Oxidants and Antioxidants in the Pathogenesis of Chronic Lung Disease of Prematurity

A Thesis submitted for the Postgraduate Degree of Doctor of Medicine, at the University of Leicester

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Julian Vyas, July 2003
“The universe is a procession with measured and beautiful motion”

Walt Whitman, The Leaves of Spring

The most beautiful thing we can imagine is the mysterious. It is the source of all true art and science”

Albert Einstein
This thesis is dedicated
to all my family
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<td>ARDS</td>
<td>Acute Respiratory Distress Syndrome (ARDS)</td>
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<td>BAL</td>
<td>bronchoalveolar lavage</td>
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<td>BALF</td>
<td>bronchoalveolar lavage fluid</td>
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<td>BPD</td>
<td>Bronchopulmonary Dysplasia</td>
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<td>Cd</td>
<td>cadmium</td>
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<td>CLD</td>
<td>Chronic Lung Disease of Prematurity</td>
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<td>DAN</td>
<td>2,3 diaminonaphthalene (DAN)</td>
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<td>DPPC</td>
<td>dipalmitoylphosphatidylycholine</td>
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<td>EDRF</td>
<td>Endothelium Derived Relaxing Factor</td>
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<td>EGF</td>
<td>Epidermal Growth Factor</td>
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<td>EGF-R</td>
<td>Epidermal Growth Factor Receptor</td>
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<td>ELF</td>
<td>epithelial lining fluid</td>
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<td>ERK</td>
<td>extracellular signal-related kinase</td>
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<td>ETT</td>
<td>endotracheal tube</td>
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<td>FiO₂</td>
<td>fraction inspired oxygen concentration</td>
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<td>GSH</td>
<td>glutathione</td>
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<td>GSSG</td>
<td>reduced glutathione</td>
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<td>HCl</td>
<td>hydrochloric acid</td>
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<td>HMD</td>
<td>Hyaline Membrane Disease</td>
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<td>HPLC</td>
<td>high pressure liquid chromatography</td>
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<td>ICAM</td>
<td>inter cellular adhesion molecule</td>
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<td>IL-1α</td>
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<td>IL-8</td>
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<td>IVH</td>
<td>intraventricular haemorrhage</td>
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<td>LLL</td>
<td>left lower lobe</td>
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<td>L-NAME</td>
<td>L-NG-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>L-NMMA</td>
<td>N(^\text{G})-monomethyl-L-arginine</td>
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<td>LPS</td>
<td>lipopolysaccharide</td>
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<td>MAP kinases</td>
<td>mitogen-activated protein kinases</td>
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<td>MPA</td>
<td>metaphosphoric acid</td>
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<td>NANC</td>
<td>non-adrenergic non-cholinergic</td>
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<td>NaOH</td>
<td>sodium hydroxide</td>
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<td>NEDD</td>
<td>N-1-naphthylethylenediamine hydrochloride</td>
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<td>NO</td>
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<td>NO(_2)</td>
<td>nitrite</td>
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<td>NO(_3)</td>
<td>nitrate</td>
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<td>NOS</td>
<td>nitric oxide synthase</td>
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<td>PBMNC</td>
<td>peripheral blood mononuclear cells</td>
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<td>PDGF BB</td>
<td>Platelet Derived Growth Factor BB</td>
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<td>PIE</td>
<td>Pulmonary Interstitial Emphysema</td>
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<td>PIP</td>
<td>peak inspiratory pressure</td>
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<td>PMA</td>
<td>phorbol myristate acetate</td>
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<td>PMN</td>
<td>polymorphonuclear cells</td>
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<td>PPHN</td>
<td>Persistent Pulmonary Hypertension of the Newborn</td>
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<td>RDS</td>
<td>Respiratory Distress Syndrome</td>
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<td>RLL</td>
<td>right lower lobe</td>
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<td>ROP</td>
<td>Retinopathy of Prematurity</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>SAPK/JNK</td>
<td>stress activated protein kinase/c-Jun-N-terminal kinase</td>
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<td>Surfactant protein – A</td>
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<td>SP-D</td>
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<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
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<td>TNF-α</td>
<td>Tumour Necrosis Factor -α</td>
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<tr>
<td>TNM</td>
<td>tetranitromethane</td>
</tr>
<tr>
<td>TRH</td>
<td>thyrotropin-releasing hormone</td>
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<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>VLBW</td>
<td>very low birth weight</td>
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Abstract

Reactive oxygen species and other radicals (e.g. peroxynitrite) are thought to play a role in the development of chronic lung disease of prematurity (CLD). I hypothesised that serial BAL may "washout" surfactant and cause persistent radiological changes. I also hypothesised that cellular antioxidants were depleted, and nitric oxide production was increased, in bronchoalveolar lavage fluid (BALF) from infants who developed CLD. Furthermore, my aim was to develop a technique to quantitate nitrite and nitrate in small (<50 microlitres) samples of BALF. I studied three groups of infants (those who developed CLD, those who recovered from respiratory distress syndrome (RDS), and control infants ventilated for surgical reasons) and (a) determined the safety of the BAL procedure, (b) developed methods to estimate nitric oxide (NO) products, (c) estimated nitrate and nitrite in BALF and (d) measured glutathione, urate and ascorbate in BALF and plasma. My data showed that serial BAL did not adversely affect radiological appearances. I studied several techniques for reducing nitrite to nitrate, and the Griess reaction and fluorometry to quantitate nitrite. I applied a modified enzymatic method, with fluorometric detection to measure NO products. BALF nitrate concentration was similar in all groups during the first week of life. Thereafter, nitrate concentration was significantly higher (p<0.05) in infants who developed CLD compared to those who did not. Nitrite concentration did not show any trends. There was a delayed increase in BALF ascorbate in the CLD group when compared to the RDS and Control groups. The BALF: plasma ratio of ascorbate was higher in the RDS group at 4 days of age than in the CLD group, suggesting that more mature infants have a better ability to concentrate ascorbate in their lungs. In summary, I noted differences in nitrate and ascorbate concentrations between infants who developed CLD, and those who didn't.
Chapter 1

Introduction
1. Chronic Lung Disease of Prematurity

1.1 Definitions of Bronchopulmonary Dysplasia and Chronic Lung Disease

Bronchopulmonary dysplasia (BPD) was first described by Northway et al (Northway 1967). They described the clinical, radiological and histopathological features of a condition seen after recovery from respiratory distress syndrome (RDS; also called hyaline membrane disease or HMD). Prior to publication of this landmark paper, respiratory disease where preterm infants developed a persistence of oxygen need and radiological changes had been described (Wilson 1960, Shepard 1964). Northway and colleagues described BPD by clinical and pathological criteria. They suggested that BPD involved “...all the tissues of the lung in the pathologic process”, and even speculated as to its aetiological factors. They described infants of between 900 and 2466 grammes birth weight, and between 36 and 39 weeks gestation, who progressed through 4 stages of radiological and histological changes.

Stage 1: “Period of acute respiratory distress syndrome” (2 to 3 days)

This was indistinguishable from RDS. The infants required mechanical ventilatory support and supplemental oxygen therapy. The chest radiographs showed a ground glass appearance and air bronchograms. Histology of the lungs of seven infants who died during this stage showed the expected hyaline membrane, along with atelectasis, loss of ciliated cells and bronchiolar mucosal necrosis.

Stage 2: “Period of regeneration” (4 to 10 days)

Most of the infants were weaned from the ventilator during this period. Radiology of the chest showed marked opacification of both lung fields and loss of clarity of the cardiac border. Lung histology demonstrated necrosis and repair of alveolar epithelium, some persistence of hyaline membranes, an emphysematous coalescence of the alveoli as well as
thickening of capillary basement membranes, increased pericapillary reticulin, patchy bronchiolar necrosis and an eosinophilic exudate in the lumen of the airway.

Stage 3: “Period of transition” (10 to 20 days)

Only two infants had an ongoing need for mechanical ventilation by this stage. The chest radiographs changed from opacification to ‘cystic’ appearances interspersed with atelectatic shadowing. Histology confirmed the radiographic appearances. There was also bronchial and bronchiolar metaplasia, marked mucus secretion, and exudation of alveolar macrophages into the airway. The alveolar emphysematous change had progressed to groups of alveoli, and there was further basement membrane thickening.

Stage 4: “Period of chronic disease” (beyond 1 month)

Eleven infants had an ongoing oxygen requirement at this stage. Fine inspiratory crackles were heard on auscultation of the lungs. Chest radiography showed larger cystic areas, and occasional cardiomegaly. Histology showed that emphysematous alveolar groups had associated bronchioles with hypertrophy of the peribronchial smooth muscle. In addition, alveolar epithelia were highly heterotypical. Increased macrophages, increased basement membrane thickening, increased reticulin, collagen, and elastin fibrils were present. Vascular lesions of a pulmonary hypertensive type were also seen. In essence, Northway described a group of infants who had required mechanical ventilatory support with supplemental oxygen therapy, who have a specific evolution of radiological signs, and who had histological appearances of fibrosis. This description gave little insight into the underlying disease processes in this novel disease. Furthermore, since lung biopsy is hardly, if ever, performed on preterm neonates, the histopathological elements of this condition may be considered to be unhelpful when applied to a clinical context.
1.2 Pathological Features of Chronic Lung Disease of Prematurity

Following Northway's initial description Toti (Toti 1996) and Hussain (Hussain 1998) described pathological changes seen in infants treated with surfactant. Toti described increased intra-alveolar haemorrhage as well as persistence of hyaline membranes in 10 surfactant treated infants who died before 28 days of age. The pathological changes of CLD were also seen. It is unclear how many of these infants would have received a clinical diagnosis of CLD if they had lived beyond 28 days of age, or 36 weeks post-conceptional age. Hussain examined radial alveolar counts, mean line intercepts (both measures of alveolarisation of the lung), and the amount and extent of fibrosis in infants with CLD, and who had received surfactant (S-BPD) and had not received surfactant (NS-BPD) (Hussain 1998). He found that alveolar septal fibrosis appeared to be less severe but more diffuse in the S-BPD group. There was partial to complete arrest of acinar development in both groups, to a similar degree of severity. These findings suggest that the pathological changes of CLD have not substantially altered with surfactant replacement therapy.

1.3 Clinical Diagnosis of CLD

Those infants studied by Northway (Northway 1967) and other groups had died, and so post-mortem histological observations could be included within the description of the disease entity. Lung biopsy is not a technique used in living preterm infants, and so Northway's histological criteria cannot be strictly applied to live infants. For this reason different diagnostic criteria were required to allow a clinical (i.e. pre-mortem) diagnosis to be made. Bancalari and colleagues (Bancalari, 1979) subsequently proposed diagnostic criteria based upon clinical features and radiological appearances:

1. Positive pressure ventilation in the first week of life, for a minimum of three days.
2. Clinical signs of chronic respiratory disease, including tachypnoea, recession and inspiratory crackles, after twenty-eight days of age.
3. An oxygen requirement to normalise PaO₂, beyond 28 days of age.

4. A chest radiograph which shows persisting areas of hyperinflation and atelectasis.

Bancalari (Bancalari, 1979) described a population of infants who were more premature and of lower birth weight than Northway’s group (Northway 1967). It is possible that in the absence of histological data that Bancalari’s group represented a different disease, or a different part of a disease spectrum from that of Northway. These criteria from Bancalari have gained acceptance amongst neonatologists.

Subsequently, Shennen et al (Shennen, 1988) proposed that an oxygen requirement beyond 36 weeks post conceptual age (i.e. regardless of gestational age at birth), in conjunction with the other criteria, was a more sensitive marker of predicting abnormal pulmonary outcome in preterm infants. It is possible Shennen’s criteria merely describe a clinically more severe subgroup of Bancalari’s population.

With time, CLD has evolved to be a disease of the more preterm infants than those in Northway’s original cohort. As a consequence, the diagnostic criteria for CLD have had to be revised. Most recently, Jobe and Bancalari (Jobe 2001) suggested differing criteria for infants born before and after 32 weeks post conceptual age. Furthermore, they have also proposed categories of severity of disease (Table 1.1).
<table>
<thead>
<tr>
<th>Gestational age</th>
<th>&lt; 32 weeks</th>
<th>&gt; 31 weeks</th>
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<tbody>
<tr>
<td>Time point of assessment</td>
<td>36 weeks post menstrual age or discharge ( whichever comes first)</td>
<td>&gt;28 days but &lt;56 days postnatal age or discharge, whichever comes first</td>
</tr>
<tr>
<td>Mild BPD</td>
<td>Breathing room air at 36 weeks PMA or discharge, whichever comes first</td>
<td>Breathing room air by 56 days postnatal age or discharge, whichever comes first</td>
</tr>
<tr>
<td>Moderate BPD</td>
<td>Need for &lt;30% oxygen at 36 weeks PMA or discharge, whichever comes first</td>
<td>Need for &lt;30% oxygen at 56 days postnatal age or discharge, whichever comes first</td>
</tr>
<tr>
<td>Severe BPD</td>
<td>Need for &gt;30% oxygen and/or positive pressure (PPV or NCPAP) at 36 weeks PMA or discharge, whichever comes first</td>
<td>Need for &gt;30% oxygen and/or positive pressure (PPV or NCPAP) at 56 days postnatal age or discharge, whichever comes first</td>
</tr>
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**Table 1.1:** Definition of Bronchopulmonary Dysplasia: Diagnostic Criteria. Jobe 2001. Abbreviations: BPD = bronchopulmonary dysplasia; NCPAP = nasal continuous positive airway pressure; PMA = postmenstrual age; PPV = positive-pressure ventilation.

BPD is a discrete clinical entity, with typical clinical, radiological and histological features. More recently the term *chronic lung disease of prematurity* (CLD) has been applied to infants with a persistent oxygen requirement. This is a less discrete diagnosis since it relies on clinical and radiological criteria. The term appears to have arisen in the late 1980’s, and was applied to preterm infants who had a chronic oxygen requirement and persistent chest radiograph changes after the first month of life. CLD may be considered to contain infants who fit the diagnostic criteria for a number of different, but clinically similar diseases (Table 1.2).
<table>
<thead>
<tr>
<th>Disorder</th>
<th>Clinical Features</th>
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<tbody>
<tr>
<td>BPD-type I</td>
<td>Small ‘grey’ lungs on chest radiograph</td>
</tr>
<tr>
<td>BPD-type II</td>
<td>Northway type IV</td>
</tr>
<tr>
<td>Wilson Mikity syndrome</td>
<td>Similar to type II BPD but without preceding RDS</td>
</tr>
<tr>
<td>Chronic pulmonary insufficiency of prematurity</td>
<td>Similar to type I BPD but without preceding RDS</td>
</tr>
<tr>
<td>Recurrent pulmonary aspiration</td>
<td>Gastro-oesophageal reflux</td>
</tr>
<tr>
<td>‘Ogawa’ subtypes (see table 1.3)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.2:** Suggested sub-types of chronic lung disease that are contained within a diagnosis of chronic lung disease of prematurity. Modified from Kotecha S, Silverman M: Chronic Respiratory Complications of Prematurity. In: Pediatric Respiratory Medicine, Mosby 1998 St Louis

Infants with Wilson-Mikity syndrome, Chronic Pulmonary Insufficiency of Prematurity (CPIP), BPD, recurrent aspiration syndromes, or congenital pulmonary hypoplasia may be diagnosed as having CLD. In addition the clinical picture of CLD may be a result of more than one of the above conditions e.g. a preterm infant who has had RDS and progressed to ‘Northway Stage 4 BPD’ may also have recurrent aspiration from gastro-oesophageal reflux. Furthermore, caution is needed about simple comparison between infants who appear to have the same ‘sub-group’ of CLD. The clinical and radiological end points utilised may arise because of a number of different pathways. The clinical and radiological features of ‘classical’ Northway Stage 4 BPD may have arisen due to barotrauma, oxygen free radical damage, infection, ischaemia-reperfusion injury, abnormal pulmonary haemodynamics or any permutation of these factors in differing amounts. A similar clinical end point in each case may not be proof that the same pathophysiological route has been taken to reach the aforementioned end point. Other authors have attempted to tease out sub-groups of infants with possibly different types of CLD. Hyde (Hyde 1989) suggested division of cases of BPD into BPD type I, and BPD type II based on the chest radiograph...
appearances (type I having homogenous alveolar shadowing; and type II having the 'classical' features of hyperinflation and atelectasis). Subsequently, Ogawa et al suggested a more refined classification of CLD into 5 sub types (Ogawa 1997). They divided infants with CLD according to their historical and radiological features (Table 1.3). It is unclear whether these different sub-groups describe genuinely different disease entities.

Charafeddine (Charafeddine 1999) described 'atypical' patterns of chronic lung disease. This paper distinguishes between infants who had RDS and remained in supplemental oxygen until after 28 days of age, and infants who were in air after their RDS and then developed an oxygen requirement. The papers from Northway (Northway 1967), Bancalari (Bancalari 1979) and Shennen (Shennen 1988) do not comment on the pattern of supplemental oxygen therapy. Northway talks of a stage of regeneration between 4 and 10 days of age. He does not stipulate whether there was an oxygen requirement during this phase. Likewise Bancalari only mentions a need for positive pressure ventilation beyond 3 days of age. Charafeddine (Charafeddine 1999) also describes a much smaller group of infants who did not appear to develop RDS at all, but who required oxygen at 28 days of age. On initial inspection this group appear similar to those described by Wilson and Mikity (Wilson 1964). There is no mention of the chest radiograph findings in the group. The paper by Charafeddine (Charafeddine 1999) fails to adequately define clinical patterns of sub-types of CLD. The classification proposed by Ogawa (Ogawa 1997), and that of Hyde (Hyde 1989), are very similar to the initial description by Northway.

For both the proposed classifications, diagnosis of a particular sub-type of CLD depends upon the presence or absence of RDS (with or without antenatal infection), and upon particular radiological changes.
<table>
<thead>
<tr>
<th>Type</th>
<th>Description</th>
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<tbody>
<tr>
<td>I</td>
<td>History of RDS and CXR showing lucency and atelectasis</td>
</tr>
<tr>
<td>II</td>
<td>History of RDS, CXR diffusely hazy</td>
</tr>
<tr>
<td>III</td>
<td>History of intrauterine infection (raised IgM at birth or histology of chorioamnionitis; CXR as type I</td>
</tr>
<tr>
<td>IV</td>
<td>No history of RDS or infection; CXR as type I</td>
</tr>
<tr>
<td>V</td>
<td>History as type IV; CXR as type II</td>
</tr>
</tbody>
</table>

Table 1.3: Table of proposed subtypes of CLD (Ogawa 1997).

It can be seen that despite a large body of research into the immunology, microbiology and pathogenesis of CLD (which I shall consider below), this very recent suggestion for classification is based on a few simple parameters. The re-classifications described above may be thought of as merely fine-tuning the original description made over 30 years ago. This suggests that detailed research has not yet been able to significantly enhance our ability to predict or explain the development of CLD on an individual patient basis.

Over the 30 years since Northway’s original paper, neonatal intensive care has become increasingly refined. Practises that were at one time considered groundbreaking are now common place, and infants of a birth weight that was considered to have virtually 100% mortality, are now regularly surviving to discharge from the neonatal intensive care unit. For this reason a number of leading authorities refer to ‘CLD in the pre-surfactant era’, and ‘CLD in the post-surfactant era’. The differentiation made by these phrases implies that with the advent of exogenous surfactant replacement therapy, the interplay of factors that contribute to CLD may be different. Nonetheless, the clinically defined end point (development of CLD) remains the same.
Interpretation of published work on CLD must take into account the different diagnostic criteria for BPD over the last 30 years; possible clinical differences between BPD and CLD; and differences between animal and human experiments. This is essential in order to prevent a simplistic evaluation of the published evidence on the evolution and pathogenesis of this disease.

1.4 Epidemiology of Chronic Lung Disease of Prematurity

As has been previously discussed, definitions of CLD vary, and so measures of the incidence and prevalence will be varied. In addition, rates of incidence are given with different denominators (all births, births of less than 32 weeks gestation, births where infants develop RDS, births of certain birth weight thresholds). Each presentation of data contributes to our understanding of the epidemiology of CLD, but restricts the ease with which data may be compared.

In 1990-1991 the incidence in a regional population in the UK (Trent Health Region) was reported as 15.5% (infants of <1500g or <32 weeks gestation, in supplementary oxygen at 28 days of age) and 6.1% (in supplementary oxygen at 36 weeks post conceptual age) of ventilated infants (Fenton 1996). The same study described incidences of 22.6% and 17.3% for the same respective groups, in a comparable population from British Columbia. These differences were not statistically significant at a 5% level. Other studies examining the incidence of CLD have shown a similar incidence to this (Reynolds 1974, Bancalari 1979, Greenough 1985).

1.5 Clinical Course of Chronic Lung Disease of Prematurity

Infants with CLD may survive with some or no respiratory morbidity. Conversely they may succumb to the CLD, being either unable to be weaned from ventilatory support, or
suffer a subsequent deterioration in respiratory status, possibly in association with superadded infection.

Those infants who do not survive may have developed other complications of extreme prematurity (e.g. intraventricular haemorrhage, necrotising enterocolitis, or sepsis) and so it may be difficult to determine exactly to what extent the patient’s death was attributable to CLD per se. Approximately 10% of infants with CLD who survive the initial neonatal period will die in the subsequent 2 years (Smyth 1981). The cause of death is often thought to be an intercurrent respiratory illness (e.g. acute viral bronchiolitis with respiratory syncitial virus), although right ventricular hypertrophy and consequent sudden death from a presumed dysrhythmia is a recognized sequel to CLD.

In infants who do not die, the expected course is one of gradual improvement, providing that adequate supportive therapy is given. This consists of supplemental oxygen therapy, adequate nutrition, diuretics, bronchodilators, and inhaled or systemic corticosteroids. Survivors still have increased rate of admission to hospital with respiratory illness. Studies performed on infants with BPD when they were able to perform lung function tests have shown that they have abnormal lung function persisting to much later in life (Northway 1990, Kimbournis 1996, Giacoia 1997). The abnormalities of lung function include increased airway resistance and air trapping.

1.6 Management of CLD

Given the large body of evidence suggesting that inflammation plays a key role in the development of CLD, much attention has been focused on possible benefits from glucocorticoids.
Other therapies have also been tried in an attempt either to prevent CLD/BPD, or to treat it once it has become established. I shall consider them in relation to whether they prevent CLD/BPD, or treat it.

