarettes**? yes ☐ no ☐ don't know ☐

If yes, during which periods?

- During pregnancy with this child yes ☐ no ☐ don't know ☐
- If yes:** how many cigarettes per day? less than 1 ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20 ☐
- When the child was 0-3 years old yes ☐ no ☐ don't know ☐
- If yes:** how many cigarettes per day? less than 1 ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20 ☐
- When the child was 3-10 years old yes ☐ no ☐ don't know ☐
- If yes:** how many cigarettes per day? less than 1 ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20 ☐
- Currently yes ☐ no ☐ don't know ☐
- If yes:** how many cigarettes per day? less than 1 ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20 ☐

21. Did the child's **father** ever **smoke cigarettes**? yes ☐ no ☐ don't know ☐

If yes, during which periods?

- During mother's pregnancy with this child yes ☐ no ☐ don't know ☐
- If yes:** how many cigarettes per day? less than 1 ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20 ☐
- When the child was 0-3 years old yes ☐ no ☐ don't know ☐
- If yes:** how many cigarettes per day? less than 1 ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20 ☐
- When the child was 3-10 years old yes ☐ no ☐ don't know ☐
- If yes:** how many cigarettes per day? less than 1 ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20 ☐
- Currently yes ☐ no ☐ don't know ☐
- If yes:** how many cigarettes per day? less than 1 ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20 ☐

22. Did any **other household members** ever **smoke cigarettes**? yes ☐ no ☐ don't know ☐

If yes, during which periods?

- During mother's pregnancy with this child yes ☐ no ☐ don't know ☐
- If yes:** how many cigarettes per day? less than 1 ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20 ☐
- When the child was 0-3 years old yes ☐ no ☐ don't know ☐
- If yes:** how many cigarettes per day? less than 1 ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20 ☐
- When the child was 3-10 years old yes ☐ no ☐ don't know ☐
- If yes:** how many cigarettes per day? less than 1 ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20 ☐
- Currently yes ☐ no ☐ don't know ☐
- If yes:** how many cigarettes per day? less than 1 ☐ 1 to 10 ☐ 11 to 20 ☐ more than 20 ☐

*If the child does not live with his/her parents or has moved away from Leicestershire, **please enter a contact address, telephone number or e-mail address.***

QUESTIONNAIRE 2:

Helium³ magnetic resonance Questionnaire on breathing problems

First Name: _____ No: 2008_He_under14

Please complete the questionnaire

Please complete by either ticking the appropriate box: ☒ or filling in a number

Persons completing questionnaire: Mother ☐ Father ☐ Other ☐

(tick all who helped to fill in) if other who

⇒ Date questionnaire completed: day _____ month _____ year _____ (please fill in today's date)

Wheezing

By "wheezing" we mean breathing that makes a high-pitched whistling or squeaking sound from the chest, not the throat

1. Has your child ever had **wheezing or whistling in the chest** at any time in the past? yes ☐ no ☐

2. Has your child had **wheezing or whistling in the chest** in the last 12 months? yes ☐ no ☐

⇒ If you answered "no" to these questions please skip to question **11**

3. In the last 12 months, has your child had wheezing or whistling in the chest **during or soon after a cold or flu**? yes ☐ no ☐

4. In the last 12 months, has your child had wheezing or whistling in the chest even **without** having a cold or flu? yes ☐ no ☐

5. **How many attacks of wheezing** has your child had during the last 12 months?

none ☐ 1 to 3 ☐ 4 to 12 ☐ more than 12 ☐

6. Do these attacks cause your child to be **short of breath**?

yes, always ☐ yes, occasionally ☐ no, never ☐

7. In the last 12 months, how often, on average, has his/her **sleep been disturbed due to wheezing**?

never woken with wheezing ☐ less than one night per week ☐ one or more nights per week ☐

8. In the last 12 months, has wheezing ever been **severe enough to limit his/her speech** to only one or two words at a time between breaths? yes ☐ no ☐

9. In the last 12 months, has his/her **chest sounded wheezy during or after exercise**? yes ☐ no ☐

10. During the last 12 months, did your child **miss school, college or work because of asthma or wheezing**?

yes ☐ no ☐

If yes: how many **days** did your child miss during the last 12 months (all missing days counted together)?