1.6.1 Preventative

A number of preventative strategies for CLD/BPD have been tried. The majority of the antenatal measures have been intended to enhance lung maturity. Those used in the postnatal period have been considered to enhance lung maturity, or other aspects of the preterm infant that are immature and may be instrumental in the development of CLD/BPD.

1.6.1.1 Ante-natal therapy

Therapeutic agents that have been tried are corticosteroids and thyrotropin-releasing hormone (TRH). Corticosteroids have been extensively studied. The earliest report of their benefit was in 1972 (Liggins 1972). Other studies have also shown a benefit, and meta-analysis by Crowley (Crowley 1995) showed that corticosteroids reduced the incidence of RDS by half. The maximal effect was when the steroids were given between 48 hours and 7 days before birth. Crowley did not analyse the effect of steroids on reducing CLD/BPD.

Van Mater (van Marter 1990) showed some benefit from antenatal steroids in reducing the incidence of CLD/BPD. Although Doyle (Doyle 1986) did not show any decrease in CLD per live birth, he did observe a reduction when the incidence of CLD/BPD was determined per survivor. A number of effects from antenatal corticosteroids have been described.

1.6.1.1a The effects of corticosteroids on lung growth

Studying the specific effects of corticosteroids on human infants is difficult since diseases such as CLD themselves may affect lung growth (Hislop 1987, Margraf 1991). Thus most
studies have concentrated on animal models to determine both beneficial and adverse effects of these drugs on lung growth. When given antenatally corticosteroids result in acceleration of lung growth (Massaro 1985). The normal thinning of the double capillary loops to form the thin gas-exchanging walls of alveoli is accelerated resulting in rapid alveolisation (Bunton 1984). The maturation of surfactant producing type II pneumocytes is also hastened (Adamson 1988). Although the alveolisation occurs rapidly due to corticosteroids, the total number of alveoli is decreased as was reported by Beck et al who treated preterm Rhesus monkeys between 66% and 85% of term with antenatal corticosteroids (Beck 1981). This decrease in eventual numbers of alveoli is in part due to suppression of the formation of secondary septa which is a necessary step in division of alveoli. This observation of accelerated alveolisation with ultimate reduction in total number alveoli has been also reported by Plopper’s group (Bunton 1984). In addition, this group demonstrated decreased body weight in Rhesus monkeys treated with antenatal steroids. This reduction in body weight was most marked when the drugs were used early in gestation than when given later, and when higher dose of corticosteroids were used. Thus antenatal corticosteroids improve alveolisation in preterm animals at a critical time when delivery may result in marked acute respiratory failure. However, lung growth and body weight may be affected in the long term by such treatment.

1.6.1.1b The effect of antenatal and postnatal corticosteroids on surfactant

Since antenatal corticosteroids accelerate maturation of type II pneumocytes, enhancement of the pulmonary surfactant system is expected. Glucocorticoids have a dose-dependent increase in mRNA of surfactant proteins B and C in rats (Schellhase 1991) and human fetal lung explants (Liley 1989). However, data for surfactant protein A (SP-A) from animal models is at best confusing and at worst conflicting; SP-A has a major role in organisation of phospholipids in tubular myelin and in regulation of phospholipid secretion and
reuptake (Weaver 1991). In human fetal lung explants glucocorticoids have both inhibitory and stimulatory effects on the concentration of SP-A and its mRNA, which are dose-dependent (Iannuzzi 1993). Dexamethasone has a stimulatory effect on SP-A transcription but the same concentrations result in decreased total amount of SP-A mRNA due to a shortened half-life of the mRNA (Iannuzzi 1993). To add to the complexity the effects of corticosteroids differ with the stage of fetal lung development.

1.6.1.1c The effect of antenatal and postnatal corticosteroids on the antioxidant system

Prenatal treatment of pregnant rats with dexamethasone enhances fetal lung antioxidant enzymes together with a parallel acceleration of fetal surfactant production (Frank 1985). Improved survival to hyperoxic exposure may provide a more convincing argument for a role for antioxidant enzymes in improving respiratory outcome in new-borns whose mothers were treated with corticosteroids. This question was addressed by Frank's group (Frank 1992) who demonstrated improved survival of full term newborn rat pups exposed to hyperoxia from birth having been delivered to mothers treated with antenatal corticosteroids. At 7 days, 97% in the antenatal dexamethasone-treated group survived compared with only 71% in the placebo group. Survival at 14 days was 55% and 31% in the treated and untreated groups respectively. However, antioxidant enzymes were increased at 24 hours in the group treated with corticosteroids but were similar to those of the untreated group after 24 hours of age. Since the survival remained better in the antenatal treated group, it may be the rate of increase in antioxidant enzymes which may be of greater importance than simple increases in these enzymes. Prematurely delivered rat pups were studied by Keeney et al (Keeney 1993) who showed that the survival of the preterm pups in the antenatal treated group was significantly better than in the untreated group at 24 hours of age (91% vs. 57%). Increases were seen at 24 hours of age in antioxidant enzymes in the treated group, as in Frank’s study, with no significant
differences being seen after this age in both the treated and untreated groups. However, Keeney and her colleagues were unable to demonstrate improved survival in the term newborn pups exposed to hyperoxia treated and untreated groups. Similar data for the role of antenatal corticosteroid treatment in humans is lacking.

Thyroid hormones are thought to play a role in lung development \textit{in vitro} and in animals. Thyroidectomy will result in lung hypoplasia and decreased type II pneumocyte differentiation (Thurlbeck 1992). Interest has focussed therefore on the effects of prenatal administration of TRH. Two recent studies have failed to show any additional benefit for preterm lung disease when antenatal TRH is given as an addition to antenatal corticosteroids (Ballard 1998, Collaborative Santiago Surfactant Group 1998). Rodriguez (Rodriguez 1991) demonstrated that in the newborn rat, TRH and dexamethasone delayed antioxidant enzyme maturation, but did not inhibit a response of these enzymes to hyperoxia. The significance of his finding is unclear.

\textbf{1.6.1.2 Post-natal therapy}

A number of postnatal treatments have been tried in an attempt to ameliorate the severity of CLD/BPD. Glucocorticoids have been shown to reduce the severity of CLD. Halliday and Ehrenkranz have reviewed the published data for the Cochrane Library (Halliday 1999a, Halliday 1999b, Halliday 1998). They have conducted three meta-analyses of the use of steroids. Steroid efficacy, when used in the first 96 hours, was studied (Halliday 1999a) and the conclusion was that there was a significant fall in the incidence of CLD/BPD when defined by the 28 days of age, or the 36 weeks post conceptual age criteria (Odds Ratio [95% confidence interval] (28 days): 0.54 [0.39,0.74]; Odds ratio [95% CI] (36 weeks) 0.62 [0.46,0.82]). However, there were a number of side effects that were more likely if steroids were given. These were gastrointestinal bleeding, hypertension, and hyperglycaemia.
Halliday also conducted meta-analyses on the efficacy of steroids given between day 7 and 14 (Halliday 1998), and also after 3 weeks of age (Halliday 1999b). When steroids were administered between 7 and 14 days of age, Halliday concluded that there was no direct reduction in CLD/BPD (RR 1.01, 95% CI 0.87, 1.17). However, there was a decrease in death or CLD at 36 weeks post-conceptional age (PCA) (RR 0.65, 95% CI 0.52, 0.80), and ‘failure of extubation’ was also less likely. A higher incidence of adverse events (e.g. hypertension, left ventricular hypertrophy, hyperglycaemia and gastrointestinal bleeding) was recorded.

Similarly, in the meta-analysis of steroid administration after 3 weeks of age (Halliday 1999b), failure to extubate by 14th day of steroids was less likely in the steroid group. However, the control group was favoured when extubation at 7 days and 28 days of steroid therapy were considered. Again, there was a high incidence of adverse effects, similar to those seen in the earlier study groups. With these three studies, the overall recommendation of the authors was that the benefit may not outweigh the potential adverse effects of the treatment, and caution must be exercised when prescribing steroids.

1.6.1.2a The effects of corticosteroids on lung growth

The effects of postnatal steroids are similar to those when given antenatally, and have been demonstrated in some elegant studies by Burri’s group (Tschanz 1995). When dexamethasone was given postnatally to newborn term rats for the first two weeks of life, acceleration of the alveolar wall thinning and microvascular maturation were seen together with partial suppression of formation of secondary septa. A week after cessation of treatment, the accelerated lung maturation was partially reversed with the inter-airspace septa regressing towards a more immature thickened state. A secondary burst of alveolisation followed but the eventual result of postnatal corticosteroid treatment was an “emphysematous” lung with larger and fewer airspaces.
Although animal models suggest that alveolisation may be affected both in prenatal and postnatal corticosteroid treatment, a similar causal effect in humans has not been directly observed. Morphometric study of the lungs of living humans is not feasible but small observational physiological studies of children at a mean age of 7.5 years ± 0.3 (SEM) born to mothers who had received antenatal steroids suggests that such treatment does not have adverse effects on pulmonary function (Wiebicke 1988). However, lung function is not considered a good indicator of lung growth. Similarly, antenatal corticosteroids do not appear to have a clinical effect on somatic growth (Collaborative Group on Antenatal Steroid Therapy 1984) or neurodevelopment (MacArthur 1982).

1.6.1.2b Effects of postnatal corticosteroids on the surfactant system

Clinically, the most convincing data to date for the role of dexamethasone in improving surfactant protein – A (SP-A) concentrations in tracheal fluid from ventilated infants was recently published by Wang et al (Wang 1996). Infants receiving early dexamethasone had increased SP-A and surfactant protein – D (SP-D) in tracheal fluid which was paralleled by improvement in the respiratory status and decrease in albumen in tracheal fluid when compared to infants who received placebo. By contrast, a clinical study by Ashton et al did not see an improvement in the hydrophobic components of surfactant, assessed by fractions of dipalmitoylphosphatidylcholine (DPPC) in bronchoalveolar lavage fluid, when infants at high risk of developing CLD were treated with corticosteroids at 14 days of age (Ashton 1994). This lack of improvement is very likely to be due to the late treatment with corticosteroids. Similar data for infants treated early with corticosteroids is currently lacking.

1.6.1.2c Antioxidant replacement therapy

Replacement of various molecules considered to be deficient has been suggested. These include inositol, vitamin A, vitamin E, superoxide dismutase, essential fatty acids. Hallman
observed an improvement in incidence of CLD/BPD when inositol was given during the first 5 days of life (Hallman 1990). Meta-analysis by Howlett (Howlett 1999) was confined to just three studies. She showed that inositol supplementation was associated with a significant reduction in mortality, death or BPD (28 day definition), retinopathy of prematurity requiring treatment, and Grade III or IV intraventricular haemorrhage. The reduction in CLD/BPD was almost significant (RR 0.68, 95% CI 0.45, 1.02).

Aerosolised Mn-SOD has been shown to decrease hyperoxic lung injury in a hyperoxic baboon model (Simonson 1997). This study demonstrated that recombinant human Mn-SOD (rhMnSOD) improved pulmonary haemodynamics and surfactant phospholipid composition, and reduced lung oedema in baboons. Similarly, evidence of a beneficial effect from administration of non-enzymatic antioxidants to preterm infants is lacking. Berger (Berger 1998) supplemented ascorbate and tocopherol in hyperoxia exposed preterm baboons. He demonstrated an increase in concentration in plasma and tracheal aspirate, but no concomitant reduction in conjugate dienes (a marker of lipid peroxidation), nor any improvement in lung compliance or histological appearance. More recently, Davis (Davis 2003) has reported that treatment of preterm infants, for up to one month of age, with recombinant human copper-zinc superoxide dismutase is associated with improved clinical status at one year post expected date of delivery.

Fardy and Silverman (Fardy 1995) advocated caution when considering antioxidant supplementation. They cited instances where an imbalance in antioxidants (e.g. a raised ascorbate acid concentration in the presence of decreased caeruloplasmin, or absent ascorbate and raised urate) may promote radical formation.
1.6.2 Supportive therapy

Once CLD/BPD is established, there are a number of therapies that will help to lessen the severity of the disease. Oxygen therapy, aimed at keeping the pulse oximetry saturations >90% has improved the clinical picture. Oxygen is continued until the child can be weaned. Opinion differs as to when the time for weaning is reached. The benefits of supplemental oxygen are in reduction of the basal work of breathing, and in decreasing pulmonary arterial hypertension. These result in fewer calories being consumed for resting function, and thus more nutrition can go to promoting growth. In addition, maintenance of oxygen saturations overnight promotes better sleep efficiency, which may in turn aid somatic growth.

Diuretics may be of benefit in CLD. There are theoretical reasons why CLD may be associated with an increase in pulmonary fluid. Interstitial and peribronchial oedema was first described by Northway (Northway 1967), and others (Abman 1987). Diuretic therapy may reduce the severity of this oedema. There is also evidence that furosemide may prevent bronchoconstriction when given via the inhaled route (Bianco 1989). When diuretics are given to infants with a chronic oxygen requirement, an improvement in markers of lung function has been shown by several authors (Engelhardt 1986, Engelhardt 1989, Albersheim 1989, Rush 1990). Most showed an improvement in compliance following either spironolactone and hydrochlorothiazide, or frusemide alone. These effects have been shown to be maximal when diuretics are given for only a short time. In spite of the described improvements in physiological parameters, there are no studies demonstrating longer term clinical benefit from diuretic therapy. This deficiency in the published data was highlighted in the recent Cochrane meta-analysis of diuretic use in CLD (Brion 1999).
If bronchodilators are given to infants who have received mechanical ventilation, lung compliance and resistance have been shown to improve (Wilkie, 1987). Longer term benefits of administration of bronchodilators whilst ventilated have yet to be demonstrated, however. There are studies that have demonstrated short term benefits to older infants with an established diagnosis of CLD. de Boeck (de Boeck 1998) observed a decrease in airway resistance in a population of 1 year old children. However, this effect was confined to approximately half of the study population. The other half of the cohort did not demonstrate any such improvement in airway resistance. Similar findings were reported by Kimbourlis (Kimbourlis 1996) in older children with evidence of small airway obstruction. Kimbourlis recommends that if there is no discernible improvement in a child’s clinical state, routine use of a bronchodilator should be abandoned. There is some evidence to suggest there may be a detriment to the use of bronchodilators in these infants. Pandya and Kotecha (Pandya 2001) and Hislop (Hislop 1989) have both described smooth muscle hypertrophy in the airways of infants with CLD. It has been speculated that such hypertrophy helps to support airway that may otherwise be excessively compliant due to dysplastic airway development. In this situation, dilatation of the hypertrophic muscle, may actually reduce the degree of support of the airway wall. This may increase wall compliance, to the extent that some malacic dysfunction may occur i.e. collapse of the airway on expiration.

Ipratropium bromide has also been investigated in infants who are ventilated, and those who have developed CLD. Yuksel (Yuksel 1991) examined the use of ipratropium in ventilated infants, and in infancy. He observed an improvement in some, but not all, of the infants in his cohort.

The efficacy of different inhaler devices (nebulisers, large volume spacers, other spacing devices) has not been compared in children with CLD. In clinical practice, the selection of
such a device is informed by the evidence available for children with asthma (SIGN/ BTS Asthma Guideline 2003). However, studies similar to those in children with asthma have yet to be undertaken in children with CLD.

1.7 The pathophysiology of chronic lung disease of prematurity

CLD is primarily a disease of preterm human infants. In order to circumvent some of the problems of ethical approval for invasive research on these preterm infants, and the relatively small numbers of infants with RDS or CLD in any one institution, many animal models of BPD have been developed. These allow much easier access to biological specimens obtained by bronchoalveolar lavage, biopsy and autopsy from animals that have been subjected to environmental conditions known to produce pathological changes similar to those seen in infant lungs with CLD.

Animals other than humans do not undergo natural preterm labour. Following preterm delivery by surgery, a lung disease analogous to respiratory distress syndrome can occur. The preterm pups are then subjected to some of the modalities of treatment given to preterm infants. Excessive amounts of one or more therapy (e.g. oxygen or positive pressure ventilation) are administered. In addition pharmacological therapies (e.g. corticosteroids) can be administered as well to determine whether they will ameliorate the disease process. A wide variety of animals have been used including rodents (rats, rabbits), sheep, piglets, or primates (Macaque monkeys, baboons). Research that has examined techniques to produce histological changes like those described by Northway (Northway 1967), has allowed workers in this field to develop models of the pathogenesis of CLD based on single modalities of injury e.g. barotrauma, oxygen toxicity, infection. From these models a more catholic paradigm for the development of CLD has been suggested. All of the models depend upon the initiation of an acute inflammatory response in the lungs of
the animals under investigation. This is because of the large body of evidence that demonstrates inflammation to underpin the development of CLD.

1.8 Inflammation in Chronic Lung Disease of Prematurity

Northway’s description of the pathological changes in BPD was highly suggestive of an inflammatory process occurring during the development of the disease (Northway 1967). Subsequently, a large body of evidence has evolved to support the hypothesis that CLD is a disease of disordered inflammation within the preterm lung.

1.8.1 Cellular migration in CLD

1.8.1.1 Neutrophils

D’Ablang (D’Ablang 1975) and Merritt (Merritt, 1981) both suggested that the eventual development of radiological BPD was heralded by increases within the first 6 days of life in polymorphonuclear leucocytes within the tracheal aspirate from susceptible preterm infants. Merritt et al studied tracheal aspirates in preterm infants. They reported on cytopathological features in infants who developed BPD, or recovered from RDS. There was a degree of overlap in findings but the trend was for those with radiological changes of BPD to demonstrate features of regeneration (multiple nuclei, and an increased nuclear:cytoplasm ratio in respiratory epithelium), and polymorphonuclear leukocytes and macrophages to be the primary reactive cell types. There was no clear-cut distinction in cytological appearance between infants who recovered from RDS and those who were surgical ‘controls’ (infants who did not have primary lung disease). No tests for statistical significance were performed on these results. Later work by Ogden (Ogden 1984) and Jackson (Jackson 1987) demonstrated similar cellular migratory events when sampling by bronchoalveolar lavage. Ogden observed a rise in neutrophil and macrophage counts
(expressed per kg of body weight) that reached a maximum at 96 hours of age. In the infants who recovered from RDS the neutrophil count fell by 1 wk of age, whereas it persisted in the CLD infants. The macrophage count was also at a peak in the RDS infants at 1 week (at which time sampling ceased in this group). In contrast, in the CLD group, the macrophage count reached a maximum at 96 hours and then fell. Comparison of the ratio of elastase to proteinase inhibitor (corrected for BAL albumin concentration) showed a significant difference between the BPD and RDS infants within the first week of life.

Proteinase inhibitor concentration in all three groups was similar when corrected for BAL albumin concentration. The elastase/proteinase inhibitor ratio showed an increase in BPD infants. This suggested that pro-inflammatory events were occurring in the lungs of those who developed BPD. Jackson et al studied RDS in Macaque monkeys delivered preterm, rather than studying CLD in human infants. They showed that in lung homogenate the fraction of cells that were neutrophils or macrophages increased during the first 120 hours of the condition. A number of other studies have also shown that in those infants who develop CLD, there is an influx of inflammatory cells in the first 10 days of life.

Watterberg et al (Watterberg 1994) found that in the first week of life, infants who developed BPD had a significantly higher neutrophil count in BAL fluid than RDS infants. Contreras (Contreras 1996) showed a maximal difference in BAL neutrophil count between BPD and RDS infants on days 3 and 4. Munshi (Munshi 1997) studied the relationship between the neutrophil influx and concentration of interleukin-8 (IL-8) and interleukin-6 (IL-6) in tracheal aspirate. He reported that neutrophil counts rose by the end of the first week, but did not sample beyond this time. He also reports that the differences between group data (BPD vs. non-BPD) did not significantly alter, whether correction using urea for epithelial lining fluid (ELF) dilution was performed or not. Kotecha (Kotecha 1995) showed an increase in BAL neutrophil yield, but in the second week of life. In Contreras’ study approximately half of the infants studied (7/15 RDS; 8/13 CLD)
were given exogenous surfactant therapy (brand not specified), and in Kotecha’s study all infants had porcine surfactant (Curosurf, Serono Laboratories). It is difficult to predict how such therapy would affect cellular migration into the lung in RDS and CLD. It is thought that surfactant proteins A and D have an immunomodulatory effect (Wright JR 1993, Crouch 1995). These data suggest an effect upon alveolar macrophages rather than neutrophils. Surfactant therapy derived from animal surfactants has some associated surfactant proteins within their “cocktail”, whereas synthetic surfactant has none. The effect of administering surfactant lacking in the normal concentrations of associated surfactant proteins is unknown. This paucity of data prevents any meaningful comparison between data from the pre-surfactant era, and that from the surfactant era.

1.8.1.2 Alveolar macrophages

Macrophages also play a vital role in the acute inflammatory response. They are able to express a wide repertoire of cytokines, chemokines, and other inflammatory mediators. One known function of the macrophage is to resolve the acute inflammatory response or to modify it into a chronic inflammatory state (Stites 1997).

Ogden (Ogden 1984) observed an increase in macrophages in tracheal fluid from animals with CLD and RDS when compared with his control group (term pups, with no lung disease). This rise was maximal by day 4. Subsequently, the macrophage concentration declined in the CLD animals but not in those with RDS. This suggests that macrophages may aid in the resolution of lung injury. Kurland reported that macrophages from neonatal rhesus monkeys were deficient in their phagocytic and chemotactic functions when compared with adult Rhesus monkey macrophages (Kurland 1988). Other evidence to suggest a role for alveolar macrophages in the development of CLD has been described by Clement et al (Clement 1998), and Kojima et al (Kojima 1996). Clement showed that
alveolar macrophages from infants with CLD released more hydrogen peroxide than those from infants without lung disease. In addition when the harvested macrophages were stimulated with phorbol myristate acetate (PMA), there was an even greater difference in hydrogen peroxide production between CLD and non-CLD infants. They also showed a relationship between clinical improvement, the administration of glucocorticoids and a decrease in the ability to generate hydrogen peroxide. Kojima showed that in rabbits without lung disease, superoxide production from alveolar macrophages was induced by the addition of PMA but was further enhanced when tracheal aspirate from infants with CLD was added. They went on to show that anti-endothelin-1 antibody, or an endothelin receptor antagonist, prevented this enhancing effect, suggesting that the effect of the BAL was via an endothelin-dependent mechanism. Surfactant replacement therapy has been shown to result in an earlier rise in macrophage influx (Arnon 1993). Recent work from Tino and Wright (Tino 1999) has suggested that a glycoprotein (gp-340) that co-purifies with surfactant proteins A and D promotes macrophage chemokinesis. This evidence suggests that the pathogenesis of CLD may be related to an increase in macrophage mediated pro-inflammatory effect, and/or a possible deficiency in its phagocytic (‘housekeeping’) role.

1.8.1.3 Other cells

The evidence for a role for lymphocytes and mast cells in CLD is much more limited than that for neutrophils and macrophages. Ohtsu observed that in a mouse-hyperoxia model of BPD, there was a pulmonary lymphocytic infiltrate apparent at 18 days of age, and this infiltrate was diminished by the administration of dexamethasone (Ohtsu 1989).

Lyle (Lyle 1995) demonstrated that in infants who had died of CLD/BPD there were a significantly greater number of tryptase positive mast cells on post-mortem histological
specimen, when compared with infants who had died of sudden infant death syndrome. It is unknown whether a similar increase of tryptase positive mast cells occurs during the development of CLD/BPD.

1.8.2 Cytokines in Chronic Lung Disease of Prematurity

The complex interplay between the cytokines and other inflammatory mediators is not fully understood in the preterm infant lung. Previous studies have examined those cytokines about whose role in inflammation, most is understood. These include: Interleukin-1β (IL-1β), IL-6, IL-8, Tumour Necrosis Factor-α (TNFα), and Transforming Growth Factor-β (TGF-β). It must be remembered that by placing emphasis upon the role of these cytokines, it is not the intent of this thesis to suggest that other cytokines are not significant in the pathogenesis of CLD.

1.8.2.1 Interleukin-1α and Interleukin-1β

The interleukin-1 family consists of 2 peptides, interleukin-1α (IL-1α) and interleukin-1β (IL-1β). They are 159 and 153 amino acids long respectively. They possess almost identical potency and biological activity, despite only sharing 26% homology for their amino acid sequence and being coded for on different chromosomes. Since they have a high degree of functional homology it may be appropriate that they are considered together when assessing their role in CLD. Rozycki (Rozycki 1994) described the concentration of IL-1β in BAL fluid from 4 infants with BPD, taken at day 14. These infants were found to have a higher concentration of IL-1β than infants who had required oxygen therapy for less than a week. Differences in IL-1β concentration between those who required long term ventilation and those who did not were detectable within the first 24 hours of life. Other groups have shown an association between IL-1β concentration in tracheal lavage and
chorioamnionitis (Watterberg 1996) or Ureaplasma infection (Patterson 1998). In both studies, infants who developed CLD were shown to have increased concentration of IL-1β on day 1 when compared with those who did not develop CLD. Patterson’s study expressed their IL-1β concentration as a ratio between that and IL-6. It is to be expected that both IL-1β and IL-6 would be increased in CLD. It is therefore unclear how expressing the results as such a ratio will be more informative than presentation of simple values. Although Watterberg did observe an association between chorioamnionitis and BPD, Patterson’s study did not discover a similar association between Ureaplasma colonisation and development of BPD. In contrast, Kotecha et al (Kotecha 1996) observed that differences in IL-1β concentration between CLD and RDS infants were maximal at day 10 of life. They also noted that immunocytochemistry demonstrated IL-1β production to be located predominantly within macrophages. IL-1β binds to IL-1 receptor. An endogenous receptor antagonist (interleukin-1 receptor antagonist [IL-1ra]) competes with IL-1β at the binding site. Simple determination of the IL-1β concentration may not truly reflect IL-1 bioactivity. A study examining IL-1β bioactivity (Rindfleisch 1996) demonstrated that in lung lavage fluid from infants who developed BPD, IL-1β concentration increased during the first week of life. At the same time, IL-1ra concentration remained unchanged. The conclusion was drawn that overall IL-1β bioactivity was increased. IL-1α has been found to increase in mice exposed to an hyperoxic stimulus (85% oxygen for 28 days), but no human studies in CLD infants are known. Inhaled sodium cromoglycate has been shown (Viscardi 1997) to decrease IL-1β concentration in infants at risk of CLD, although not to a statistically significant degree. In spite of this, and significant decreases in other inflammatory markers (TNF-α, and IL-8), no clinical difference was seen between the placebo and cromoglycate groups. In contrast, Groneck demonstrated that ‘early’ dexamethasone (starting on day 10) improved clinical measures (fraction inspired oxygen concentration (FiO₂) and peak inspiratory pressure
and promoted a decrease in BAL IL-1β. Bry (Bry 1997) has shown that intra-amniotic administration of IL-1α to rabbits promoted an increase in surfactant protein-A (SP-A) and surfactant protein – B (SP-B) mRNA, and improved dynamic lung compliance when postnatally ventilated. She did not observe such an effect from administration of IL-1ra.

1.8.2.2 Interleukin-6

IL-6 has many biological activities on a variety of cells. It synergises with IL-1 and TNF to stimulate T cells. It also induces the acute phase response within the liver and pyrexia in the hypothalamus (Stites 1997). In vitro studies of fibroblast culture suggested that exposure to Ureaplasma urealyticum, oxygen and lipopolysaccharide (LPS) resulted in significant secretion of IL-6 and IL-8 (Stancombe 1993). Several groups have demonstrated that IL-6 concentration in BAL fluid is increased in infants who develop CLD. The time at which this difference is most marked is less clear-cut. Bagchi (Bagchi 1994) showed IL-6 concentration to increase during the first 2 weeks of life, and to be approximately maintained at 28 days of age. In contrast, IL-6 activity as measured by in vitro bioassay was maximal on day 1, and decreased to a minimum by day 28 (Bagchi 1994). Kotecha (Kotecha 1996) showed IL-6 to be maximally raised in the second week of life (day 10) and then to decline. Kotecha found the IL-6 concentration to be approximately 10x that found by Bagchi. The reason for this difference is not clear from comparison of the papers. Parton’s group has presented data that has shown IL-6 to be raised in the latter half of the first week of life (Munshi 1997, Niu 1998). Unfortunately, they did not measure IL-6 or other inflammatory markers beyond day 7.
1.8.2.3 Interleukin-8

As stated above, Stancombe induced IL-8 production from fibroblast culture exposed to hyperoxia, LPS and Ureaplasma (Stancombe 1993). Human studies found IL-8 concentration to be raised in BAL from infants who developed BPD. The timing of the peak IL-8 concentration appears variable. It has been described as maximal at day 1 and day 3 (Munshi 1997), day 10 (Kotecha 1995), day 14 (Little 1995) and day 15 (Groneck 1994). Jones et al compared term and preterm infants with respiratory failure and found a greater concentration of IL-8 in lavage fluid from term infants than preterm infants. Jones’ findings do not necessarily contradict those of the other groups. The term infants in Jones’ study had acute respiratory failure and radiographic evidence of ARDS, but the exact diagnoses are not recorded (Jones 1996). Beresford has also shown that IL-8 increases in the first week in those infants who develop CLD (Beresford 2002).

1.8.2.4 Tumour Necrosis Factor-alpha

Murch reported that tumour necrosis factor-α (TNF-α) concentration was increased in lavage fluid from infants who develop CLD/BPD (Murch 1992). Bagchi (Bagchi 1994) demonstrated an increase in TNF-α concentration, but not TNF-α activity, in BAL fluid from infants who developed CLD. Sodium cromoglycate (Viscardi 1997) has been shown to reduce TNF concentration in infants at risk of CLD.

1.8.2.5 Other Interleukins

Data on the presence and putative roles of other interleukins in CLD are very limited. Interleukin-2 receptor concentration (IL-2R) was increased in plasma and tracheal aspirate from infants who are at risk of developing CLD, or who have developed it (Stefano 1992). Peripheral blood mononuclear cells (PBMNC’s) derived from infants with BPD show evidence of increased interleukin – 2 (IL-2) and interleukin – 3 (IL-3) secretion.
Dexamethasone in vitro reduced the IL-2 and IL-3 increase (Bessler 1996). Jones observed interleukin-10 (IL-10) concentration, and IL-10 mRNA, to be undetectable in neutrophils and macrophages harvested by BAL from infants (Jones 1996). Beresford (Beresford 2002) has shown that IL-10 concentration in infants who develop CLD is significantly lower on day one than in the non-CLD group, but by day 4 the differences have reversed, and the CLD group then have a significantly higher concentration of IL-10 than the non-CLD group. This suggests that the development of CLD may rest (at least in part) on failure of down-regulation of the acute inflammatory response. Waxman (Waxman 1998) has shown differing protective effect against hyperoxia in term transgenic mice that were bred to be either interleukin-11 (IL-11) knockout, or IL-11 enhanced. The knockout mice had 100% mortality when exposed to 100% oxygen for 72-96 hours, whereas the IL-11 enhanced mice had greater than 90% survival after 10 days in 100% oxygen. IL-11 is known to be of the IL-6 superfamily, but to have effects on immunoglobulin synthesis, and inhibition of metalloproteinase-1 and the acute phase response.

1.8.2.6 Transforming Growth Factor -beta (TGF-β)

This is a potent pro-fibrotic growth factor, that also has down-regulatory effects upon the inflammatory process (Stites 1997). Total and active TGF-β1 has been shown to be elevated as early as day 4 in BAL fluid from infants who develop CLD (Kotecha 1996b). Kotecha, and Toti (Toti 1997) attempted to localise sites of TGF-β production. Kotecha demonstrated that pan-TGF-β could be localised to macrophages obtained by BAL. Toti examined lung tissue from post mortem, and demonstrated staining for TGF-β in monocyte/macrophages present in lung section capillaries, and also in myofibroblasts within alveolar walls.
1.8.2.7 Other Growth Factors

Recent work does not clarify the role of vascular endothelial growth factor (VEGF) in the pathogenesis of CLD. Currie observed that VEGF was undetectable at birth in infants who developed CLD, those who recovered from RDS, and those ventilated for non-respiratory reasons (Currie 2001). He did not observe an association with gestational age, but in all three groups VEGF concentration increased with postnatal age. In contrast, Lassus reported VEGF to be lower in tracheal aspirate of infants who developed CLD than those who did not (Lassus 1999). There was an increase in tracheal aspirate VEGF over the first week of life; more so in infants whose mothers had chorioamnionitis. Currie's study population did not include infants where chorioamnionitis was present. This difference between Currie and Lassus may account for the differences between their findings.

Catterton (Catterton 1979) demonstrated that epidermal growth factor (EGF) promotes maturation of lung epithelium in preterm rabbit pups. Stahlman (Stahlman 1989) showed that EGF, and TGF-β, were produced by the epithelium and alveolar macrophages of infant with CLD. Whitsett and Zhou have recently reviewed the role of EGF and other growth factors in normal lung morphogenesis (Whitsett 1996). Evidence suggests that EGF influences branching morphogenesis, cell proliferation and type 2 pneumocytes differentiation. In a review of EGF and its receptor (EGF-R), Miettinen (Miettinen 1997a) described lung immaturity, with a 50% reduction in alveolar number in an EGF-R knockout mouse, and has suggested (Miettinen 1997b) that this may be a valid animal model for CLD. Evidence that the reduced alveolar number may be related to an effect of EGF on maturation of type II pneumocytes has been described by Yasui (Yasui 1993). Currie (Currie 2001) found that EGF concentration correlated with post conceptual age, but not with the development of CLD per se.
1.8.2.8 Other Inflammatory Markers

Both prostaglandins and other lipid mediators (e.g. platelet-activating factor) have been shown to be increased in infants who develop CLD. Watterberg (Watterberg 1996) showed that prostaglandin E2 and thromboxane B2 were increased in BAL fluid in the first week of life in infants who developed BPD compared with those who had RDS. Leukotriene-B4 (LTB₄) was lower in the BPD infants over the same period, although the reason for this is unclear. Urinary leukotriene-E₄ (LTE₄) concentration is elevated in infants who were 28 days old, and ventilator dependent (Davidson 1995). Gaylord (Gaylord 1996) found that blood and lavage PAF concentration, and blood PAF-acetylhydrolase activity were increased in the first week of life in infants who developed CLD. Groneck (Groneck 1995) described increased C5-derived anaphylotoxin in BAL fluid from infants with CLD.

1.9 Nitric Oxide

Nitric oxide (NO) is a small molecule (molecular weight 30 kDa). Since it was first suggested that it may be endothelium derived relaxing factor (EDRF) by both Moncada’s group (Palmer 1987) and Ignarro (Ignarro 1987) it has been recognised to have many different roles in a number of organ systems within the human body.

NO is synthesised by the action of nitric oxide synthase (NOS) upon arginine. This two-step reaction is summarised in figure 1.1. It is catalysed by NOS. There are three isoforms of NOS. They were previously classified as neuronal, inducible and endothelial, but are currently referred to as types I, II and III respectively.

Once it is secreted NO can affect a number of physiological/organ systems depending upon its site of production. NO can be produced by the central and peripheral nervous system (from type I NOS), endothelial cells (from type III NOS), and other cells some of which
are involved in the inflammatory response (type II NOS). The production of nitric oxide by respiratory cells and those of the inflammatory system will be discussed in further detail below. NO has a variety of physiological actions and acts upon many organ systems (Mupanemunda 1995).

Fig 1.1: Diagram showing production of nitric oxide (NO) from L-arginine and its action on smooth muscle relaxation via cGMP. NOS nitric oxide synthase.

1.9.1 Nitric oxide and the lung

Nitric oxide has a variety of physiological effects on different cell lines within the lung. Although there are thought to be over 40 different cell types within the lung, the existence of NOS has only been shown in the major cell types including airway epithelium, bronchial smooth muscle, pulmonary vascular smooth muscle, fibroblasts, and non-adrenergic non-cholinergic (NANC) neurones (Kobzik 1993). All three isoforms of NOS are thought to be present in the human lung, but not all isoforms are found in all cells within the lung. Xue (Xue 1996) demonstrated the presence of the different isoforms in fetal and postnatal rat lung. He described that type I NOS and type II NOS were also found in the epithelium, and type III NOS was only present in lung endothelium. Shaul et al (Shaul 1994) demonstrated type III NOS in human bronchial epithelial cell culture. Asano
demonstrated 'constitutive' and 'inducible' NOS in human lung epithelial culture, although this group did not differentiate 'constitutive' NOS into type I and type III (Asano 1994). There is no direct evidence of NOS in human airway smooth muscle, however Boota (Boota 1996) has shown, in vitro, that rat pulmonary vascular smooth muscle cells do produce NO in response to 'cytomix' (a cocktail of IL-1β, TNF-α, and IFN-β).

1.9.2 Nitric oxide and the inflammatory response

I shall briefly examine the production of NO by each cell type, and the effects of NO on the inflammatory response

1.9.2.1 Neutrophils

Kubes (Kubes 1991) described the secretion of NO from neutrophils. It is thought that neutrophils contain only type II NOS. The function of NO in relation to neutrophils is very complex and is not fully elucidated. It has been shown that NO-releasing yeast can reduce the F-actin component of neutrophils, thus decreasing phagocytosis in human neutrophils (Forslund 1997). The relevance of this to the intrinsic inflammatory response is unclear. Tassiopoulos (Tassiopoulos 1998) showed that inhibition of NO synthesis by a variety of compounds (aminoguanidine, dexamethasone and TNF binding protein), in rats that had had aortic cross clamping, resulted in decreased sequestration of neutrophils into the rat lung. In contrast to this Bloomfield et al in a pig model of ARDS, demonstrated that inhaled NO inhibited neutrophil migration and oxidative burst within the lung. Whether the different results are due to the concentration of NO present in the lung (in Bloomfield’s study 20 parts per million [ppm] were used), or represent unknown species differences, is unclear. Kinsella and Abman found a similar effect with 5 ppm NO in preterm sheep with severe experimental respiratory distress syndrome. They showed that a low human therapeutic dose (5ppm) was associated with less neutrophil accumulation, as shown by
myeloperoxidase staining. Su et al (Su 1996) showed that ‘cytomix’ stimulated NO production from a rat lung epithelial cell line. The result of increased NO secretion (as measured by nitrite concentration) was to attenuate $^{51}$Cr release from the epithelia following incubation with activated polymorphonuclear cells (PMN). $^{51}$Cr is a marker of epithelial cell damage. Addition of L-NMMA ($N^G$-monomethyl-L-arginine), an inhibitor of NOS resulted in a loss of the protective effect previously seen. In this paper Su also examined the presence of ICAM (inter cellular adhesion molecule) by Immunoblot analysis (a semiquantitative assay technique). Their data suggest that when NO production is upregulated, there is a concomitant increase in ICAM-1. ICAM-1 promotes the margination of neutrophils prior to migration into an injured area (Stites 1997).

1.9.2.2 Macrophages

Investigation of NO action on, and secretion from, alveolar macrophages often utilises PBMNC’s as a surrogate for alveolar macrophages. For a long time, it has been known that rat alveolar macrophages posses type II NOS, but it could not be demonstrated in human PBMNC’s, or human alveolar macrophages. Nicholson was able to demonstrate that type II NOS was present in alveolar macrophages obtained by bronchoalveolar lavage from patients undergoing bronchoscopy for Mycobacterium tuberculosis infection (Nicholson 1996). Lincoln et al (Lincoln 1997) speculate that the reason why there is so little evidence for type II NOS in human alveolar macrophages is because many human studies preferentially used PBMNC’s.

NO has been described to mediate murine peritoneal macrophage apoptosis (Albina 1993). It is unknown whether it has a similar effect on alveolar macrophages.
1.9.2.3 Other inflammatory cells

Type II NOS has been described in a variety of lymphocytes including natural killer cells, and \( T_{\text{H}}1 \) cells (Barnes 1995). There is no published evidence to demonstrate secretion of NO, or the presence of NOS mRNA in mast cells. However, depletion of NO has been observed to increase oxidative stress within mast cells, with the result that neutrophil adhesion to endothelium is increased (Niu 1996). These data suggest that in inflammation, NO may modulate mast cell activity.

1.9.3 Nitric Oxide in Neonatal Lung Diseases

A number of studies have observed a beneficial effect from inhaled NO (iNO) on oxygenation in near-term infants (Finer 2000), and preterm infants with RDS (Desandes 2004, Finer 2001, Jaillard 2000, Hoehn 2000). It is less clear whether iNO can reduce the incidence of CLD. Schreiber (Schreiber 2003) and Kinsella (Kinsella 1999) both reported such an effect, but Hoehn’s findings run counter to this (Hoehn, 2000). These studies have failed to show a difference in mortality between groups of infants who have, and have not, received iNO (Hoehn 2000). Infants who develop meconium aspiration syndrome, respiratory distress syndrome, or pneumonia may benefit from the therapeutic administration of exogenous NO. It is believed that these diseases may be complicated by a defect of transition of the circulation from the fetal configuration (via the foramen ovale and the ductus arteriosus - whereby blood by-passes the lungs) to the postnatal ‘adult’ circulation. In this circumstance, therapeutic NO is administered via the ventilator circuit, at a dose of 5-20 ppm, and may improve oxygenation by improving ventilation/ perfusion mismatching in the diseased lung. Recent meta-analysis by the Cochrane Neonatal group has suggested that inhaled NO is beneficial in the short term (Barrington 2003).

The, now widely, recognised expectation of clinical benefit from iNO cannot be assumed to provide proof that the underlying disease is partly mediated by a failure of endogenous
NO production. To date, there are no data to clarify whether the benefit from administering inhaled NO is due to a failure of endogenous NO production, a defect of NO receptor activity, or some other (as yet unknown) action of NO when given in supra-physiological amounts. It is perhaps interesting to remember that measurements of exhaled NO report concentrations of the order of parts per billion. The concentration of eNO measured may be proportionately representative of the overall amount of NO secretion occurring within the lung, but the absolute amount of NO secreted may be significantly higher close to source (where endogenous NO exerts its physiological effects), than just outside the respiratory tract. Oxidative reactions to produce peroxynitrite, nitrate, nitrite, nitrosothiols and other NO metabolites all may reduce the amount of native NO present in exhaled gases. The dose administered with exogenous, iNO therapy is of the order of parts per million. It is not known by what order of magnitude (if any) the dose of iNO is decreased during its passage to the terminal bronchioles and the alveoli. It is conceivable that iNO is present in the distal areas of the lung at concentrations some one thousand times greater than those occurring naturally. These differences between physiological concentrations of NO, and those administered therapeutically, must undermine any assumptions that are made that the efficacy of iNO therapy in ameliorating some preterm lung diseases is definite evidence of a failure of the intrinsic NO system. No data exist to help clarify possible confusion arising from this difference of orders of magnitude in NO concentration between iNO and eNO.

The only study to date of preterm lung disease which provides any evidence of an alteration in endogenous NO production is that of Banks et al (Banks 1998). His data suggest that NO secretion may be increased in CLD/BPD. He describes an increase in the plasma concentration of 3-nitrotyrosine in infants with CLD. 3-nitrotyrosine is thought to be a product of the action of peroxynitrite (an oxidation product of NO and superoxide radicals) on tyrosine residues in proteins. Bank’s findings support the hypothesis that
infants with CLD have an increased endogenous production of NO. However, the presence of 3-nitrotyrosine within the plasma cannot provide any information as to the anatomical source of the NO.

For this reason I considered the next important step in determining the role of NO in the pathogenesis of CLD was to collect samples from the lung milieu, in order to localise NO production to the lung with a greater degree of confidence than previous studies could properly permit. By performing BAL on ventilated preterm infants who subsequently developed CLD, I considered that I would be able to obtain samples as least likely as possible to be "contaminated" by NO products from a non-pulmonary source. Whilst this assumption appears intuitively to be correct, there are no data to state categorically that various BALF constituents such as NO (and nitrite and nitrate), cellular antioxidants, nitrotyrosine etc are solely produced in the lung. However, the likelihood of this being the case is far greater than when plasma is analysed, as Banks' group did.