less than 1 week ☐ 1 to 2 weeks ☐ 3 to 4 weeks ☐ more than 4 weeks ☐

11. Have you ever been told by a doctor or a nurse that **your child has asthma**? yes ☐ no ☐

12. Has he/she had **any other chest or breathing problems** over the last years? yes ☐ no ☐

If yes: please describe

Ears, nose and throat

13. In the last 12 months, **how many times** has your child had a **cold or flu**?

never ☐ 1 - 3 times ☐ 4 - 6 times ☐ 7 or more times ☐

14. In the past 12 months, has your child had **hayfever**? yes ☐ no ☐

15. In the past 12 months, has your child had **allergic rhinitis** (sneeze, red eyes) caused by things other than grass pollen (for example pets or dust)? yes ☐ no ☐

16. In the past 12 months, has your child had **eczema**? yes ☐ no ☐

Coughing

17. Do you think that your child coughs **more than other people**? yes ☐ no ☐

18. Does your child usually have **a cough with colds**? yes ☐ no ☐

19. Does your child usually have **a cough even without having a cold**? yes ☐ no ☐

20. Does your child usually **bring up phlegm (spit, sputum) from your chest**? yes ☐ no ☐

(a) If yes, about how many **days a week**? 1 ☐ 2 - 3 ☐ more than 4 ☐

(b) Do you bring up phlegm **almost days** over at least 3 consecutive months of the year? yes ☐ no ☐

21. In the last 12 months, has your child had a **dry cough at night**, apart from a cough associated with a cold or a chest infection? yes ☐ no ☐

Treatment

22. Has your child taken **any inhalers or other treatment for asthma** in the last 12 months? yes ☐ no ☐

If yes: did your child take any of the following drugs during the last 12 months?

- Salbutamol, Ventolin, Bricanyl or any **blue** inhaler yes ☐ no ☐ don't know ☐
- Pulmicort, Flixotide, Becotide, Qvar or other **brown or orange** inhaler yes ☐ no ☐ don't know ☐
- Serevent or Oxis (a **green** or green-white inhaler) yes ☐ no ☐ don't know ☐
- Seretide or Symbicort (a **violet** or **red**-white inhaler) yes ☐ no ☐ don't know ☐
- **Singulair tablets** (Montelukast) yes ☐ no ☐ don't know ☐
- **Steroid tablets** (prednisolone) **for attacks** yes ☐ no ☐ don't know ☐

23. Has your child had **any other disease** for which he/she has been taking **regular** (at least for a period of 2-3 months) **treatment** in the last 12 months? yes ☐ no ☐

If yes: please specify

(a) the disease _____ (please discuss with the study team if you are unsure)

(b) and the treatment _____

24. Has your child taken **oral steroid tablets** (prednisolone) for any disease (e.g. renal disease, rheumatoid arthritis, tuberculosis, asthma) in the last 12 months? yes ☐ no ☐
If yes: please specify for which disease: _____ (please discuss with the study team if you are unsure)
 and for how long weeks or months (please fill in number)

Household and environment

25. How many rooms are there in the house where you live now? (not counting kitchen, bathroom and toilets)? (please fill in a number)

26. How many people usually live in your household, including yourself? (please fill in numbers)

(a) aged 16 and under (b) aged 17 and over

27. Which fuel is mainly used for cooking in your home? (tick as many as apply)

electricity ☐ gas ☐ other fuel ☐ if other, what _____

28. How is **your home heated**? (tick as many as apply)

central heating ☐ gas heaters in rooms ☐ coal or wood fire ☐ other ☐ if other, what _____

29. What is the **highest qualification** of the child's parents?

Mother

Father

- | | | |
|--|--------------------------|--------------------------|
| • No qualifications at all..... | ☐ | ☐ |
| • Other qualification: level unknown..... | ☐ | ☐ |
| • GCSE/O level grade A*-C, vocational level 2 or equivalent..... | ☐ | ☐ |
| • Trade apprenticeships (or equivalent)..... | ☐ | ☐ |
| • A levels, vocational level 3 or equivalent..... | ☐ | ☐ |
| • Other higher education below degree level..... | ☐ | ☐ |
| • Degree or degree equivalent, and above..... | ☐ | ☐ |

30. Has your child had any **brothers** and **sisters (including half-siblings)**? yes ☐ no ☐

If yes: (a) how **many brothers**?

what years were they born? _____

(b) how **many sisters**?