1.9.3.1 Postnatal Circulatory Transition

Experimental evidence suggests that NOS is present in fetal lung (Shaul 1993). In ovine lung amount of type III NOS is thought to be maximal during fetal and early postnatal life (Halbower 1994). Thereafter there appears to be a decrease in type II NOS immunostaining. This finding supports those of Abman (Abman 1990) and Zellers (Zellers 1991). Abman described an effect of L-arginine (an NO antagonist) on pulmonary vascular resistance in the ovine lung. Following administration of L-arginine to newborn sheep, postnatal pulmonary vasodilatation was selectively inhibited. This suggested that an L-arginine sensitive process (NO secretion) was important in the changes in pulmonary vascular tone at birth. Zellers subsequently showed that piglet endothelium had increasing sensitivity to sodium nitroprusside (an NO donating compound) after birth. This suggests that NO secretion needs to be maximal at birth and can decrease subsequently, without
adversely affecting pulmonary vascular tone. The term ‘acute lung injury’ refers to a pathological state of increased epithelial permeability, and is considered synonymous with acute respiratory distress syndrome (ARDS). Models of acute lung injury have been developed in animals in order to study the molecular and cellular events that occur during the pathogenesis of ARDS. Hyperoxia is sometimes used to provoke an acute lung injury model (Waxman 1998, Haddad 1994). Since hyperoxia is also used to generate a CLD-like condition in animal models it is possible that some of the pathological processes in the evolution of CLD mimic those in ARDS. Acute lung injury in infants, that is not associated with pulmonary immaturity and surfactant deficiency, is often due to meconium aspiration or pneumonia.

Animal models require the lung to be exposed to other insults e.g. tracheal instillation of lipopolysaccharide (endotoxin) (Mikawa 1998) or hydrochloric acid (Ohara 1998). This review will not consider the use of therapeutic NO in ARDS, but rather the role of endogenous NO in the pathophysiology of acute lung injury.

Adults who developed ARDS following sepsis or pneumonia were shown to have upregulation of iNOS expression in alveolar macrophages obtained by BAL (Kobayashi 1998). In this study iNOS expression was increased in patients with sepsis, although it is unclear which of the ARDS patients had systemic sepsis and which had pneumonia. Yang (Yang 1998) instilled lipopolysaccharide (LPS) into rats’ trachea, and observed an increase in epithelial permeability (as measured with $^{125}$I-labelled albumin). Inhibition of NOS by L-NMMA resulted in a decrease in the epithelial permeability. Brett (Brett 1998) described a decrease in exhaled NO in adults with ARDS. This finding may be explained by the rapid interaction of NO with superoxide to form peroxynitrite or ONOO⁻, and in forming other
oxides of nitrogen e.g. nitrite and nitrate. Haddad (Haddad 1994) described increased nitrotyrosine in sections of lung from children and adults who died following ARDS.

From the above evidence it would seem likely that the pathogenesis of CLD would be associated with an increase in NO production within the lung.

1.9.3.2 Determining Nitric Oxide Production in small volumes of biological fluid

Various methods have been described for the measurement of NO in a biological system. These include direct quantitation of NO evolved into surrounding gas, and measurement of a surrogate marker of NO production (usually an oxidative metabolite of NO e.g. nitrate, nitrite, nitrotyrosine). For my research I wished to use a cheap, reliable method to provide accurate results from small (<50 microlitre) samples of BALF. Direct measurement of exhaled NO (eNO) was not feasible. The equipment is very expensive, and is not easily portable. The infants’ severity of disease (they all had sufficiently severe respiratory disease to warrant mechanical ventilatory support) meant that I would have had to take the equipment to the cot side. At the time of the study recruitment (1996-1998), there was no portable, inexpensive method of measuring eNO in ventilated, preterm infants. Therefore, although several children may have been suitable for inclusion into the study, use of complex, non-portable, eNO measuring equipment would have prevented all of them being recruited at any given time. This would have either meant that the total number of infants included in the study would have to be reduced (since the research project was only funded for 2 years), or that the duration of the project would have had to be for a much longer period (possibly more than five years for recruitment of a similar number of subjects to that which we actually obtained).

Furthermore, no methods of preparation of BALF, to enable it to discharge its NO content, were known. I decided, therefore, to utilise a method which measured the concentration of
nitrate and nitrite, as proxy measures of NO secretion per se, using either the Griess method, or a fluorometric method (v.i.). Review of the published methodologies demonstrated that the previously described techniques for quantitating nitrite and nitrate used volumes of 100 microlitres (Misko 1993, Gilliam 1993), or more. Tsiakis (Tsiakis 1997) did describe a technique which utilised a sample volume of only 1 microlitre in a gas chromatograph-mass spectrometer stage. However, it was not feasible for me to use this technique for three reasons:

1. The methodology was not published until 1997, approximately 6 months after I started my research.
2. I did not have access to a mass spectrometer.
3. Although the final stage of the technique only requires 1 microlitre, volumes in excess of 500 microlitres were used for the prior stage of reduction of nitrate to nitrite.

It has previously been recognised that enzymatic reduction of nitrate to nitrite requires cofactors which can (and often do) interfere with the fluorometric assay. This problem was also recognised by me in preliminary experiments with a commercial kit for fluorometric analysis (Cayman Chemicals, Ann Arbor, MI). It was for these reasons that I decided that I had first to develop an assay technique which I could reliably use to test my hypothesis on endogenous nitric oxide production in children who develop CLD. The technique I wished to develop had to fulfil the criteria given above, i.e. it should be suitable for reduction of BALF sample volumes of <50 microlitres, be reliable, inexpensive, and not be prey to problems of interference from co-factors in the enzymatic reduction of nitrate to nitrite.

1.10 Antioxidants

Infants with respiratory failure are often treated with increased concentration of inspired oxygen. It is known that high concentrations of ambient oxygen (>60%) have an injurious
effect upon on respiratory tract epithelium resulting in reactive oxygen species (ROS) production (Freeman 1981). This injury will result in an acute inflammatory response.

Acute inflammation results in the production of a number of free radical molecules (Stites 1997) that may also be injurious to the native lung.

Animal models for CLD/BPD have been created by exposure to hyperoxia. A number of species have been used by different groups, including guinea-pigs (Kelly 1991), baboons (DeLemos 1987), and rats (Randell 1990). In all these studies, exposure of a particular animal species to hyperoxia results in histological changes very similar to those seen in human infants with CLD/BPD. Randell (Randell 1990) observed lower alveolar surface area, enlarged alveolar ducts and a difference in alveolar size class distribution (a measure of the range of alveolar numbers), but a lack of fibrosis in the peribronchial and septal areas that is seen in CLD/BPD. Kelly’s guinea-pig model demonstrates that term and preterm pups are susceptible to hyperoxic lung injury, as defined by BALF protein, histological changes (atelectasis, pulmonary oedema, fibrin deposition and inflammatory cell infiltrate) (Kelly 1991). There was a difference in survival favouring those kept in air, and those pups delivered at term. Inflammatory cell infiltrates were seen in both groups submitted to 95% O₂, but not in the preterm, air group. Thus the preterm guinea pigs may not have a period of initial surfactant deficiency, akin to RDS in humans. It is unclear how this possible lack of RDS affects the validity of Kelly’s guinea pig model. DeLemos’ preterm baboon model did experience a form of RDS, with hyaline membranes seen on the lung histology of sacrificed animals (DeLemos 1987). Hyperoxia results in metaplasia/hyperplasia of the airway epithelium, airspace atelectasis/hyperinflation and airway wall fibrosis by 7 days of age or older. These features are typical of CLD/BPD. None of the animals given oxygen as required, to maintain the arterial PaO₂ between 55 and 85 mm Hg, had histological evidence of BPD. This model appears to provide a more
elegant paradigm for CLD/BPD. Ames suggested primates, and other mammals, have a less well-developed antioxidant system in comparison to humans (Ames 1981). It is possible therefore that these animal models demonstrate a susceptibility to hyperoxia that is greater than that found in preterm human infants. It is possible that the end stage histology of CLD/BPD represents a common final pathway from a combination of insults, of which hyperoxia is only one.

As outlined above, ROS can injure a range of molecules essential to the normal functional integrity of a cell. Lipids (Haynes 1990, Ogihara 1991, Supnet 1994, Varsila 1994), DNA synthesis (Han 1996), proteins (Kelly 1995, Lubec 1997, Varsila 1995, Gladstone 1994) isoprostanes (oxidation products from prostaglandins) (Cotton 1996) have all been demonstrated to be damaged by oxidants. In addition, lipid peroxides are oxidants in their own right, and damage proteins and DNA (Halliwell 1996). An important group of lipids in the context of lung disease are the surfactant phospholipids. Disruption of these lipids has an implication for normal gas transfer function at the alveolar surface. Since surfactant deficiency is a feature of RDS, which precedes the onset of CLD, oxidant damage to surfactant lipids may be another factor in the development of CLD. Coalson has described oxidant damage to surfactant protein mRNA in baboons exposed to hyperoxia ± E. coli intratracheal instillation (Coalson 1995). She demonstrated a qualitative decrease in intensity of expression of SP-A in baboons exposed to hyperoxia regardless of whether they had undergone E.coli tracheal instillation. A similar decrease in SP-B and surfactant protein – C (SP-C) mRNA was not seen in these groups. It is interesting to note that despite these differences in mRNA, there was no qualitative difference in immunostaining for SP-A in lung sections.
1.11 Antioxidant mechanisms

The antioxidant system found in humans can be considered to be composed of two broad
groups of antioxidants: non-enzymatic and enzymatic antioxidants. I shall consider the
non-enzymatic antioxidants.

There are many different molecules that may be considered to reduce the oxidative stress
load incurred with lung inflammation. Some may directly scavenge free radicals, whilst
others act to reactivate oxidised antioxidants. Many of these antioxidants have been studied
in the context of the pathogenesis of CLD/BPD. As will be discussed below, this thesis
will be concerned in part with the concentration of ascorbate, urate and glutathione in
BALF. I shall discuss the evidence for their roles in the antioxidant system first.

1.11.1 Ascorbate

Ascorbate has many important roles in humans. Banhegyi comprehensively reviewed
ascorbate metabolism (Banhegyi 1997). Humans cannot synthesise ascorbate, and so relies
upon dietary intake for maintenance of an adequate amount. Ascorbate can be recycled, by
a glutathione (GSH) dependent process (May 1994).

Ascorbate is a key component of the antioxidant system, and is also required for collagen
formation during wound healing, carnitine biosynthesis and tyrosine metabolism. The role
of ascorbate in relation to oxidants is particularly complex. It can act as a free radical
scavenger (Banhegyi 1997), and has been described to aid regeneration of tocopherol
(Heffner 1989). It is also thought to help maintain transition metals in a reduced oxidation
state (Padh 1990). The Fenton reaction involves generation of hydroxyl radicals from
hydrogen peroxide, by a metal ion transferring from a low oxidation state to a higher one.
Since ascorbate may maintain ions in a lower oxidation state it is possible that ascorbate may promote the Fenton reaction.

Silvers (Silvers 1994) observed that a high plasma vitamin C concentration at birth was associated with lower antioxidant status, in those preterm infants who died. No exact causes of death were given. Silvers observed that a high plasma ascorbate to caeruloplasmin ratio at birth was associated with lower antioxidant status. Caeruloplasmin is thought to maintain iron in its ferric (+3) oxidation state. Silvers suggested that the high ration of ascorbate to caeruloplasmin may predispose to iron being in the +2 state, and so promote OH⁺ formation. Oxidant stress was measured using a marker of overall oxidant damage, thiobarbituric acid reactive substances.

Berger et al (Berger 1997) demonstrated in vivo that plasma with a high ascorbate concentration (the same order of magnitude as in Silvers’ study) had equal amounts of protein carbonyls and F₂-isoprostanes whether ‘bleomycin detectable iron’ was present or not. This suggests that lipid peroxidation (as identified by isoprostane concentration) was not associated with high ascorbate concentration, whether in the presence of ‘bleomycin detectable iron’ or not.

Jonas (Jonas 1993) demonstrated that ascorbate has a dose related effect on reducing neutrophil-endothelial cell adhesion. There was also a decrease in chemiluminescence from oxygen radicals. An inflammatory response has mechanisms for auto-amplification of the response. ROS are known to be pro-inflammatory. It is unclear whether the effect of ascorbate on neutrophils is one of a direct effect on cell-cell adhesion, or one mediated via an antioxidant effect. The neutrophils in this study (Jonas 1993) had been previously stimulated with LPS, suggesting that the ascorbate affected intrinsic neutrophil-endothelial
cell adhesion. Data from Scorza (Scorza 1997) suggests that ascorbate and plasma thiols may affect release of NO from plasma S-nitrosoalbumin and S-nitrosothiols. These nitroso-compounds are formed by the adduction of NO to albumin or glutathione and are described to be NO donors. They are considered to play a role in transport of NO from its source to distant sites.

Ascorbate is important in wound repair. It regulates the formation of collagen and elastin, albeit differentially. Ascorbate is a cofactor for prolyl hydroxylase, the enzyme which hydroxylates proline residues in procollagen. This hydroxylation process is vital for the collagen to adopt its triple helix formation. However, although proline hydroxylation also occurs in the formation of elastin, ascorbate has been observed to have a dose related, negative effect on elastin formation (Davidson 1997). Davidson’s data was derived from skin and vascular smooth muscle. It is unclear whether ascorbate has the same effect within the lung.

Van Zoren-Grobben (van Zoren-Grobben 1994) described serial blood ascorbate concentrations at intervals from day 1 to day 42 of life in infants fed either preterm formula or breast milk. He showed a high initial concentration, and a subsequent fall to a concentration similar to adult concentrations also described by that group (Lindeman 1989). Moison (Moison 1997) described the concentration of ascorbate in plasma from infants who developed CLD or RDS. He showed that on days 1, 4 and 28 the RDS group had significantly higher (p<0.05) concentration of ascorbate than a control group (minimal respiratory distress for <48 hours; FiO₂<0.03). He did not describe similar differences between the CLD group, and the control or RDS groups. This finding may suggest that the plasma ascorbate concentration in the CLD infants was low, given that such infants are likely to be exposed to oxidant stress, for the reasons given above. This group also
determined the ascorbate to dehydroascorbate ratio (as a marker of oxidant stress) but did not observe any group differences. Ogihara (Ogihara 1998) observed no difference in ascorbate: dehydroascorbate, between a CLD group and a non-CLD (RDS) group. Ogihara and Moison also found a high initial concentration of ascorbate that decreased in the first few days of life. Silvers (Silvers 1994) observed that a high plasma vitamin C concentration at birth was associated with those preterm infants who died, although no causes of death were given. He speculated that this was due to a prooxidant effect from ascorbate.

Data relating to the concentration of ascorbate in BAL fluid of preterm infants is limited. Moison (Moison 1997) performed a single BAL on eleven infants, aged between 1 day and 164 days old. Seven infants had a diagnosis of RDS and CLD, 3 only had RDS, and 1 had been ventilated for apnoea. They presented the ratio of ascorbate: dehydroascorbate. They were unable to demonstrate any correlation between diagnosis and redox ratio, nor between maximal FiO$_2$, a/A ratio or duration of oxygen therapy, and redox ratio. Data from Ogihara’s group (Ogihara 1998) from a population of 20 infants (10 with CLD and 10 with RDS) showed that a diagnosis of CLD was not associated with a difference in the ascorbate: dehydroascorbate ratio.

1.11.2 Urate

Ames speculated that urate may have a crucial role in preventing oxidant damage (Ames 1981). He cited the fact that mammals all have a shorter life expectancy than humans, and that they have a greater risk of developing malignant disease. He suggested that the reason for the longer survival and lower incidence of malignancy in humans may be due to the higher concentration of urate in humans. The serum concentration of urate in humans is approximately 10 times that of rats or prosimians (Ames 1981). This raises the speculation
that animal models of CLD do not accurately reflect the true disease process in preterm infants. At the concentrations described in humans, urate is very close to its limit of solubility within plasma. It is tempting to speculate that for the human body to maintain urate at such a high concentration (approximately 5 times the molar concentration of ascorbate) it is likely to possess an important function.

Urate formation is catalysed by xanthine dehydrogenase, and requires NAD+ as a co-substrate (and electron acceptor) (Supnet 1994). Ischaemia (and possibly other injuries) results in conversion of xanthine dehydrogenase to xanthine oxidase via sulphydryl oxidation. Xanthine oxidase also converts hypoxanthine to urate, but utilises molecular oxygen as the electron acceptor, and so produces a single oxygen radical during the process (Brown 1988).

Urate is irreversibly oxidised when it scavenges radicals (Becker 1991). It also acts as an anti-oxidant by forming stable metal ion-urate complexes to ultimately reduce lipid peroxidation (Davies 1986), and it binds to peroxynitrite to prevent oxidation/nitration (Hooper 1998).

Raivio (Ravio 1976) described a ‘neonatal hyperuricaemia’ in the first 3 days of life in preterm infants with RDS, and term infants which then fell to low adult concentration. Lindeman (Lindeman 1989) observed a similar phenomenon of high cord blood urate, but did not determine urate concentration subsequently. van Zoren-Grobben (van Zoren-Grobben), Ogihara (Ogihara 1998) and Moison (Moison 1997) all observed high initial concentrations of urate. van Zoren-Grobben and Moison determined serial concentrations of urate, which fell over the first month. Moison, and Ogihara also determined the urate to allantoin ratio in plasma (a marker of urate oxidation; a higher ratio suggests increase urate
oxidation to allantoin). Moison reported a higher initial urate to allantoin ratio in CLD infants compared to control infants. This difference disappeared over the first month. Ogihara (Ogihara 1996) also reported raised initial allantoin: urate in plasma of CLD infants. Confusingly, Ogihara (Ogihara 1998) later showed the converse. No initial difference in plasma urate to allantoin ratio between CLD and non-CLD infants. By day 6 a significant difference was noted between the two groups. It is unclear why this difference between the two groups’ data should be, but it may reflect differential exposure to endogenous oxidants, and environmental hyperoxia, over a period of time. In both studies surfactant replacement therapy was used. The Japanese surfactant (Surfactant TA, Tokyo Tanabe Co, Japan) is a bovine surfactant with lipid augmentation, and can be considered as essentially the same as Survanta (Abbott Laboratories, The Netherlands) that was used in Moison’s study (Suwabe 1998).

Saugstad suggested that xanthine oxidase was associated with oxygen-induced lung injury in infants (Saugstad 1984). Supnet observed increased xanthine oxidase activity in infants who had a “poor outcome” (defined as “…early neonatal death, pulmonary haemorrhage within the first week…, intra/ periventricular haemorrhage grade III or IV, necrotizing enterocolitis…”). More recently, Conteras (Conteras 1996) determined epithelial lining fluid (ELF) xanthine oxidase concentration in preterm infants. She found that the concentration on days 2 and 3 of life was significantly higher in ELF from infants who developed CLD/BPD.

Moison also determined urate: allantoin ratio in the group described above (Moison 1997). They were unable to demonstrate any correlation between diagnosis and redox ratio, nor between maximal FiO₂, a/A ratio or duration of oxygen therapy, and redox ratio. Data from
Ogihara’s group (Ogihara 1998) demonstrated an association between a diagnosis of CLD and an increased allantoin: urate in BAL fluid at days 4-6.

### 1.11.3 Glutathione

Glutathione (GSH) is a cornerstone of the antioxidant system. GSH is a tripeptide, composed of glutamate-cysteine-glycine (Bernard 1997). Its predominant role appears to be via the glutathione redox cycle (Heffner 1989). It also acts as a free radical scavenger in its own right (Heffner 1989), and may promote ascorbate regeneration (see Section 1.11.1). GSH redox activity is relatively high in liver, lung and heart, but it is present throughout the body, predominantly in the cytosol (Marklund 1982). The other thiol containing antioxidants (N-acetyl cysteine, cysteine and cysteamine) are thought to increase serum cysteine concentrations, which in turn may increase GSH concentration (Fardy 1995).

White (White 1994) determined the concentration of plasma cysteine in infants who developed persistent pulmonary hypertension of the newborn (PPHN) and RDS. He found that infants who developed RDS had lower plasma cysteine concentration than fasted or fed control subjects. Similar differences in plasma methionine and homocysteine were also seen. Lavoie et al (Lavoie 1997) refined this observation by describing a positive relationship between gestational age, and also gender, and GSH reductase activity. They noted that lower GSH reductase activity was associated with lower gestational age. GSH reductase activity was determined in umbilical cord endothelium, and tracheal aspirate cells. They did not report data for plasma GSH or reduced glutathione (GSSG) concentration, or GSH: GSSG ratio. Nemeth (Nemeth 1994) advocated the use of blood GSH redox ratio (ratio of GSSG to GSH) as a better marker of GSH ability to counteract oxidative stress, and showed that infants with RDS had lower serum GSH, but increased GSSG concentration (as might be expected). These data were obtained in infants with
varying \( \text{FiO}_2 \) requirements. They did not identify any differences in GSH or GSSG in initial blood samples. Darlow (Darlow 1995) observed a relationship between plasma selenium concentration and respiratory outcome, in preterm infants. Selenium is a known cofactor for GSH peroxidase. The maximal difference was seen at 28 days. Concentrations of maternal selenium, cord selenium and selenium were the same at 48 hours of age. This suggests that the differences noted may have been an effect of poor nutrition and/or increased oxidative stress, and that the gestational age did not exert an effect on selenium concentration. van Zorren-Grobben (van Zorren-Grobben 1997) observed a different picture of GSH related compounds. No differences were observed between a control group and an RDS group for GSH peroxidase, GSH reductase, or GSH concentrations. They did demonstrate lower concentrations of SOD and GSSG in infants with RDS. Smith et al (Smith 1993) compared infants of 23-33 weeks gestation with those of 34-42 weeks gestation. The more immature group had higher \( \text{FiO}_2 \), alveolar oxygen partial pressure \( (P_{A}O_2) \), and arterial oxygen partial pressure \( (P_aO_2) \). The more immature group also had lower plasma (arterial) GSH and higher GSSG concentrations than in venous blood. This suggests that GSH was being oxidised to GSSG within the lung, although whether this was happening within the vascular compartment or alveolar compartment is not clear from these data. Moison (Moison 1997) did not demonstrate any differences in erythrocyte sulphydryl groups between RDS and CLD infants. However, when erythrocyte sulphydryl concentration was corrected for plasma protein concentration, as advocated by Quinlan (Quinlan 1994), the CLD infants had a significantly lower amount than the Control group. This difference was only statistically significant at 28 days. This suggests the differences were an effect of ongoing oxidant stress rather than an inherent, premorbid difference that was causal to CLD. van Zoren-Grobben (van Zoren-Grobben 1994) did not demonstrate a difference in plasma sulphydryl groups, urate, ascorbate, or tocopherol between preterm infants (33.5 ±1.5 weeks) fed breast milk, preterm formula or mixed feed (increasing
preterm formula as maternal milk volume decreased). They did observe an increase in bilirubin in the breast fed infants and a concomitant increase in total peroxyl trapping capacity (TRAP) (an in vitro assay of the plasma ability to prevent lipid and linoleic acid peroxidation). The three feed groups were all of the same gestational age range, and so differences described by other authors, would not be expected in this study. The initial TRAP study (Lindeman 1989) did not show any differences in cord blood concentrations of sulphhydryl groups, urate, ascorbate, tocopherol, or bilirubin in preterm (32 weeks) and term (39 weeks) infants. These data also suggest that between RDS and CLD infants, differences in plasma GSM and sulphhydrals are not inherent at birth. Rather they appear to occur postnatally, and may be related to nutritional intake and exposure to ongoing oxidative stress.

Grigg et al (Grigg 1993) performed BAL upon preterm infants that were ventilated. He found that those infants who developed CLD had a lower total glutathione on day 1 than those who did not develop CLD. The difference had disappeared by day 5. Grigg did not relate total GSH concentration to FiO\(_2\) on each day. Reise (Reise 1997) did relate GSH to FiO\(_2\). She found that there was a non-linear inverse relationship between GSH concentration and oxygenation index (a measure of ability to oxygenate a mechanically ventilated individual; calculated from [mean airway pressure x FiO\(_2\)/PaO\(_2\)]). Neither Grigg nor Reise were able to demonstrate the inverse relationship with gestational age that Lavoie (Lavoie 1997) suggested. Data derived from BAL suggest that GSH concentration may be associated with the development of CLD. The data does not enable us to distinguish whether the low GSH concentration is causal, or a result of oxidant stress.
1.12 Non-bronchoscopic bronchoalveolar lavage

With the development of techniques in cellular and molecular biology, it has been possible to begin to understand the pathophysiology that underlies many respiratory disorders of newborn infants. In practice we are severely restricted in human infants to which lung tissues are available for study. Lung tissue obtained at autopsy represents the extreme end of the disease process, and is affected by artefacts inherent in post-mortem sampling. Lung biopsies are hazardous and unethical in newborn infants. Adequate direct vision bronchoalveolar lavage is currently not possible due to the small size of the suction channel of neonatal bronchoscopes. Thus many groups have relied on non-bronchoscopic bronchoalveolar lavage (BAL) (Grigg 1992, Kotecha 1996) to study respiratory disorders of newborn infants.

1.12.1 Technique of non-bronchoscopic bronchoalveolar lavage

The technique of BAL is essentially the same, although few groups have attempted to standardise non-bronchoscopic lavage, and there are many variations to the technique. The method utilised in this study was first described by Grigg (Grigg 1992). In it the infant is placed supine, with the head turned to the left. An endotracheal tube suction catheter with a rounded tip and an end hole is used. The size is 4 or 5 Fr gauge, as will be admitted along the endotracheal tube. The catheter is then introduced along the lumen of the endotracheal tube, until resistance is felt. At this point the catheter is thought to be ‘wedged’ in a segmental bronchus. Normal saline at 1ml / kg is instilled over a period of 3-5 seconds, and then aspirated by suction into a sterile trap. Aspiration is performed using a negative pressure of 50 mmHg. The catheter is gradually withdrawn as the instilled saline is aspirated. The infant is then observed to ensure that they have tolerated the procedure. If the transcutaneous saturations and heart rate are acceptable, the process is repeated. The two aliquots obtained this way may be pooled (as they were for the studies in this thesis).
Some groups collect bronchoalveolar lavage fluid (Bagchi 1994, Gerdes 1988, Grigg 1992, Kotecha 1996) whilst others collect tracheal aspirate (Heikinheimo 1992, Lotze 1992, Sluis 1994). Some have used a fixed volume of saline (Ogden 1984, Murch 1992, Watts 1992) whilst others administer a weight-related quantity (Grigg 1992, Kotecha 1996). Some use syringes to suck back the instilled saline whilst others use automated suction units with or without reported suction pressures. The positioning of the infant is often not described, and thus the area of the lung sampled is unknown. Despite such variations most research work utilising BAL has one thing in common: BAL is performed repeatedly over a number of days or even weeks. Important information has been generated by such studies using BAL. Pulmonary inflammation is now widely accepted as a feature in the development of CLD. The majority of the work that supports this observation was done using BAL.

1.12.2 Adverse effects associated with BAL

BAL in infants is considered to be safe (Kotecha 1999), although few studies have specifically examined infants for adverse effects from this technique. Tachycardia and an increase in blood pressure (Grigg 1992), a decrease in transcutaneous oxygen saturation (Kimbourlis 1993), and bradycardia, increased blood pressure and hypoxia (Belai 1997) have all been described. Transient bradycardia due to a vasovagal response to insertion of the catheter may also occur.

Repeated large volume bronchoalveolar lavage of animals such as rabbits has been described to induce surfactant deficiency (Lachman 1980), and so concern has been raised that repeated BAL of the same area of the lung may result in constant removal of surfactant from that region. Simple quantitation of phospholipid or surfactant protein in lavage fluid will not resolve concerns about depletion of surfactant. A low surfactant concentration in lavage fluid may indicate a low concentration within the epithelial lining fluid or that the technique of BAL does not remove significant amounts of surfactant. Conversely, a high
surfactant concentration may indicate adequate alveolar surfactant concentration or significant washing-out of surfactant by the lavage process.

1.12.3 Interpretation of BAL fluid results and dilutional factors

The large number of studies in neonates that have used sampling from the lower respiratory tract have used either tracheal aspirate or BAL. There is a difference between the two samples, in that BAL may be considered to retrieve ELF from a more distal portion of the bronchial tree. Even within the technique of BAL, it is thought that the first aliquot is predominantly from the large airways, and that subsequent aliquots yield cells and ELF from more distal airways (Rannard 1990). Thus, the findings from a study that used tracheal aspirate would be reporting findings from a different part of the respiratory anatomy, than would a study that used BAL. This difference must be borne in mind when comparing such studies. The yield from a lavage is of the order of 30-70% of the volume instilled. The proportion of the recovered fluid that is ELF will vary. Whilst this may not be of relevance if proportions of cell types are described, if absolute concentrations are reported there may be significant spurious effect from variation in ELF content of the recovered fluid. A number of techniques have been described to help determine the extent of dilution of the ELF within the recovered fluid. None are ideal, and all have their relative problems. ELF protein has been advocated. Since there is protein leak into the alveolar space in lung injury in preterm infants, its usefulness in newborn infants is likely to be limited as there will be an underestimation of the quantity of ELF recovered. Secretory component of IgA has also been advocated. The developmental profile of this protein, is thought to be unrelated to gestational age, although there is no published evidence. Determination of a normal range would be helpful, but this would require intubation of infants of the appropriate gestational age. Preterm infants are often intubated for respiratory disease. The lower the gestational age, the more likely they are to have
respiratory failure. It would be very difficult, if not impossible, to derive a normal range (i.e. a range in healthy infants) for the gestational ages most at risk of CLD/BPD since this group is most likely to have respiratory failure at birth requiring ventilation. Urea dilution has been proposed as a marker for ELF dilution. It has the advantage of being related to serum concentrations, and is not actively secreted into the lungs. However it is affected by dwell times. It is not known how much difference a few seconds would make to the concentration.

Since there is uncertainty, even with urea dilution, many groups express their findings ‘per ml of aspirate’ in accordance with the suggestions by European Respiratory Society Task Force on bronchoalveolar lavage of children (de Blic 2000).
1.13 Specific Aims of this thesis

The specific aims of this project were to:

1. Determine whether serial non-bronchoscopic lavage in mechanically ventilated preterm infants is associated with significant morbidity.

2. Develop an appropriate technique to quantify NO-derived products (nitrite and nitrate) in small volumes of BAL fluid (less than 50 microlitres).

3. Use this technique to determine the concentration of nitrite and nitrate (as markers of NO production) within the lungs of infants who develop CLD.

4. Determine the concentration of humoral, non-enzymatic antioxidants ascorbate, urate and total glutathione in BAL, and serum from infants who develop CLD.
Chapter 2

The Safety of Serial Non-Bronchoscopic Bronchoalveolar Lavage
2.1 Background

A study was performed in order to examine the effect of serial BAL upon ventilated preterm infants at risk of lung disease (RDS and CLD). I hypothesised that repeated serial BAL of the right lower lobe may "wash-out" surfactant and thus result in a localised collapse/consolidation on a chest radiograph. I therefore compared a group that had undergone BAL with a historical group that had not undergone any formal lavage procedure.

Although immediate effects from BAL have been described there is no data on the cumulative effects of repeated BAL. It was not possible to conduct a prospective, randomised trial of serial lavage, as at the time of this study, all patients suitable for BAL were recruited into the CLD study described in this thesis. For this reason it was decided to perform a comparison with an historically derived control group. The study was performed on the Neonatal Unit of the Leicester Royal Infirmary.

A marker of disease severity was needed to compare the lavaged group with the historical control group. It was decided not to rely upon documented clinical measurements within the case notes, as the accuracy of this information could not be verified. In addition, any clinical parameters recorded in the case notes may not have been at the true extreme point of the parameter (e.g. recorded maximum or minimum FiO2). For these reasons the use of a surrogate marker of clinical outcome was considered appropriate for this study.

Radiological scores have been demonstrated to correlate well with clinical outcomes in CLD (Toce 1984, Maconochie 1991, Weinstein 1994). It was decided to use a radiographic score of chest x-ray appearances in infants who had and had not been serially lavaged. Of the scores described above, that of Maconochie et al was most suitable to detect changes of
acute surfactant deficiency (RDS) as well as chronic changes of CLD. The components of Maconochie's scoring system are shown in Table 2.1.

2.1.1 Objectives /Hypothesis

I hypothesised that repeated serial BAL of the right lower lobe may be expected to "wash out" surfactant from that area of the lung. Any clinically significant deleterious effect from this hypothesised "washing out" effect should have an accompanying radiological change, which may be detected on review of chest radiographs taken during the serial lavage period. The objective of this study was to establish if there were differences between chest radiographs from infants who underwent serial lavage, and infants (of very similar gestational age and birth weight) who did not.

2.2 Patient Groups

The two patient groups in this study were infants from the CLD study described in this thesis, and an historically derived control group. Lavaged infants consisted of those infants who underwent serial lavage, and who had acquired retrospective diagnoses of CLD or RDS (section 2.2.2, 2.2.3). Infants in the non-lavaged group also acquired a diagnosis of CLD or RDS using the same definitions.

2.2.1 Group of infants undergoing BAL

Patients admitted to the unit, at birth, and who required endotracheal tube (ETT) insertion for mechanical ventilation, were enrolled. The presence of the ETT was necessary because the technique of non-bronchoscopic bronchoalveolar lavage requires access below the vocal cords via an indwelling ETT.
Patients were lavaged according to the schedule discussed below. Segregation of the patients into the CLD and RDS diagnostic groups was done after the threshold of oxygen requirement for a diagnosis of CLD was reached.

### 2.2.2 Definition of CLD

Infants were diagnosed as having developed CLD if they fulfilled the criteria of Bancalari (Bancalari 1979). This states that a child may be diagnosed as having CLD if they have:

a. A requirement for mechanical ventilation in the first week of life, and lasting for at least 3 days.

b. Chronic respiratory distress characterised by tachypnoea, intercostal retraction and crackles persisting longer than 28 days of age.

c. An oxygen requirement lasting for longer than 28 days of age.

d. An abnormal chest radiograph, demonstrating areas of increased shadowing (atelectasis) and increased radiolucency (hyperinflation).

All infants in these studies who are diagnosed as having CLD fulfil the above criteria.

### 2.2.3 Definition of RDS

Infants put into this category were those who, in the first week of life, had respiratory failure requiring an FiO₂ of >0.3, and with a typical time course of increasing respiratory distress over the first 4-6 hours, and resolution within 2 to 5 days. Typical changes in the chest radiograph were also seen: a granular, ‘ground glass’ appearance to the lung fields, the presence of air bronchograms, and loss of the clarity of the cardiac borders (Bancalari 1999).
2.3 Protocol for performing BAL

Bronchoalveolar lavage was performed regularly at the time of routine endotracheal tube suction until 28 days of age or extubation. The infants were lying supine, with their head turned to the left. A six French gauge suction catheter was introduced into the endotracheal tube, until resistance was felt. An aliquot of 1 ml per kg of N-saline was instilled via the catheter and immediately aspirated back with a negative pressure of fifty mmHg. The process was repeated and the two aspirated aliquots pooled. The BAL fluid was then centrifuged at 400g and the supernatant separated and stored at –70°C until further analysis.

<table>
<thead>
<tr>
<th>Volume of thorax</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degree of inflation</td>
<td>normal</td>
<td>Reduced</td>
<td>Increased</td>
<td></td>
</tr>
<tr>
<td>of lung field</td>
<td>normal</td>
<td>Under inflated</td>
<td>Over inflated</td>
<td></td>
</tr>
<tr>
<td>Opacification</td>
<td>normal</td>
<td>Perihilar or regional</td>
<td>Perihilar and regional</td>
<td>complete</td>
</tr>
<tr>
<td>Air bronchogram</td>
<td>absent</td>
<td>Present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIE</td>
<td>absent</td>
<td>Present</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cystic elements</td>
<td>none</td>
<td>Single cyst</td>
<td>Multiple in one zone</td>
<td></td>
</tr>
<tr>
<td>Size of cysts</td>
<td>none</td>
<td>&lt;1/3 ipsilateral lung field</td>
<td>&gt;1/3 ipsilateral lung field</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.1: Chest radiographic scoring system of Maconochie et al (Maconochie 1991). Maximum score = 13. PIE = pulmonary interstitial emphysema
2.3.1 Frequency of BAL

The initial BAL was performed as soon after birth as possible. This was usually after the infant had been admitted to the neonatal intensive care unit, and before intra-tracheal surfactant administration. A second lavage was performed 12 hours after the first dose of intra-tracheal surfactant had been administered. This was in accordance with the local policy for no aspiration or lavage of the ETT for 12 hours after administration of surfactant. A third BAL was performed at 24 hours of age. Subsequent BAL was done daily until the seventh day. After this BAL was performed on alternate days until the infants was 28 days old. Extubation at any time resulted in no further BAL being performed upon that child, unless they were subsequently re-intubated before the 28th day of life. After the first 2 days, a BAL was performed at the time when routine ETT toilet was due. The lavage was done instead of the normal clinical ETT toilet. The patient groups were derived such that there was similarity for birth weight and gestational age. The two groups were compared for birthweight, gestational age, antenatal steroid treatment, and incidence of severe (≥ Grade III) IVH and severe (≥ Stage 3+) ROP. This was to ensure that the two groups could be considered comparable. Chest radiographs taken for clinical indications were retrieved for scoring. Radiographs taken on day 1, day 7 and day 28 were scored.

2.4 Chest radiograph scoring

Chest radiographs were performed on the infants only when clinically indicated. Radiographs taken closest to day 1, day 7 and day 28 of age were selected for comparison. The first radiograph preceded any lavage procedure thus represented the severity of the respiratory illness of the infant. Two paediatric radiologists independently scored the radiographs using the scoring system devised by Maconochie et al (Maconochie 1991) (Table 2.1). Only the right and left lower lobes were scored since these represented the
regions of interest. A high score suggested worse disease than a low score. Each patient’s mean radiographic score was calculated from the two radiologists’ scores. Group mean values were then determined from these patient mean scores.

2.5 Statistical Analysis

Statistical analysis was performed using the Minitab software (Minitab Inc., State College, PA, USA). Parametric data was described by mean and standard error of the mean. Non-parametric data was described by median and range. Comparison of parametric group data was done by t-test, and non-parametric data was tested for comparison using the Kruskal-Wallis test. Logarithmic mean values for non-parametric data were also used to compare group data.

2.6 Results

2.6.1 Patient characteristics

Chest radiographs taken at 1, 7 and 28 days from 54 infants who had received ventilatory respiratory support at Leicester Royal Infirmary Neonatal Unit were assessed. All infants had developed RDS and had received surfactant therapy (Survanta, Abbott Laboratories, UK) within the first 12 hours of life. Patient characteristics are given in Table 2.2.

28 infants were studied in the BAL group. 16 progressed to develop CLD and 12 recovered from RDS and were nursed in air by 28 days of age. These infants underwent a median (range) of 9 (1-22) lavages. There were 26 infants in the non-BAL (control) group who did not undergo any lavage procedure. 10 developed CLD and 16 recovered from RDS (p>0.05).
The birthweight, gestational age, duration of supplemental oxygen requirement and maximal FiO₂ in the first 24 hours of age in both groups, and in CLD and RDS sub-groups were similar. Only the maximal FiO₂ in the first 24 hours was significantly higher for the lavaged RDS group than in the non-BAL RDS group (p<0.05).
<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th></th>
<th>CLD</th>
<th></th>
<th>RDS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BAL</td>
<td>Non-BAL</td>
<td>BAL</td>
<td>Non-BAL</td>
<td>BAL</td>
<td>Non-BAL</td>
</tr>
<tr>
<td>Number of patients (n)</td>
<td>28</td>
<td>26</td>
<td>12</td>
<td>16</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>Birth weight (g)</td>
<td>1350 (156)</td>
<td>1450 (119)</td>
<td>780 (47)</td>
<td>942 (82)</td>
<td>1820 (219)</td>
<td>1650 (190)</td>
</tr>
<tr>
<td>Gestational age (weeks)</td>
<td>28.6 (0.7)</td>
<td>29.9 (0.9)</td>
<td>26.2 (0.5)</td>
<td>26.6 (0.9)</td>
<td>31.2 (0.9)</td>
<td>32 (1.2)</td>
</tr>
<tr>
<td>Maximum FiO&lt;sub&gt;2&lt;/sub&gt; in first 24 hours</td>
<td>0.6 (0.05)</td>
<td>0.51 (0.05)</td>
<td>0.70 (0.06)</td>
<td>0.68 (0.09)</td>
<td>0.41 (0.02)</td>
<td>0.34 (0.02) *</td>
</tr>
<tr>
<td>Number receiving antenatal dexamethasone (%)</td>
<td>21 (75)</td>
<td>19 (73)</td>
<td>11 (91)</td>
<td>12 (75)</td>
<td>10 (62.5)</td>
<td>7 (70)</td>
</tr>
<tr>
<td>Number of lavages</td>
<td>9 (1-22)</td>
<td>N/A</td>
<td>17 (12-22)</td>
<td>N/A</td>
<td>3 (1-14)</td>
<td>N/A</td>
</tr>
<tr>
<td>Duration of ventilation (days)</td>
<td>20.2 (4)</td>
<td>15.3 (2.5)</td>
<td>27 (5.3)</td>
<td>22.6 (4.4)</td>
<td>5.7 (1.4)</td>
<td>4.5 (1.2)</td>
</tr>
<tr>
<td>Duration of supplemental oxygen requirement (days)</td>
<td>32.9 (6.5)</td>
<td>37.5 (8.5)</td>
<td>47 (9.7)</td>
<td>51 (17.7)</td>
<td>20.9 (7.6)</td>
<td>16.5 (8.3)</td>
</tr>
</tbody>
</table>

Table 2.2: Patient characteristics for all patients: those with CLD and those with RDS. Mean (± SEM) are shown for birthweight, gestation, maximal FiO<sub>2</sub> in the first 24 hours, duration of ventilation, and duration of supplemental oxygen requirement. Median (range) is given for the number of lavages performed. CLD = chronic lung disease of prematurity; RDS = respiratory distress syndrome. * p<0.05 BAL vs. non-BAL.
2.6.2 Right Lower Lobe: BAL vs. non-BAL

The mean difference (95% confidence interval) between the two radiologist’s scores was 0.56 (0.36-0.75). The median radiological scores of the right lower lobe (RLL) of the infants undergoing BAL was compared with the RLL scores of infants in the control group who had not undergone any formal BAL procedure (Table 2.3). The day 1 radiological score of the RLL was greater in the BAL group prior to undergoing BAL when compared to the control infants (lavaged RLL 3.5; range 2-6 vs. non-lavaged RLL 2; 0-6. p<0.05). The scores at day 7 (lavaged RLL 3 [0-6] vs. non-lavaged RLL 3[0-8]. p=NS) and day 28 of age (3 [0-5] vs. non-lavaged RLL 3 [0-8]. p=NS) were very similar.

2.6.3 BAL infants; Right Lower Lobe vs. Left Lower Lobe

The radiological scores for the RLL of the infants who had undergone BAL were compared to the contra-lateral left lower lobe (LLL) of these children at days 1, 7 and 28 of age (Table 2.4). The median (range) of the radiological scores prior to any lavage procedure being performed was very similar in both the lower lobes of the BAL group (lavaged RLL 3 [range 2-6] vs. non-lavaged LLL 3.5 [2-6]. p=NS). After serial BAL of the RLL the radiological severity of the lavaged lobe did not worsen at 7 days (3 [0-6] vs. 3 [1-6]. p=NS) or 28 days of age (3 [0-5] vs. 3 [0-6]. p=NS). As expected, with improving clinical condition of the infants, the radiological scores for the lower lobes in both groups also decreased.
<table>
<thead>
<tr>
<th></th>
<th>BAL</th>
<th>Non-BAL</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1</strong></td>
<td>3.5 (2-6)</td>
<td>2 (0-6)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td><strong>Day 7</strong></td>
<td>3 (0-6)</td>
<td>3 (0-8)</td>
<td></td>
</tr>
<tr>
<td><strong>Day 28</strong></td>
<td>3 (0-5)</td>
<td>3 (0-8)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.3:** Chest radiograph scores for the right lower lobe (R LL) which had undergone serial BAL compared to the right lower lobe of infants who had not undergone any lavage procedure. Median (range) is shown.