what years were they born? _____

Parents and grandparents

31. Has **the child's mother** ever suffered from any of the following conditions?

- | | | | |
|-------------------------------|------------------------------|-----------------------------|-------------------------------------|
| • wheezing | yes ☐ | no ☐ | don't know ☐ |
| • asthma | yes ☐ | no ☐ | don't know ☐ |
| • COPD | yes ☐ | no ☐ | don't know ☐ |
| • hayfever | yes ☐ | no ☐ | don't know ☐ |
| • chronic cough | yes ☐ | no ☐ | don't know ☐ |
| • other relevant lung disease | yes ☐ | no ☐ | don't know ☐ |
| • If other, what _____ | | | |

32. Has **the child's father** ever suffered from any of the following conditions?

- | | | | |
|------------|------------------------------|-----------------------------|-------------------------------------|
| • wheezing | yes ☐ | no ☐ | don't know ☐ |
| • asthma | yes ☐ | no ☐ | don't know ☐ |
| • COPD | yes ☐ | no ☐ | don't know ☐ |
| • hayfever | yes ☐ | no ☐ | don't know ☐ |

- chronic cough yes ☐ no ☐ don't know ☐
- other relevant lung disease yes ☐ no ☐ don't know ☐
- If other, what _____

33.**Mother****Father**(a) What is the child's mother's and father's **year of birth**?(b) What is the child's mother's and father's **country of birth**?(c) If born abroad, **what year did** mother and father **immigrate**?(d) What is the child's mother's and father's **main spoken language**?(e) What is the child's mother's and father's **ethnic origin***?(f) What is the child's mother's and father's **religion**?

(*with ethnic origin we mean the ethnic group your family originally comes from, for example European, Afro-Caribbean, south Asian, Chinese...)

34. In which country were the child's grandparents **born**?(a) his/her mother's **father**?(b) his/her mother's **mother**?(c) his/her father's **father**?(d) his/her father's **mother**?

If you have moved away from Leicestershire, please enter a contact address, telephone number or e-mail address.

You can also write any other comments you might have in the space below.

Thank you very much for helping us again to study and improve health in children and young people!!

QUESTIONNAIRE 3:

Helium³ magnetic resonance

Questionnaire – Personal questions –

14 years old and over (Girls)

Your first Name:

No:

2008_He_O14_F

Please complete by either ticking the appropriate box:



or filling in a number

93

⇒ 'Please fill in these questions yourself. We would like to assure you that your answers will remain confidential in all but the most exceptional circumstances, where we believe you are at risk of significant harm. We would not show this questionnaire to your parents unless we had your full agreement'

⇒ **Date questionnaire completed:** day _____ month _____ year _____ (please fill in today's date)

Smoking

1. Have you ever smoked **cigarettes or roll ups** (or any other form of tobacco)? yes ☐ no ☐

⇒ If you answered "no" to this question please skip to question **(7)**

2. Have you ever been a **regular smoker** (smoking at least daily for a period of more than 3 months)?

yes ☐ no ☐

3. In **what form** do you, or did you, usually smoke? (tick as many as apply)

- Cigarette with tobacco ☐
- Roll ups ☐
- Other ☐

If other, please specify _____

4. How **old** were you when you **started smoking**? _____ yrs _____ months

5. Are you **still smoking**? yes ☐ no ☐

If no, how **old** were you when you **stopped smoking**? _____ yrs _____ months

6. How **many cigarettes** (or equivalent) do (did) you usually smoke **per day**? (please fill in a number)

(7) Have you ever smoked **Cannabis** (also known as Marijuana, weed, grass, pot, hash...)? yes ☐ no ☐

8. In the last 12 months, how frequently have you smoked **Cannabis** (also known as Marijuana, weed, grass, pot, hash...)? never ☐ less than once a month ☐ monthly ☐ weekly ☐ daily ☐

Hobbies, activities

9. On average, how many **hours per week** do you spend outside normal work/study periods on:

(a) **sport or vigorous physical activity**? 0-1 hr ☐ 2-3 hrs ☐ 4-5 hrs ☐ 6hrs or more ☐

(b) **TV, video or computer** activities? 0-4 hrs ☐ 5-10 hrs ☐ 10-20 hrs ☐ 20hrs or more ☐