<table>
<thead>
<tr>
<th></th>
<th>LLL</th>
<th>RLL</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 1</strong></td>
<td>3 (2-6)</td>
<td>3.5 (2-6)</td>
<td></td>
</tr>
<tr>
<td><strong>Day 7</strong></td>
<td>3 (0-6)</td>
<td>3 (1-6)</td>
<td></td>
</tr>
<tr>
<td><strong>Day 28</strong></td>
<td>3 (0-5)</td>
<td>3 (0-6)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.4:** Chest radiograph scores comparing the right lower lobe (RLL) that had undergone BAL when compared to the contra-lateral left lower lobe (LLL) which had not undergone any lavage procedure. Median (range) is shown.
2.7 Discussion

Serial NB-BAL is frequently used to study respiratory disorders of the newborn infant. In this study we asked if such repeated lavages resulted in localised radiological changes due to washing out of endogenous surfactant or due to localised trauma. Our data shows that when the lavaged RLL was compared to the contralateral unlavaged LLL no difference was found between the two sides at 7 and 28 days after NB-BAL. Similarly, when the lavaged RLL was compared to the RLL of Control infants who had not undergone any NB-BAL procedure no differences were noted at 7 and 28 days of age. This was despite the NB-BAL group having an increased radiological score at the outset prior to any lavage procedure being performed. Equally reassuring are the lack of differences found between the two groups for clinical parameters including the length of mechanical ventilation, oxygen requirements and hospital stay as well as the incidence of CLD, ROP and intraventricular haemorrhages.

Previous studies have reported adverse effects encountered during the NB-BAL procedure including bradycardia most often due to insertion of the suction catheter resulting in a vasovagal response (Belai 1997, Grigg 1992, Kimbourlis 1993). This is transient and usually recovers within a few seconds as long as the catheter is not repeatedly moved thus resulting in further bradycardia. As expected both tachycardia and increased blood pressure are also encountered as is hypoxia which again is transient and responds to an increase in inspired oxygen (Grigg 1992).

Of equal concern is the hypothesis that repeated serial lavages may remove surfactant as is often used experimentally to induce surfactant in animal experiments (Belai 1997). In human infants estimation of components of the surfactant system is possible but often the underlying disease being studied has abnormalities of surfactant eg RDS, meconium
aspiration and infection. It would be impossible to study a control group since the acquisition of lung lavage fluid would involve a lavage procedure. Lung biopsies are clearly not an option. Thus we hypothesised that direct trauma or repeated removal of surfactant following serial NB-BAL would result in localised atelectasis due to mucosal damage or due to reduced surface tension of the affected area and that this would manifest as localised radiological changes. Using a radiological score we were unable to demonstrate any changes of the lavaged right lower lobe when compared to the contralateral unlavaged left lower lobe or when compared to the RLL of infants who had not undergone any formal lavage procedure.

Since the lavage procedure is blinded the positioning of the catheter can not be certain. However, previous work by Silverman’s group has shown that repeated passage of the catheter whilst the infant’s head is turned to the left aids its placement into the right lower lobes (Kimbourlis 19935, Placzek 1983). We have obtained chest radiographs in children receiving extracorporeal membrane oxygenation to demonstrate similar placement of the catheter in the right lower lobe during the lavage process (Kotecha 1999). With the standardised procedure it is likely that the catheter is introduced into the RLL. In any case infants who had not undergone any lavages were used as a Control group thus changes would have been noted for the radiological scores of the study group when compared to the Control group.

Serial NB-BAL may result in more subtle pulmonary changes resulting in more longer term morbidity including increased length of mechanical ventilation, oxygen requirement, hospital stay or in involvement of other organs especially ROP and intraventricular haemorrhages. Belai et al described a cohort of 32 infants, 5 of whom developed CLD. They described intraventricular haemorrhages (grade III-IV) in 2 infants and no ROP but
did not have a comparison control group (Belai 1997). In our study, using a control group, we were not able to show significant differences between the two groups for any of these parameters of morbidity.

We used a historical group of infants for our Control group since all infants born during the study period were recruited for the study presented within this thesis, investigating the pathogenesis of CLD. Thus the groups are not strictly comparable. Nevertheless the results are of importance since no differences were found between the two groups for the parameters studied including the radiological scores. Furthermore, we had an “internal” control by comparing the RLL with the contralateral LLL which did not undergo NB-BAL.

In older subjects where BAL is performed usually only once with flexible bronchoscopes, it is thought to be a safe procedure (Pohunek 1996, Ratjen 1996, Reidler 1995). Tachycardia, hypertension and hypoxia are noted as with the newborn and since symptom reporting is easier, transient ‘flu-like symptoms are often described (Pohunek 1996, Ratjen 1996). Infection may be introduced but may be related more to the instruments than the procedure as long as standard anti-septic procedures are followed with NB-BAL including the use of sterile saline and catheters. In adults an increase of pro-inflammatory cytokines interleukin-1, -6 and tumour necrosis factor alpha has been described (Krause 1997) but we have previously reported decreasing serum interleukin-8 concentrations with time in infants who had undergone the NB-BAL procedure (Kotecha 1995) thus suggesting that a similar response is not seen in newborn infants.

In summary, we have used a radiological score of the lavaged lobe as a surrogate measure for surfactant removal and/or direct trauma following NB-BAL and were unable to show any persisting radiological changes in the lobe undergoing formal, repeated and standardised NB-BAL. In addition, there were no differences for longer term measures of
morbidity (including length of mechanical ventilation, oxygen requirement and hospital stay), nor in ROP or intraventricular haemorrhages. It is unlikely that by serial NB-BAL of ventilated newborn infants we are exacerbating the underlying disease.

Access to the alveolar milieu in preterm infants is limited. The development of non-bronchoscopic bronchoalveolar lavage in infants has permitted study of cellular and humoral events in neonatal lung diseases. Further work is essential for gaining a greater understanding of the pulmonary pathophysiology of RDS and CLD. The technique of BAL will prove invaluable in such research.
Chapter 3
Development of Methods for Quantitation of Nitric Oxide Products
3.1 Background

Production of NO can be determined by a number of different techniques. Some directly measure the concentration of NO in a gaseous phase, whilst others measure activity of NOS, or detect the presence of metabolites of NO.

Since NO readily forms a number of oxides there is a theoretical risk that simple quantitation of evolved NO (in a gaseous phase) will not provide an accurate measure of the absolute quantity of NO produced. Quantitation of NO in the gaseous phase is performed by chemiluminescence. The equipment required for this procedure is necessarily sophisticated, and therefore very expensive. Alternative methods for measuring oxidative products of NO are well described, and are much cheaper.

3.1.1 Objective

There were two objectives for these experiments.

1. To develop a reliable assay for measurement of low concentration of nitrite (c 5-20 micromoles/litre) in small sample volumes (<50 microlitres) i.e. to accurately detect picomolar quantities of nitrite.

2. To develop a reliable, reproducible means to reduce nitrate to nitrite, without the cofactors within the enzymatic reduction kits causing interference with the colourimetric or fluorometric processes.

3.1.2 Colorimetric Assay (The Griess Reaction)

This reaction was first described by Griess in 1879, and was one of the first azo-dye reactions to be determined (Griess 1879).
Sulphanilimide reacts with N-1-naphthylethylenediamine hydrochloride (NEDD) in the presence of nitrite (NO$_2^-$) to produce an azo-dye (figure 3.1). The intensity of the azo dye is proportional to the concentration of NO$_2^-$ in the original sample. Thus by constructing a standard curve, it is possible to determine the concentration of NO$_2^-$ in a sample.

The procedure is as follows:

- 100 µl 1% sulphanilimide solution (1g in 100 mls 5% orthophosphoric acid) is mixed with NO$_2^-$ standard solutions, or the unknown sample.
- This mixture is incubated in the dark, at room temperature, for 10 minutes.
- 100 µl NEDD (0.1% w/v) solution is then added.
Figure 3.1: The Griess reaction between sulphanilimide, and N-1-naphthylethylendiamine hydrochloride (NEDD) in the presence of nitrite.
After a further 5 minutes incubation the samples are read in a colourimeter (MRX Platereader, Dynatech Laboratories Inc., Chantilly, VA, USA) set for 540 nm wavelength.

This technique is very robust, and has been used for many biological fluids. However, the limit of detection with this is nanomolar quantities of nitrite. The volume of fluid recovered by BAL of preterm infants is very small (approximately 500 µl for a 1kg infant). The quantity of nitrite produced in BALF samples was below the limit of detection of the Griess reaction. For this reason a more sensitive assay was required.

3.1.3 Fluorometric Assay

Misko (Misko 1993) described the use of a fluorometric assay to quantitate NO$_2^-$.

In this reaction 2,3 diaminonaphthalene (DAN) reacts with NO$_2^-$ to produce 1(H)-naphthotrazole (figure 3.2). When excited by light at 360 nm wavelength, 1(H)-naphthotrazole will fluoresce at 460 nm. The degree of fluorescence is linearly proportional to the quantity of nitrite in the original sample. As with the Griess reaction (described in Section 3.1.1), the concentration of NO$_2^-$ can be derived by the use of NO$_2^-$ standard solutions. The sensitivity of the fluorometric assay is reported to be at the level of picomolar quantities of NO$_2^-$.

In the initial experiments conducted in this research project, a commercial kit was used for fluorometric analysis (Caymen Chemicals, Ann Arbor MI, USA). At first fluorometry was performed using a Denley Wellfluor fluorometric plate reader (Denley, Life Sciences Int. Ltd, Basingstoke, Hampshire). Subsequently the reagents were prepared from the basic chemicals. The standard salts (NO$_3^-$ and NO$_2^-$, sulphanilimide, NEDD) were obtained
from Sigma (Sigma, Bournemouth UK). The DAN was from Acros Fisher (Fisher, Loughborough UK).
Special Note

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Special Note

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Figure 3.2: The reaction between nitrite and 2,3 diaminonaphthalene (DAN) to produce the fluorescent compound 1(H)-naphthotriazole
The composition of the reagents was as suggested by Schmidt (Schmidt 1996). The reagents were made up as follows:

- Nitrite standard solutions: 200 μM stock solutions made from ‘Analar’ sodium nitrite (Sigma, Bournemouth).

- DAN: conc. 50μg /ml, in 0.62M HCl; 10μl per assay

The DAN was incubated with the NO$_2^-$ for 10 minutes, in a darkened environment. The reaction was then quenched with 2.8M sodium hydroxide (NaOH) solution. Data from the nitrite fluorometry is shown in figure 3.3. 20μl of NO$_2^-$ standard solutions were used in 141 μl of double deionised water. 20μl of DAN was incubated with this mixture, and the reaction quenched with 10μl of NaOH.

### 3.1.4 Conversion of nitrate to nitrite

NO$_2^-$ readily oxidises further to form NO$_3^-$. Neither the Griess reaction, nor the fluorometric assay, is able to detect NO$_3^-$ per se. Instead the NO$_3^-$ must be reduced to NO$_2^-$ in order to be quantified by the above methods.

A number of methods have been described to convert nitrate to nitrite. Two commonly used techniques are reduction by cadmium catalyst, or by enzymatic methods. A variety of methods based upon these two principles were trialled during the course of this research. I shall discuss the use of each method tested in the subsequent paragraphs. In all methods described as part of the research project, double deionised water was always used for dilution. The results from them are presented below.
**Figure 3.3:** Graph showing fluorometry from nitrite standard solutions using prepared DAN. The $R^2$ value, and trendline equation are shown adjacent to the trend line.

$y = 207.54x + 1846.8$

$R^2 = 0.9984$
It should be noted that these results are given as examples and do not represent all the experiments performed. These experiments are detailed within the laboratory notebooks kept during the experiments.

3.1.4.1 Cadmium reduction

Cadmium is able to catalyse the reduce NO$_3^-$ to NO$_2^-$.

I examined several techniques based on cadmium’s catalytic action during this research project, which are described below.

3.1.4.1a World Precision Instruments Nitrate Reductor

This instrument (World Precision Instruments, Sarasota, Florida) consists of a glass vial that is coated inside, with a roughened cadmium surface. A buffer of aqueous ammonium chloride (5M) was prepared and the pH adjusted to 9 by sodium hydroxide. Buffer and sample were added to the nitrate reductor in a ratio of 1:9 buffer: sample. The reductor was then agitated by hand, or by rotator, for 5 minutes. An aliquot of the reduced solution was then reacted with sulphanilimide and NEDD for quantitation of NO$_2^-$.

The results of a standard curve derived by this method are shown in figure 3.4.

As can be seen, the standard curves are of similar magnitude. The ‘Nitrate Reductor’ appears be an effective means of reduction of nitrate prior to its determination by the Griess method.

However, this technique has a major drawback. Once the sample and buffer are in the reductor, they need to be agitated for 5 minutes. It was anticipated that there would be at least 200 samples to be processed. This would mean that the last sample would be agitated
16 hours after the first one, not including time to wash and re-prime the reductor vials. It is known that in aqueous solution, nitrite is unstable and will gradually oxidise to nitrate. Thus there is a potential risk of the nitrite in the reduced sample, re-oxidising back to nitrate.

It was not practical to assay each sample immediately after reduction, as this would have required a standard curve to be derived each time. This would have greatly increased the consumption of the reagents, and consumable items (e.g. microtitre plates). For these reasons, the ‘Nitrate Reductor’ vials were not used for reduction of the BAL samples.
Figure 3.4: Standard curves for nitrite (open diamonds) (solid trendline), and nitrate after reduction by 'Nitrate Reductor' (open circles) (dashed trendline). $R^2$ values and trendline equations for each trendline are shown adjacent to respective trend line.
3.1.4.1b Cadmium Beads for Reduction of Nitrate

This technique used a commercially available kit (Biogenesis, Poole UK). This kit uses cadmium beads to reduce NO$_3^-$ to NO$_2^-$. The method is similar to that of the “Nitrate Reductor”. 0.5g of cadmium (Cd) beads were washed in 2 aliquots of double deionised water. Then they were washed twice in 0.1 M hydrochloric acid (HCl), followed by 2 washes in 0.1M ammonium hydroxide (NH$_4$OH). Once the beads had been washed, 100 μl of NO$_3^-$ solution was added. This mixture was then agitated for 4 to 6 hours. After this the solution was removed and assayed for NO$_2^-$ concentration by the Griess reaction. Results from assays of freshly made standard solutions of NO$_2^-$ and NO$_3^-$ are shown in figure 3.5.

The graph shows the degree of reduction of NO$_3^-$ to NO$_2^-$ by the Cd bead method. Although there was a strong linear relationship for the conversion of NO$_3^-$ to NO$_2^-$ ($R^2 = 0.987$), the slope of the trendline was much less than that of the NO$_2^-$ standard curve. This suggested that not all the NO$_3^-$ was being catalysed to NO$_2^-$, or that a component of the reduction process (or contaminant of the Cd beads) was interfering with the subsequent fluorometry. A further experiment determining fluorometric intensity for a range of standard solutions suggested that there may be some interference but that this was more manifest with NO$_3^-$ samples. Standard solutions of NO$_2^-$ and NO$_3^-$ were incubated overnight with the Cd beads, and the NO$_2^-$ concentrations were measured by fluorometry. A fresh standard solution of NO$_2^-$ was also measured at the same time. The results are shown in figure 3.6. Compared with the fresh solution of NO$_2^-$, both samples incubated overnight demonstrated less fluorometry. The effect was most marked in the NO$_3^-$ standards. It appeared that the Cd beads were not able to reliably reduce NO$_3^-$ to NO$_2^-$. 

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Figure 3.5: Graph to show conversion of nitrate to nitrite by the Biogenesis cadmium bead kit, as measured by the fluorometric assay. Nitrite (control) results are shown as open diamonds (solid trendline); nitrate results (after reduction to nitrite by the Cd beads) are shown as open circles (dashed trendline). $R^2$ values and equations are shown adjacent to their respective trend lines.
Figure 3.6: Graph showing fluorometric intensity of standard solutions of nitrite and nitrate that were incubated with cadmium beads overnight, and compared with fluorometric intensity of a fresh solution of nitrite. The key to the symbols and trendline styles are shown on the chart.
3.1.4.1c HPLC - Cadmium Column Reduction of Nitrate

This technique also utilises the reducing properties of cadmium, and was first described by Green (Green 1982). The basic principle is that a column packed with copper coated cadmium is inserted into a high-pressure liquid chromatography circuit, as shown in figure 3.7.

The column (Alltech direct connect refillable column, Deerfield, IL USA) was attached to the circuit by means of 1/8th to 1/16th inch fritted reducing unions. The HPLC circuit was run by means of a Waters 600E controller. The flow rate was set at 0.1 mls/minute.

An initial experiment was performed with a NO$_2^-$ standard to establish the length of time for passage of sample. It was determined that the sample passed through the system by 2 minutes (figure 3.8).

After passage through the circuit, NO$_2^-$ concentration was determined by the Griess reaction. The samples were collected for 2 minutes after injection into the circuit. Between sample injections, double deionised water, and then NH$_4$Cl buffer was pumped through the circuit to clean it and maintain the Cd column.

The column was shown not to affect Griess reaction colourimetry, nor fluorometry. The next experiment was to determine the catalytic ability of the column. Both Griess reaction and fluorometry were tested. Data from the fluorometric assay is shown in figure 3.9. As can be seen from these results, the passage of NO$_3^-$ through the column resulted in fluorometry that was almost identical to that of fresh NO$_2^-$ . The fluorometric intensity of the 200 µM standard was almost identical, and there was no background contamination of then fluorometric reaction, as was seen following the Cd bead process.
3.1.4.2 Enzymatic reduction of nitrate

Nitrate may be enzymatically reduced to nitrite by nitrate reductases. The procedure was described by Wu (Wu 1992). He reported an efficiency of conversion of 95%. The method involves the use of a purified Aspergillus species nitrate reductase. The reduction catalysed by this enzyme requires NADPH, and FAD as co factors. It can be summarised as:

\[
\text{NO}_3^- + \text{NADPH} + \text{H}^+ \rightarrow \text{NO}_2^- + \text{NADP}' + \text{H}_2\text{O}
\]

Verdon (Verdon 1995) reported a lesser degree of conversion efficiency, and also that NADP' appeared to be interfering with the Griess reaction. He advocated using glucose–6-phosphate dehydrogenase to reduce the NADP' back to NADPH. This additional step appeared to reduce the interference. Using the Cayman Chemicals kit, this problem was not observed with the Griess reaction (figure 3.10)
Figure 3.7: Schematic diagram of the HPLC circuit used. Ammonium chloride (NH$_4$Cl) buffer is pumped through the system at a continuous rate. A known amount of sample is introduced via the insertion port. The sample passes over the HPLC column which is packed with copper coated cadmium beads. During this passage, the nitrate is reduced to nitrite. Previous experiments dictated that sample passage and distal recovery took two minutes. Samples were recovered and then stored on ice until sufficient were recovered to be analysed by the fluorometric method of nitrite quantitation.
Figure 3.8: Graph showing the time for all of 20 microlitres of a 200 micromolar standard nitrite solution to pass through the high performance liquid chromatography pump, when set at a speed of 0.1 ml/minute. ° denotes the colourimetric intensity of a water-filled blank well.
It should be noted that the assay only gives a 5-fold increase over the range of standard concentrations. Similar problems were observed during this research, with fluorometry. Initially, I used a commercially available kit (Cayman Chemicals) for nitrate reduction. This kit was identical to the one available from Cayman Chemicals for the Griess assay of NO$_2^-$ and NO$_3^-$; except that the assay is performed using fluorometry and not Griess colourimetry. Reduction of NO$_3^-$ was by nitrate reductase and a cofactor solution. The manufacturers would not divulge information as to the exact composition of the enzyme and co-factor solutions. Since they referred to Misko (Misko 1993) it is assumed that the cofactor solution contained NADPH and FAD. The kit gave poor NO$_3^-$ conversion at first. An attempt was made to determine whether the interference was coming from the NADPH, the FAD, or the nitrate reductase, or a combination of these cofactors. The results are shown in figure 3.11.

A number of modifications were attempted in order to improve the signal, and/or decrease the background fluorometry for NO$_3^-$ enzymatic conversion. It should be noted that during this period of modification of the kit method, it was necessary to use a different fluorometer because the original needed to be returned to the manufacturer for servicing (Dynatech Microfluor, Dynatech Industries Inc, Chantilly, VA, USA).

Previously, Verdon (Verdon 1995) has described similar problems with interference from NADP following reduction of NO$_3^-$ to NO$_2^-$. They described a technique whereby lower initial concentrations of NADPH were used and then were regenerated from NADP by G-6-P and LDH. I did not find this method to provide reliable reduction of NO$_3^-$ to NO$_2^-$. Likewise attempts to find optimum concentration of NADPH which promoted reduction of NO3 but did not interfere in the fluorescence were unsuccessful.
Figure 3.9: Fluorometric intensity for nitrite and nitrate following passage through the Cd filled microcolumn. Open diamonds and a solid trendline represent nitrite data. Open circles and a dashed trendline represent nitrate data. $R^2$ values and trendline equations are adjacent to their respective trendlines.
Figure 3.10: Graph to show the conversion of nitrate to nitrite by the Cayman Chemicals nitrate reductase kit. Crosses and the solid trendline represent nitrate data, open circles and the dashed trendline represent the nitrite data. Equations and $R^2$ values are adjacent to trendlines. Note that the initial concentrations were lower than previously used, and also only 40ul of sample was used.
It was eventually possible to reduce background fluorescence by the following method:

1. Standards solutions were prepared with double deionised water, in decreasing concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0 μM).
2. 20 μl of standard solution were added to 50μl of buffer.
3. 10 μl of cofactor was added and the plate gently agitated.
4. 10μl of nitrate reductase was added and the plate gently agitated.
5. The plate was incubated for 2 hours at 37° C.
6. 20 μl of the post incubation mixture (enzyme, cofactor, standard and buffer) was transferred to another 96 well plate, and made up to 100μl with buffer.
7. 20μl DAN was added to this second plate.
8. 10 minutes incubation in the dark
9. 10 μl 2.8M NaOH
10. Plate read by fluorometer.

A sample NO₃⁻ standard curve is shown in figure 3.12.

By the technique of transferring a fraction (20μl) of the original reduced nitrate/buffer solution, I speculate that the concentration of NADPH and/or FAD was reduced sufficiently low enough to not interfere with the fluorescent reaction.

Having modified the nitrate reductase method to work for fluorometric analysis of NO₂⁻ in the above manner, the technique was used to determine the concentration of NO₂⁻ and NO₃⁻ in samples of BAL collected from infants in the CLD study. The results of this assay are presented in Chapter 4.
3.2 Summary

Of the different methods for reduction of nitrate to nitrite assessed, most were either not amenable to use with many samples (e.g. the use of the “nitrate reductor” [World Precision Instruments], or were not robust enough for adequate conversion to nitrite without interference to the quantitation process (nitrate reductase in commercial kit form).

The preferred methods of reduction of nitrate was via the use of copper coated cadmium bead in the HPLC column, and via the use of nitrate reductase, and transfer of a post reduction aliquot for fluorometry. The HPLC method was technically very simple, and enabled a large number of samples to be processed. Furthermore, washing the circuit through with ammonium chloride buffer ensured that the cadmium catalyst was constantly being regenerated. Unfortunately, the fluorometer was not adjacent to the HPLC circuit, and this limited the number of samples that could be processed at any time. Analysis of the samples for this thesis was performed done using the modified enzymatic reduction of nitrate (described in section 3.1.3.2), and then the fluorometric method of nitrite quantitation. Assessment if this technique showed it to provide excellent conversion of nitrate to nitrite without interference from cofactors. It was a time consuming technique and so analysis of samples in duplicate took approximately three hours for forty-eight samples. In contrast the HPLC technique took two hours for the same number of samples. It is suggested that the HPLC column method of conversion of $\text{NO}_3^-$ to $\text{NO}_2^-$ should be considered for future experiments. However, the proviso is added that there must be a fluorometer present within the laboratory.
**Figure 3.11**: Graph to show effect upon nitrite fluorescence from combinations of cofactors used in the reduction of nitrate. The key to which data points and trendlines represent which data are shown in the graph.
Figure 3.12: Graph showing standard curve for nitrate, after reduction using normal reagent volumes, and subsequent transfer of 20 µl of reduced mixture for reaction with DAN. Trendline equation and $R^2$ values are adjacent to trend line.
Chapter 4

Production of Nitric Oxide in Chronic Lung Disease of Prematurity
4.1 Background

Since pulmonary inflammation is associated with the development of CLD, and since pro-inflamatory cytokines increase the expression of iNOS, I postulated that infants who developed CLD would have increased production of endogenous NO. To assess the production of endogenous NO, I determined the concentration of nitrate and nitrite in BALF obtained from infants who had not received inhaled NO, and who did and did not develop CLD.

4.2 Methods

4.2.1 Patient Groups

Newborn infants requiring mechanical ventilation were recruited to the study. Three groups of infants were studied:

(a) **Chronic Lung Disease Group**: infants who were initially ventilated for respiratory distress syndrome (RDS) and who were oxygen dependent and had chest radiographic changes at 28 days of age (Bancalari 1979).

(b) **Respiratory Distress Syndrome Group**: infants who were ventilated for RDS, but who were nursed in air, and had a normal chest x-ray by 28 days of age.

(c) **Control Group**: infants who required ventilation for non-respiratory reasons and required an FiO₂ of less than 0.28.

Infants who were diagnosed as having persistent pulmonary hypertension of the newborn by echocardiography or who received inhaled nitric oxide therapy were excluded from the study. Infants of mothers who had infection or prolonged rupture of membranes (of greater than 48 hours) were also excluded as were infants with infection at any time verified by positive blood or endotracheal secretion culture. Informed consent was obtained from the parents and the
study was approved by the Local Research Ethics Committee.

4.2.2 Bronchoalveolar Lavage

Bronchoalveolar lavage was performed at times during the day when clinically necessary endotracheal tube suction was required. The frequency was as previously described in Chapter 2. Such intermittent sampling was performed until extubation or 28 days of age. The fluid obtained was centrifuged within 10 minutes of collection at 500 g for 10 min and the supernatant stored at -70°C for subsequent analysis of nitrite and nitrate.

4.2.2.1 Use of dilutional correction

As stated in Chapter 1 (v.s.), the use of a correction factor for dilutional effects is not standardised. Various molecules have been used as a denominator in calculating the proportion of ELF present in a recovered sample. ELF protein may be adversely affected by the protein leak seen in RDS/CLD. Use of secretory IgA is hindered by a paucity of knowledge of changes in its concentration during the last trimester (or its post-conceptual age equivalent in preterm infants), which would allow greater understanding of its expected serum concentration at a given gestational age. Urea dilution is thought to be affected by the dwell time of the saline after instillation. It is unknown what difference even a few seconds will make to the eventual urea concentration in a sample.

A study by Reise (Reise 1997) described glutathione concentration in BALF from preterm infants. The study also reported the observation that qualitative differences between diagnostic groups were maintained, whether urea dilution was used or not. In the studies in this thesis, the BALF was used for descriptive analysis of differences in concentration of antioxidants, and NO products, between diagnostic groups. Reise’s observations suggest that use of dilutional correction is less important in this context, in contrast to the use of
BALF analysis as part of the process to make a diagnosis. If used for diagnostic purposes, accurate values for constituent concentration are of greater importance.

Due to these unknown variables, the European Respiratory Society Task Force Recommendations (de Blic 2000) advocated that absolute values be recorded, and analysed. The studies presented in this thesis have followed that advice.

4.2.3 Assay Methods

4.2.3.1 Nitrite Assay

The samples were thawed on ice to prevent degradation of nitrite and were centrifuged in 12 kDa molecular weight cut-off ultrafiltration microcentrifuge tubes (Whatman, Maidstone) at 12,000 rpm for 35 minutes to remove proteins which may interfere with the assay. The concentration of nitrite was determined using the Fluorometric assay described in Chapter 3. The limit of detection for this assay was 0.2 μM nitrite.

4.2.3.2 Nitrate assay

Nitrate was measured as described in Chapter 3. The limit of detection was also 0.2 μM.

4.2.4 Statistical methods

The median (± range) is shown for gestation and birth weight of the infants studied. Because of skewed data, the mean of the log transformed data (±SEM) for the concentration of nitrite and nitrate was calculated. Groups were compared by the non-parametric test Kruskal-Wallis and paired groups were compared by Mann-Whitney. A p value of <0.05 was considered significant.
4.3 Results

4.3.1 Patient characteristics

The patient characteristics are shown in Table 4.1. One hundred and thirty six lavages were performed on 37 infants receiving mechanical respiratory support. 12 infants (5 female, 7 male) developed CLD, 18 had acute RDS (8 female, 10 male) and 7 infants (1 female, 6 male) were ventilated for non-respiratory reasons (4 for abdominal surgery, 1 for tracheo-oesophageal fistula with oesophageal atresia, 1 for mild asphyxia at birth and 1 for apnoea). Antenatal dexamethasone had been given to 11 of 12 mothers in the CLD group, 11 out of 18 in the RDS group and 1 of 7 in the control group. The birth weight and gestational age of the CLD group was significantly lower than those of the RDS or Control group (birth weight: CLD vs. RDS p<0.05, CLD vs. Control p<0.05; gestational age: CLD vs. RDS p<0.05, CLD vs. Control p<0.05).

Surfactant (Survanta, Abbott Laboratories, Abingdon, Oxon.) was given to all the infants in both the CLD and RDS groups but none was given to infants in the control group. The first bronchoalveolar lavage was performed before the first dose of surfactant was administered. There were no differences between the CLD and RDS groups on day 1 for maximum peak inspiratory pressure, maximum FiO₂, mean airway pressure or oxygenation index (Table 4.1). As expected, the control group had a significantly lower maximum FiO₂ and mean airway pressure (p<0.05) than the other two groups.

4.3.2 Concentration of BAL nitrate

The concentration of nitrate in BALF for all three groups during the first week of life was between 25 and 30μmol/l with little differences seen between the groups (figure 4.1). By 14 days of age, the concentration of nitrate in lavage fluid had decreased in the RDS and control groups to 14.2 μmol/l and 5.5μmol/l respectively. In contrast, it remained at
<table>
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<th>RDS</th>
<th>Control</th>
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<tr>
<td>Number</td>
<td>12</td>
<td>18</td>
<td>7</td>
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<tr>
<td>Female: male</td>
<td>5:7</td>
<td>8:10</td>
<td>1:6</td>
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<td>2230</td>
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<td></td>
<td>(630-1070)</td>
<td>(970-3480)</td>
<td>(2180-2970)</td>
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<tr>
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<td>29.5</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>(23-28)</td>
<td>(27-39)</td>
<td>(31-39)</td>
</tr>
<tr>
<td>Antenatal dexamethasone</td>
<td>11</td>
<td>11</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(92%)</td>
<td>(61%)</td>
<td>(14%)</td>
</tr>
<tr>
<td>Surfactant administration</td>
<td>12</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(100%)</td>
<td>(100%)</td>
<td>(0%)</td>
</tr>
<tr>
<td>Day 1 maximum peak inspiratory pressure /cm H₂O</td>
<td>18</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>(14-36)</td>
<td>(13-22)</td>
<td>(15-20)</td>
</tr>
<tr>
<td>Day 1 maximum FiO₂</td>
<td>0.46</td>
<td>0.35</td>
<td>0.25</td>
</tr>
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<td>(0.24-1.0)</td>
<td>(0.25-0.76)</td>
<td>(0.21-0.31)</td>
</tr>
<tr>
<td>Day 1 maximum mean airway pressure /cm H₂O</td>
<td>8.5</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>(5-18)</td>
<td>(5-13)</td>
<td>(4-8)</td>
</tr>
<tr>
<td>Day 1 maximum oxygenation index</td>
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<td>7.9</td>
<td>2.4</td>
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<td>(1.2-14.2)</td>
<td>(2.2-3.6)</td>
</tr>
</tbody>
</table>

Table 4.1: Patient characteristics. Median values (± range) are shown. CLD chronic lung disease of prematurity; RDS respiratory distress syndrome. CLD group is the same as group in chapter 5. The RDS group is that of Chapter 5, plus an additional two patients. The Control group is that of Chapter 5, plus an additional patient.
approximate the same level in the CLD group until 28 days of age (31.3 μmol/l at day 14, p<0.05 when compared to the RDS group and p<0.05 with Control group).

### 4.3.3 Concentration of BAL nitrite

The mean concentration of nitrite in bronchoalveolar lavage fluid obtained from all three groups throughout the study period was less than 1.2 μmol/l with no differences noted between the groups (figure 4.2).

### 4.4 Discussion

My results have shown that nitrate concentration in BALF from all three groups was very similar during the first week of life. During the second week of life, nitrate concentration remained increased in the infants who progressed to CLD. In contrast, it decreased in the
RDS and control groups. Nitrite concentration in BALF was less discriminatory between the three groups being less than 1.2 μmol/l throughout the study period.

The similar concentration of nitrate in all three groups during the first week of life suggests that there is a common cause for its production independent of gestational age. In animal models, NO is thought to have a role in transition of the fetal circulation to the adult circulation (Abman 1990, Zellers 1991). Abman et al have shown in newborn lambs that inhibition of NO synthase by the L-arginine analogue nitro-L-arginine prevents the normal physiological decrease in pulmonary arterial resistance thus suggesting that NO may have a role in transition from fetal to adult circulation (Abman 1990). It is tempting to speculate that the nitrate I have detected reflects such a circulatory adaptation after birth in all groups. Further studies will be necessary to determine if this is indeed the case. There is evidence that the sensitivity of newborn piglet pulmonary arterial smooth muscle cells to NO increases after day 3 of life thus a lower concentration of NO is required for equivalent vasodilatory effect in the pulmonary circulation (Zellers 1991). This observation may explain the decrease in BALF nitrate that I have seen during the second week of life in the RDS and control infants. The persistence of BALF nitrate in the CLD group after the first week may be due to a failure of change in sensitivity of smooth muscle cells to NO. This may result in maladaptation of the fetal circulation in this group of babies. Alternatively, the increase in BALF nitrate in the CLD infants during the second week of life may be due to increased pulmonary inflammation (Barnes 1995). As described in Chapter 1, pulmonary inflammation has shown by many groups to be increased in the lungs of infants who progress to CLD when compared to those who recover from RDS. It is likely that certain pro-inflammatory cytokines found in BALF in CLD, and which promote lung inflammation, will induce the expression of the inducible form NO synthase (iNOS) as has been shown in asthma (Barnes 1996) and sepsis (Carraway 1998).
Figure 4.1: Mean concentration (± SEM) of nitrate in bronchoalveolar lavage fluid of infants who developed chronic lung disease of prematurity (CLD), infants who had respiratory distress syndrome and were nursed in air by day 28 (RDS) and infants ventilated for non-respiratory reasons (Control). * p<0.05 when CLD compared to RDS and compared to Control infants.
Figure 4.2: Mean concentration (± SEM) of nitrite in bronchoalveolar lavage fluid of infants who developed chronic lung disease of prematurity (CLD), infants who had respiratory distress syndrome and were nursed in air by day 28 (RDS), and infants ventilated for non-respiratory reasons (Control).
The persistence of nitrate in the CLD group during the second week of life may be due to inflammation in the lungs of babies who develop CLD.

Further evidence for lung inflammation contributing to the production of NO is provided by animal models. Hyperoxia, which newborn preterm infants who progress to CLD are exposed to, has been shown to increase the production of nitrate and nitrite in the lungs of animals (Arkowitz 1997). In Arkowitz’s model, inhibition of NOS by L-NG-nitro-L-arginine methyl ester (L-NAME) worsened hyperoxic acute pulmonary injury thus suggesting that NO may protect the lungs from such injury. However, the mechanism of increased nitrate and nitrite production in BALF with hyperoxia is unclear since the expression of iNOS was not increased. It may be that there is a balance of beneficial against adverse effects of NO depending on other prevailing factors.

In the inflammatory environment of the lung of infants who develop CLD, endogenously produced NO, as shown by our study, is likely to react with reactive oxygen species to form pro-oxidant products such as peroxynitrite. Peroxynitrite is an unstable product which rapidly nitrates tyrosine residues to form nitrotyrosine (Beckman 1996). Nitrotyrosine has been used as a marker of the presence of peroxynitrite in many inflammatory disorders including rheumatoid arthritis (Kaur 1994), ARDS (Haddad 1994), and atherosclerosis (Morie 1997). Further preliminary studies by our group demonstrate that infants who develop CLD (and are not treated with inhaled NO) have increased nitrotyrosine in proteins obtained from BALF. This finding has been confirmed by others (Hallman 1998). More importantly, the formation of nitrotyrosine may interfere with phosphorylation of tyrosine residues in critical cell signalling pathways which result in cell proliferation and differentiation (Yu 1997).
As stated in Chapter 1 (v.s.), the use of a correction factor for dilutional effects is not standardised. Various molecules have been used as a denominator in calculating the proportion of ELF present in a recovered sample. ELF protein may be adversely affected by the protein leak seen in RDS/CLD. Use of secretory IgA is hindered by a paucity of knowledge of changes in its concentration during the last trimester (or its post-conceptional age equivalent in preterm infants), which would allow greater understanding of its expected serum concentration at a given gestational age. Urea dilution is thought to be affected by the dwell time of the saline after instillation. It is unknown what difference even a few seconds will make to the eventual urea concentration in a sample.

A study by Reise (Reise 1997) described glutathione concentration in BALF from preterm infants. The study also reported the observation that qualitative differences between diagnostic groups were maintained, whether urea dilution was used or not. In the studies in this thesis, the BALF was used for descriptive analysis of differences in concentration of antioxidants, and NO products, between diagnostic groups. Reise's observations suggest that use of dilutional correction is less important in this context, in contrast to the use of BALF analysis as part of the process to make a diagnosis. If used for diagnostic purposes, accurate values for constituent concentration are of greater importance.

Due to these unknown variables, the European Respiratory Society Task Force Recommendations (de Blic 2000) advocated that absolute values be recorded, and analysed. The studies presented in this thesis have followed that advice.

In conclusion, our data show that, during the first week of life, nitrate in BALF from ventilated infants was similar in all three groups that I have studied. This suggests that NO may play a role in the transition of the fetal circulation to the post-natal circulation. Pulmonary NO production appears to be persistent in infants who develop CLD, possibly reflecting maladaptation of the pulmonary circulation in these infants or reflecting on-
going pulmonary inflammation that has been noted in the lungs of infants who subsequently develop CLD.
Chapter 5

Non-enzymatic Antioxidants in Chronic Lung Disease of Prematurity
5.1 Background

As described in Chapter 1 (Section 1.10, and 1.11) there is evidence of intracellular antioxidant enzyme status depends largely on the stage of lung maturation (Frank 1987, Frank 1992). ROS arising from increased electron chain leakage are particularly likely to be harmful in very immature infants. ROS, arising from recruited neutrophils, are dealt with to a large extent, by extracellular antioxidants present in the ELF. The developmental profile or impact of oxidative lung disease on ELF antioxidant defences is unknown. To address these issues, I determined the concentration of ascorbate, urate and total glutathione in BALF and plasma obtained from infants who did, or did not develop CLD following treatment for neonatal RDS.

5.2 Methods

5.2.1 Patient Groups

The same patient groups used in Chapter 4 (Section 4.2.1) were also used for this study. Thirty-four newborn infants requiring mechanical ventilation were recruited to the study. These were divided into diagnostic groups for CLD, RDS and control as in Chapter 4, and from the criteria of Bancalari (Bancalari 1979).

Infants of mothers who had either infection or prolonged rupture of membranes (of greater than 48 hours) were excluded as were infants with sepsis verified by positive blood cultures at birth. Informed consent was obtained from the parents and the Hospital Ethics Committee approved the study.
5.2.2 Bronchoalveolar Lavage

Bronchoalveolar lavage, as described in Chapter 2 (Section 2.3) was performed twice weekly at the time of routine endotracheal tube suction until 28 days of age or extubation. Two aliquots of 1 ml per kg of N-saline were instilled via the catheter and immediately sucked back. The pooled aspirate was centrifuged at 500g for 10 minutes at room temperature and the resultant supernatant obtained. Of this, 300 μl was combined with 34μl of 16% metaphosphoric acid (MPA) for the subsequent measurement of ascorbate and urate. The remainder of the sample was stored at -70°C and subsequently used to estimate total glutathione and protein concentrations.

5.2.3 Collection of blood samples

Plasma samples were taken for ascorbate and urate analysis at the time of routine clinical phlebotomy usually within 4 hours of the bronchoalveolar lavage. As with lavage samples, the plasma aliquot for urate and ascorbate estimation were prepared in the presence of MPA and stored at -70°C for up to 6 weeks before analysis.

5.2.4 Urate and ascorbate determination

The HPLC determination of urate and ascorbate was based on the method of Iriyama and colleagues (Iriyama 1984). The assays were performed by Professor Kelly’s group at St Thomas’ Hospital, London. 100 μl HPLC-grade heptane was added to each sample and after vigorous mixing for 40 seconds, the samples were centrifuged at 13 000g for 5 minutes. The resultant lower layer was removed and transferred to a 1ml HPLC vial. Aliquots of 20μl were injected using a Gilson 231 autosampler. A 10 x 250mm, 5-μm C18 column was eluted with a 0.2 mol/L K₂HPO₄-H₃PO₄ running buffer, pH 2.1, containing 0.25 mmol/L octanesulfonic acid at a flow rate of 1.0 ml/min. An EG&G
electrochemical detector (Jones Chromatography, Hengoed, Wales) was used for
detection, with E set at 810 mV, a time constant of 5s, a cathodic output and sensitivity
of 100nA (BALF) or 200-500nA (plasma). Minimum detection was 0.01µmol/L for both
ascorbate and urate. Both intra- and inter-assay variation of plasma and BALF fluid
sample ascorbate and urate concentrations were less than 5%.

5.2.5 Glutathione determination

Total glutathione concentration was determined using the enzyme recycling method
described by Tietze (Teitze 1969) adapted for use with a microplate reader. Standards
containing 0 - 165 pmol/50 µl oxidised glutathione, equivalent to 0 - 330 pmol/50µl
glutathione were prepared in 150 mmol/l NaCl, 1 mmol/L EDTA, pH 7.5. Fifty µl of
standard or sample were then transferred into micro-titre plate wells. 100 µl of reaction
mixture were added to each well to give a final concentration in each well of 0.15
mmol/L DTNB (5,5'-dithiobis- [2-nitrobenzoic acid]), 0.2 mmol/L NADPH and 1U of
glutathione reductase. Immediately after addition of this reaction mixture, the microtitre
plate was transferred to a plate reader (EL340, Biokinetics Reader) for analysis. The rate
of TMB formation was followed by the rate of change of absorbance at 405 nm over a 2-
minute period at 30°C. The minimum detection limit for total glutathione was 0.04
µmol/L. The intra- and inter-assay variations were less than 5% for BALF glutathione
measurements.

5.2.6 Protein determination

Protein was measured using the biocinchoninic acid method (Smith 1985) adapted for
use on a microplate reader. Minimum detection was 0.02 mg protein /ml.
5.3 Statistical methods

The median (± range) are shown for gestation and birth weight of the infants studied. The mean (± 95% confidence intervals) of the log-transformed data is shown for ascorbate, urate, glutathione and protein concentrations. Comparisons between groups were made using ANOVA and paired groups were compared by t-tests. A p<0.05 was considered significant.