10. In the last 12 months, how often, on average, did you **eat fresh fruit**?

less than twice a week ☐ 2 to 4 times a week ☐ daily ☐ twice daily ☐

11. How do you **normally sleep**?

On your front ☐ On your back ☐ On your left side ☐ On your right side ☐ On either side ☐

I do not have any preference ☐

12. Do you currently play any **wind musical instruments** (eg. trumpet, clarinet, flute...)? yes ☐ no ☐
If yes: please specify _____

13. Have you ever played any **wind musical instruments** (eg. trumpet, clarinet, flute...)? yes ☐ no ☐
If yes: please specify _____
how long did you play it? _____ yrs _____ months

Body development

The next questions are about changes that may be happening to your body. These changes normally happen to different young people at different ages. We are asking these questions since they may have something to do with how your lungs grow. If you do not understand a question or do not know the answer, just mark "I don't know".

14. Would you say that your rapid **growth in height** that occurs in teenagers

- Has not yet begun ☐
- Has only just started ☐
- Is definitely underway ☐
- Seems complete ☐
- I do not know ☐

15. Would you say that your **body hair growth** ("body hair" means hair in any other place than your head, such as under your arms)

- Has not yet begun ☐
- Has only just started ☐
- Is definitely underway ☐
- Seems complete ☐
- I do not know ☐

16. Have you noticed any **skin changes**, especially pimples?

- Skin has not yet started changing ☐
- Skin has only just started changing ☐
- Skin changes are definitely underway ☐
- Skin changes seem complete ☐
- I do not know ☐

17. Have you noticed that your **breasts have begun to grow**?

- They have not yet started growing ☐
- They have only just started growing ☐
- Breast growth is definitely underway ☐
- Breast growth seems complete ☐
- I do not know ☐

18. Have you started to have **your periods**? yes ☐ no ☐

If yes: **how old** were you when you **started** having your periods? _____ yrs _____ months

*It is common for girls and young women to be given **contraceptive pills** (birth control pills) for painful periods or other reasons. We would like to know if you are on these pills.*

19. Do you currently take **contraceptive pills** or birth control pills for any reason? yes ☐ no ☐

If yes: what age did you **start** using them? _____ yrs _____ months

20. Have you ever taken contraceptive pills or birth control pills for any reason? yes ☐ no ☐

If yes: what age did you **start** using them? _____ yrs _____ months

what age did you **stop** using them? _____ yrs _____ months

Thank you for completing the questionnaire

APPENDIX 2:

ETHICS APPROVAL DOCUMENTS

SL14 Favourable opinion following consideration of further information
Version 2, October 2004



JHL 9580
CML Resp

Leicestershire Local Research Ethics Committee One

Lakeside House
4 Smith Way
Grove Park
Enderby
Leicester
LE19 1SS



Telephone: 0116 295 7592
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22 December 2004

Prof Michael Silverman
Professor of Child Health
Division of Child Health
c/o Research Office
Leicester General Hospital

Dear Prof Silverman

Full title of study: *Measuring lung development using Helium-3 magnetic resonance*
REC reference number: 04/Q2501/114
Protocol number: 04-Q2501-114rp040719

Thank you for your letter of 10 December 2004, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

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.

The favourable opinion applies to the research sites listed on the attached form. Confirmation of approval for other sites listed in the application will be issued as soon as local assessors have confirmed that they have no objection.

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	1, 04-Q2501-114af040717.ofd	25/06/2004	25/10/2004

SL14 Favourable opinion following consideration of further information
Version 2, October 2004

Investigator CV	1, 04-Q2501-114cv-pi040719.doc	20/07/2004	25/10/2004
Protocol	2, 04-Q2501-114rp040719.doc	15/07/2004	25/10/2004
Peer Review	1	17/07/2004	22/10/2004
Letters of Invitation to Participants	1, 04-Q2501-114il-p(1)040719.doc	15/07/2004	22/10/2004
Letters of Invitation to Participants	1, 04-Q2501-114il-p(2)040719.doc	15/07/2004	22/10/2004
Letters of Invitation to Participants	1, 04-Q2501-114il-p(3)040719.doc		25/10/2004
Participant Information Sheet	2, 04-Q2501-114is-p040719.doc	19/07/2004	22/10/2004
Participant Information Sheet	2, 04-Q2501-114is-ch040719.doc	19/07/2004	22/10/2004
Participant Information Sheet	2, 04-Q2501-114is-yp040719.doc	19/07/2004	22/10/2004
Participant Information Sheet	2, 04-Q2501-114is-yc040719.doc	17/12/2004	17/12/2004
Participant Consent Form	1, 04-Q2501-114cf-p040719.doc	19/07/2004	22/10/2004
Response to Request for Further Information	10 December 2004	10/12/2004	17/12/2004
Contract for Undertaking Clinical Research in a Host Establishment	1	22/10/2004	22/10/2004
Reply Slip	1, 04-Q2501-114rep040719.doc	15/07/2004	22/10/2004

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 are listed on the attached sheet.

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.

An advisory committee to Leicestershire, Northamptonshire and Rutland Strategic Health Authority

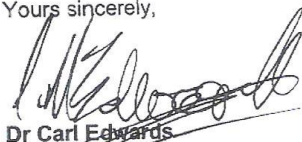
SL14 Favourable opinion following consideration of further information
Version 2, October 2004

04/Q2501/114

Please quote this number on all correspondence

With the Committee's best wishes for the success of this project,

Yours sincerely,



Dr Carl Edwards
Chair

Enclosures

Standard approval conditions

Site approval form (SF1)

APPENDIX 3:

LIST OF PUBLICATIONS/ ABSTRACTS/ PRESENTATIONS

Publications:

1. Narayanan M, Owers-Bradley J, Beardsmore C, et al. 'Alveolarization continues during childhood and adolescence: New evidence from ^3He magnetic resonance.' *Am J Respir Crit Care Med*. 2012; 185(2): 186–191.
2. Narayanan M, Beardsmore C, Owers-Bradley J, et al. 'Catch up alveolarization in ex-preterm children: Evidence from ^3He magnetic resonance'. *Am J Respir Crit Care Med* 2013; 187(10): 1104-1109
3. Narayanan M, Owers-Bradley J, Beardsmore C, Kuehni C, Silverman M. "Reply: On the Use of ^3He Diffusion Magnetic Resonance as Evidence of Neo-Alveolarization during Childhood and Adolescence", *American Journal of Respiratory and Critical Care Medicine*, Vol. 189, No. 4 (2014), pp. 502-504.

Presentations:

1. 'Emerging technologies to assess peripheral lung structure and development' at the European respiratory society (ERS) annual congress (Barcelona) in October 2010.
2. 'Evidence for acinar airway injury and alveolar catch-up growth in survivors of neonatal chronic lung disease' presented at the American thoracic society(ATS) annual congress, New Orleans, May 2010.
3. 'Evidence for continuous alveolisation during childhood, using ^3He magnetic resonance' presented at the ERS Annual Congress (Vienna), October 2009.
4. 'Let no one ever doubt, what nobody is sure about: alveolar growth' at the British Paediatric Respiratory Society summer meeting at Nottingham, July 2011
5. 'Hyperpolarized ^3He measurements of alveolar size in childhood: aging and risk factors' at the UK Pulmonary MRI workshop at the University of Sheffield on 13 April 2011.

Posters/ Abstracts

1. Narayanan M, Dogaru C, Owers-Bradley J, et al. 'Impact of early life parental smoking on acinar airway inhomogeneity': Poster discussion at ERS annual congress 2010, Barcelona.
2. Narayanan M, Dogaru C, Beardsmore CS, et al. 'Early life parental smoking is associated with enlarge alveoli in later childhood: evidence from ^3He -helium magnetic resonance'. Poster presented at ERS annual congress 2010, Barcelona.
3. Phillips J, Narayanan M, Owers-Bradley J, et al. 'Conductive and acinar ventilation inhomogeneity by nitrogen washout in healthy children.': Poster discussion at ERS annual congress 2010, Barcelona.
4. Narayanan M, Mada MO, Owers-Bradley JR, et al. 'Acinar Development after Preterm Birth': Poster discussion at the ATS Annual Conference 2008, Toronto.
5. Narayanan M, Mada MO, Owers-Bradley JR, et al. 'Pulmonary acinar structure and function after preterm birth': Poster presented at the Perinatal conference 2008, Harrogate.
6. Mada MO, Owers-Bradley JR, Narayanan M, et al. Assessment of peripheral airways development after preterm birth by hyperpolarized helium'. Poster presented at the International society of magnetic resonance in medicine May 2008, Toronto.

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