5.4 Results

5.4.1 Patient characteristics

The patient characteristics are shown in Table 5.1. One hundred and forty one lavages were collected from 34 infants receiving mechanical respiratory support. Twenty-eight infants developed RDS: 12 (5 female, 7 male) progressed to CLD and 16 (8 female, 8 male) were nursed in air by 28 days of age. Six infants (2 female, 4 male) who were ventilated for non-respiratory reasons and who required <28% oxygen were also studied: 4 who required surgery, 1 who had birth asphyxia but was ventilated in air and 1 infant who required respiratory support for apnoea of prematurity. Antenatal dexamethasone had been given to 11 of 12 mothers in the CLD group, to 10 of the 16 mothers in the RDS group, and to 1 of 6 in the control group. All infants in the CLD and RDS groups received surfactant (Survanta, Abbott Laboratories). The initial lavage was obtained before the first dose of surfactant had been administered. Significant differences were noted between the 3 groups for birth weight and gestational age; CLD vs. RDS (birth weight p<0.01 and gestational age p<0.01), CLD vs. Control (BW p<0.01, gest p<0.01) and RDS vs. Control (BW p<0.01, gestation p<0.01). Analysis of day 1 maximum peak inspiratory pressure, mean airway pressure showed no statistically significant difference between the groups.
<table>
<thead>
<tr>
<th></th>
<th><strong>CLD</strong></th>
<th><strong>RDS</strong></th>
<th><strong>Control</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>12</td>
<td>16</td>
<td>6</td>
</tr>
<tr>
<td><strong>Female: male</strong></td>
<td>5:7</td>
<td>7:9</td>
<td>1:5</td>
</tr>
<tr>
<td><strong>Birth weight (g)</strong></td>
<td>780</td>
<td>1850</td>
<td>2180</td>
</tr>
<tr>
<td></td>
<td>(630-1070)</td>
<td>(840-4160)</td>
<td>(1100-2860)</td>
</tr>
<tr>
<td><strong>Gestation (weeks)</strong></td>
<td>26</td>
<td>31</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>(23-28)</td>
<td>(26-39)</td>
<td>(32-38)</td>
</tr>
<tr>
<td><strong>Antenatal dexamethasone</strong></td>
<td>11</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(92%)</td>
<td>(63%)</td>
<td>(16%)</td>
</tr>
<tr>
<td><strong>Surfactant administration</strong></td>
<td>12</td>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(100%)</td>
<td>(100%)</td>
<td>(0%)</td>
</tr>
<tr>
<td><strong>Day 1 maximum peak inspiratory pressure (cm H₂O)</strong></td>
<td>18</td>
<td>19</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>(14-36)</td>
<td>(13-22)</td>
<td>(15-20)</td>
</tr>
<tr>
<td><strong>Day 1 maximum FiO₂</strong> *</td>
<td>0.46</td>
<td>0.41</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>(0.27-1.0)</td>
<td>(0.23-0.96)</td>
<td>(0.21-0.28)</td>
</tr>
<tr>
<td><strong>Day 1 maximum mean airway pressure (cm H₂O)</strong></td>
<td>8.5</td>
<td>9</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>(5-18)</td>
<td>(5-13)</td>
<td>(4-8)</td>
</tr>
</tbody>
</table>

**Table 5.1:** Patient characteristics. Values shown are median (± range). CLD: chronic lung disease of prematurity. RDS: infants who received ventilatory support but who were fully recovered by 28 days of age. Control: infants ventilated for non-respiratory reasons. *p<0.05 CLD vs. RDS for maximum FiO₂ in first 24 hours. Maximum FiO₂ in the first 24 hours was significantly higher in the CLD group (p<0.05). CLD, RDS and control group patients are all included in data for Chapter 4.
<table>
<thead>
<tr>
<th>Day</th>
<th>Chronic Lung Disease of Prematurity</th>
<th>Respiratory Distress Syndrome</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>18</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>12</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>11</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>10</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>14</td>
<td>10</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>21</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>28</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Table 5.2: Table to show the number of patients at each time point shown.*
5.4.2 Nutrition Status

Full enteral milk feeds were reached at a median age of 21 days (range 9 - 28d) in the CLD group, 7 days (4 - 14 d) in the RDS group and 7 days (4 - 18 d) in the Control group. All infants received intravenous fluid support whilst full enteral feeds were established. Infants in the CLD group received total parenteral nutrition prior to commencing enteral feeds, whereas those in the other groups were given dextrose solutions with added electrolytes.

5.4.3 Bronchoalveolar lavage fluid

In accordance with the recommendations of the European Respiratory Society Taskforce on BAL in children (de Blic 2000), I have not corrected any of these measurements for dilution of ELF during the lavage procedure.

5.4.4 Ascorbate concentration

The mean concentration (95% confidence intervals) of ascorbate in BALF of infants who developed CLD was 3.8 (1.3-6.3) μmol/l on day 1 and 3.4 (0.1-6.7) on day 4. Thereafter BALF ascorbate fell in these infants to 1.2 (0-2.7) (day 7) and 1.0 (0.9-3.1) (day 10) before increasing to 8.4 (0-22.2) on day 21 (figure 5.1). In RDS infants the mean ascorbate concentration in BALF was 5.6 (3.4-7.6) μmol/l (day 1) and 6.3 (1.9-10.6) μmol/l (day 4). By day 7, BALF ascorbate concentration decreased to 1.3 (0-3.3) μmol/l but then subsequently rose to 9.0 (0-67.1) μmol/l by day 14. The difference between the mean ascorbate concentrations in the CLD and RDS groups was only statistically significant on day 14 (p<0.05). BALF ascorbate levels in control infants were very similar to that seen in RDS infants: 3.7 (0-11.1)(day 1), 5.5 (0-15) (day 4), 1.6 (0-20) (day 7) and 6.1 (0-87) (day 14).
Plasma ascorbate concentration was similar in all three groups in the first week of life (figure 5.2). The day 1 concentrations, 61.6 (12.49-100.81)μmol/l (CLD), 79.2 (54.06-104.11) μmol/l (RDS), 46.0 (5.46-103.77)μmol/l (Control) decreased similarly in all groups until day 7. From day 7, RDS infants’ plasma ascorbate concentration increased to 60.1 (29.71-91.63) μmol/l (day 14) whereas the CLD infants ascorbate concentration remained at 20.5 (12.1-28.12)μmol/l (p<0.05). There was a delayed increase in the CLD infants’ plasma ascorbate concentration to 116.2 (0-232.6) μmol/l by day 28.

The ratio of BALF and plasma ascorbate was studied for the RDS and CLD infants to determine whether those infants who recover from the neonatal RDS have an increased ability to concentrate ascorbate in their ELF (figure 5.3). The ratio of BALF to plasma ascorbate showed a significant difference on day 4 between the CLD and RDS infants (CLD: 0.057 ± 0.022, RDS: 0.21 ± 0.051; p<0.05).
Figure 5.1: Mean concentration of ascorbate in bronchoalveolar lavage fluid in ventilated infants who develop chronic lung disease of prematurity (CLD), those with respiratory distress syndrome (RDS) and those who require ventilation for non-respiratory causes (Control). Data has been log transformed. Concentrations are in μmol/l. * RDS vs. CLD, p<0.05.
Figure 5.2: Mean concentration of ascorbate (± SEM) in plasma in ventilated infants who develop chronic lung disease of prematurity (CLD), those with respiratory distress syndrome (RDS) and those who require ventilation for non-respiratory causes (Control). Concentrations are in $\mu$mol/l.
Figure 5.3: Ratio of BALF to plasma ascorbate in the CLD and RDS infants. * RDS vs. CLD, p<0.05.
5.4.5 Urate concentration

At birth, the mean concentration of urate in BALF was similar in all three groups (figure 5.4). Initial concentrations were 42.1 (28.7-54.6)μmol/l (CLD), 40.8 (31.7-49.3)μmol/l (RDS) and 37.7 (14.9-51.9)μmol/l (Control infants). Thereafter BALF urate concentration decreased over the first week to approximately 20 μmol/L in all three groups. By day 14, BALF urate in control infants had fallen further to 11.7 (0-32.24) but remained unchanged in CLD and RDS infants. At day 28, BALF urate in the CLD infants was 15.6 (7.67-15.04).

Plasma urate concentrations were approximately equal at birth (332-471μmol/l) and decreased in all three groups at approximately the same rate (figure 5.5). The plasma urate concentration was approximately ten times greater than the BALF urate concentration. The BALF to plasma ratio rose over time but did not show any significant differences between the groups (figure 5.6).
Figure 5.4: Mean concentration of urate in bronchoalveolar lavage fluid in ventilated infants who develop chronic lung disease of prematurity (CLD), those with respiratory distress syndrome (RDS) and those who require ventilation for non-respiratory causes (Control). Data has been log transformed. Concentrations are in μmol/l.
Figure 5.5: Mean concentration of urate (±SEM) in plasma in ventilated infants who develop chronic lung disease of prematurity (CLD), those with respiratory distress syndrome (RDS) and those who require ventilation for non-respiratory causes (Control). Concentrations are in μmol/l.
Figure 5.6: Ratio of BALF to plasma urate in the CLD and the RDS infants.
5.4.6 Glutathione concentration

The initial, mean concentrations of total glutathione were 3.2 (0.06-6.4) (CLD), 3.6 (0.16-7.08) (RDS) and 1.0 (0-2.97)μmol/l (Control). Thereafter, the concentrations of BALF total glutathione fell in the CLD and RDS infants to control levels (figure 5.7). In the CLD infants, BALF total glutathione concentration ranged between 0.8 and 2.4μmol/l over the 28 days. No statistically significant differences were observed at any time for mean BALF total glutathione.

5.4.7 Protein concentration

The initial, mean protein concentration in BALF was 1.7 ± 0.4 mg/l in the CLD infants, 2.1±0.5 mg/l in RDS infants and 2.1±0.5 mg/l in the control infants (figure 5.8). Although an increase in protein concentration was seen in the CLD group at day 7 to 4.0 ± 1.1 mg/l, this was not significant when compared to the RDS group. Subsequent mean concentrations were between 1.6 and 2.6 mg/l in the CLD infants and between 1.1 and 2.8 mg/l in the RDS and Control groups. There were no statistically significant differences between the groups at any time.
Figure 5.7: Mean concentration of total glutathione in bronchoalveolar lavage fluid in ventilated infants who develop chronic lung disease of prematurity (CLD), those with respiratory distress syndrome (RDS) and those who require ventilation for non-respiratory causes (Control). Data has been log transformed. Concentrations are in μmol/l.
Figure 5.8: Mean concentration of protein in bronchoalveolar lavage fluid in ventilated infants who develop chronic lung disease of prematurity (CLD), those with respiratory distress syndrome (RDS) and those who require ventilation for non-respiratory causes (Control). Concentrations are in mg/ml.
5.5 Discussion

5.5.1 Ascorbate results

Plasma ascorbate concentration was similar in all three groups of infants on day 1. Other studies have shown a similar initial concentration (Moison 1997, Miller 1993, Silvers 1994, van Zoerren-Grobben 1994) and then a subsequent decrease in plasma ascorbate with time (van Zoerren-Grobben 1994). Although a subsequent increase in plasma ascorbate concentration must occur, the time course of this event in RDS and CLD infants has not previously been reported. The results of the present study indicate that normal plasma ascorbate levels are achieved more rapidly in infants who do not subsequently develop CLD.

Ascorbate is not synthesised in mammalian cells and it is likely that BALF ascorbate level is related, at least in part, to the plasma ascorbate concentration. This in turn will be related to dietary intake. Such an association is supported by the parallel increases in BALF and plasma ascorbate during the second week in RDS infants and during the fourth in the CLD infants. Determination of the ratio of BALF: plasma ascorbate concentration revealed an increase on day 4 in RDS infants that did not occur in CLD infants (p<0.05). This finding suggests that infants who have RDS, but who do not progress to develop CLD, are able to increase their lung extra-cellular ascorbate levels, despite the plasma ascorbate decreasing at this time.

The mechanism by which the concentration of BALF ascorbate increases on day 4, in the face of a decreasing plasma ascorbate concentration, is currently unclear. An ascorbate transport mechanism has been identified in human placenta, which
transports ascorbate (as dehydroascorbate) against a concentration gradient (Rybakowski 1995). A similar transport pump may exist within the lung.

Alternatively, a process by which oxidised ascorbate is reduced back to ascorbate (May 1996), as has been described in erythrocytes in adults, may be deficient or immature in those infants who develop CLD.

The absolute concentration of ascorbate in BALF of infants who develop CLD has not been reported previously although the ratio of dehydroascorbate to ascorbate has been described in tracheal aspirate (Moison 1997) and plasma (Moison 1994, Ogihara 1998). In both studies, the ratio of dehydroascorbate to ascorbate in tracheal aspirate and plasma did not show an increase in the CLD infants when compared to the RDS group suggesting that ascorbate is not subject to greater oxidant stress in the CLD infants. These findings, coupled with those reported in the present study suggest that the supply of ascorbate to the lung extra-cellular compartment is limited in CLD infants.

That the differences in BALF ascorbate are due to just the dietary differences between the different groups is simplistic. Although full enteral feeds were reached later in the CLD group when compared to the RDS or Control groups, the infants who progressed to CLD had been commenced on parenteral nutrition by 48 hours of age. It is practice in the Neonatal Unit where the study was conducted to commence gut “priming” by 48 hours of age, unless a clinical contraindication exists (e.g. gut surgery or necrotising enterocolitis). Either expressed breast milk (which is preferred) or a modified cow’s milk preterm formula is introduced via a nasogastric tube at an initial rate of 0.5 – 1 ml of milk per hour. If this is tolerated for approximately 24 hours, the
volume is increased by 0.5 ml every 8-12 hours in infants <1,000g in weight or 1.0 ml every 6-8 hours in infants >1,000g as tolerated. As establishment of full enteral feeding often takes several days or even weeks, it is our policy to commence intravenous parenteral nutrition by 48 hours of age given via an indwelling fine silastic long line. The parenteral nutrition contains both fat- and water-soluble vitamins including ascorbate at 5 mg/kg of body weight. The parenteral nutrition is decreased as the enteral feeds increase. The intravenous nutrition contains sufficient ascorbate to prevent nutritional deficiency of this important vitamin in this group of babies. Data relating to the composition of human breast milk at differing post-conceptional ages is severely limited. In addition infants will have differing degrees of prematurity, severity of respiratory disease, tolerance or intolerance to feeds, and varying ratios of expressed milk to modified preterm formulae. All of these make further analysis of ascorbate absorption from the gut difficult to interpret. For these reasons only a simple guide to when full enteral feeds commenced has been given. It is acknowledged that there are limitations to the analysis of dietary intake in our study groups. It is possible that the infants who developed CLD had less mature transport systems to transfer ascorbate from the plasma to the lung or that there is increased consumption of this agent in the lungs of infants who develop CLD. Another explanation, as described above, may be an immature system to reduce oxidised ascorbate in this group of babies (May 1996).

The role of ascorbate in the lung is complex. Ascorbate scavenges superoxide radicals, hydroxyl radicals and hypochlorous acid, which are generated by activated neutrophils and macrophages. It may also mediate the interaction between neutrophils and endothelium seen in ARDS (Jonas 1993) and enhance regeneration of the vitamin
E (McCay 1985), which is often deficient in preterm babies (Kelly 1990). Ascorbate may also enhance the decomposition of S-nitrosothiols (nitric oxide adduct compounds with similar bioactivity) (Kashiba-Iwatsuki 1997) and may protect against oxidative damage to lipids (Frei 1998). It may also initiate collagen hydroxylation following acute lung injury. Failure of adequate resolution and repair of an injury may predispose the lung to persistent inflammatory cell infiltrate. Such a persistent infiltrate is seen in CLD. Given the plethora of beneficial effects of ascorbate, the increase in BAL fluid observed during the second week of life in RDS infants may provide important protection against the adverse effects of ROS by some of the mechanisms mentioned above. It therefore follows that a delayed increase in ascorbate in the CLD infants may expose them to more severe lung injury at an early point in life.

5.5.2 Urate results

Initial BALF urate concentrations were similar in all three groups (37-42 μmol/l). After the first week, BALF urate reached an approximate steady state concentration (15-20 μmol/l). These concentrations are 2-3 times greater than those in adult BALF (Kelly 1997, Slade 1993, Mudway 1996, Kelly 1996) even allowing for the smaller lavage volumes used in babies. Whether this is an adaptive response to the relative extrauterine hyperoxia that the newborn infant is exposed to after delivery from the intrauterine hypoxic environment is unclear. Plasma urate concentrations were similar in all three groups and in the range of those reported previously (Moison 1997, Miller 1993, van Zoeren-Grobben 1994, Lindeman 1989, Schrod 1997), including values for non-pregnant adults (Giles 1997) and pregnant women (Lind 1984). Since the ratio of BALF to plasma urate was similar throughout the study period in all three groups
it is likely that the BALF urate is derived from plasma by passive diffusion. These data are in variance with those reported by Schrod et al who suggested that low urate in tracheal fluid may predict the development of CLD (Schrod 1997). This may be due to the differences in the methods for obtaining lung lavage fluid since our technique is likely to reflect the bronchoalveolar milieu whereas the tracheal aspirates obtained by Schrod may reflect the upper airways only. Interestingly though, urate levels are thought to be generally highest in the upper airways (Mudway 1996).

5.5.3 Glutathione results
In contrast to Grigg et al (Grigg 1993), I did not observe a difference in BALF total glutathione levels between the CLD and non-CLD infants. The mean value of total glutathione in their infants with CLD was lower than those reported in our study even though our CLD infants were smaller in both birthweight and gestation. This may be due to the relatively small number of infants with CLD examined by Grigg et al, or due to improved nutrition or the routine use of surfactant in current neonatal practice. Grigg’s population were derived during the pre-surfactant era. Lavoie reported that red blood cell intracellular glutathione is dependent on gestational age and sex (Lavoie 1995). I did not however find a relationship between BALF glutathione concentration and sex or gestation (data not shown) nor was there a relationship with oxygenation index (OI) as was reported by Reise et al (Reise 1997).

5.5.4 Protein results
I was unable to show any significant differences for BALF protein in the three groups. This may be due to the small numbers that I have studied or due to the neonatal
respiratory distress syndrome being modified by the regular use of exogenous surfactant, antenatal corticosteroids or modern ventilatory strategies to reduce barotrauma. It was interesting to note that there were minimal differences between the groups for oxygen requirements or for oxygenation index, raising the speculation that CLD in our group may have been suffering from a different disease process from that described by Northway (Northway 1967).

5.5.5 Conclusions

In conclusion, my data shows that infants who develop CLD are unable increase their BALF ascorbate relative to their plasma concentration as seen in the RDS infants at day 4 and there was a delayed increase of ascorbate within the ELF when compared to both the RDS and Control groups. The exact mechanism for these observations can only be speculative. Immaturity of (a possible) pulmonary transport system for ascorbate, re-absorbtion of luminal ascorbate, or poor intake may all play a role in this observed difference. I was unable to find differences for total glutathione or urate in BALF from the three groups. These data suggest that ascorbate may play an important role in our population, in the prevention of CLD within the preterm lung.
Chapter 6

Lung Lavage

volumes recovered

and cell counts
6.1 Lung Lavage recovery volumes

A total of 333 non-bronchoscopic bronchoalveolar lavages were performed during this study. For analysis of samples at certain time points (e.g. day 1, day 4 etc) 138 samples were used. The initial volume of normal saline (0.9% saline) instilled was determined by the infants current weight. One ml per kg of 0.9% saline was instilled twice. The total volume of aspirate was measured using a previously calibrated universal container with a conical floor. No studies were undertaken to determine the accuracy of the markings, but there were intervals of approximately 0.5mm between the gradations for 250 microlitres, 500 microlitres, 750 microlitres and 1 millilitre. The table shows the statistical analysis of the volume of aspirate recovered when compared with the initial volume instilled.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Volume recovered (% of amount instilled)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>59.7</td>
</tr>
<tr>
<td>Median</td>
<td>57.7</td>
</tr>
<tr>
<td>Std deviation</td>
<td>19.2</td>
</tr>
<tr>
<td>Max</td>
<td>100</td>
</tr>
<tr>
<td>Min</td>
<td>8.9</td>
</tr>
<tr>
<td>5(^{th}) centile</td>
<td>27.8</td>
</tr>
<tr>
<td>95(^{th}) centile</td>
<td>93.75</td>
</tr>
</tbody>
</table>

Table 6.1 Table to show variation in lung lavage recovery volumes
6.2 Cell counts

6.2.1 Method

Following BAL as described in the original thesis submitted (v.s.), the recovered aspirate was centrifuged, and the supernatant fluid removed for future analysis. The remaining cell pellet was re-suspended in 0.9% saline. An initial cell count was made using a haemocytometer. From this count, it was determined how much of this suspension was required to make an adequate cytospin slide preparation.

Following preparation of the slides, one was stained using the Diff-Quik (Dade, Switzerland). A differential cell count was obtained later. Cells were categorised into epithelia, neutrophils and macrophages. The absolute concentration of macrophages or neutrophils was then calculated from these data.

6.2.2 Results

6.2.2.1 Total cell count

The total cell counts are shown in Figure 1. In the RDS group of infants the total cell count (SEM) increased from 258 (74) x 10⁴/ml on day one to 368 (107) x 10⁴/ml on day 4. After this, the total cell count decreased over the following 6 days to a basal count of ≤ 60 x 10⁴/ml on day 10 and day 14. A similar order of increase in total cell count was seen in the CLD group. This increase occurred six days after that in the RDS group. The maximal total cell count was 382 (180) x 10⁴/ml on day 10. The differences between the RDS and CLD infants at day 10 were statistically significant (p< 0.05).
6.2.2.2 Neutrophils

The absolute neutrophil count decreased over the study period in the RDS group. The counts in the CLD group rose over the first 10 days to a maximal count of 326 (180) x 10^4/ml. However, the difference between the CLD and RDS group counts was not statistically significant at this or any other time in the study period. The neutrophil count is shown in Figure 2.

6.2.2.3 Macrophages

The absolute macrophage count for both the RDS and CLD infants was generally of the same order of magnitude throughout the study period (≤56 x 10^4/ml). On day 4 however, there was a marked increase in the RDS group macrophage count to 176 (40) x 10^4/ml (p<0.05). By day 10 this had returned to the previous count values. On day 10 there was a slight increase in the CLD macrophage count 56 (26) x 10^4/ml, but this was not of any statistical significance. The macrophage counts are shown in Figure 3.

6.3 Discussion

The cell count data shows a statistically significant (p<0.05) increase in macrophage count in the RDS group on day 4. There was also an increase in neutrophil count in the CLD group on day 10. This difference did not reach statistical significance. The maximal difference in the macrophage counts occurred on day 4. This time point coincided with the previously described maximal difference in BALF: plasma ascorbate ratio. It is tempting to speculate that there is a causal link between these two findings, and that this observed association correlates with the resolution of lung
disease which defined the RDS group. It is well recognized that macrophages have a role in resolution of inflammation (Stites, 1997). Similarly, it is also well known that ascorbate plays a prominent role in wound healing, by initiating collagen hydroxylation. It is possible that the cell count and ascorbate data demonstrate evidence of a failure of healing of acute lung injury in those children who later develop CLD.

The studies described in this thesis were not devised to test this hypothesis, and therefore further experimental work, specifically designed to test this hypothesis, is necessary.

The neutrophil count does not show any statistically significant differences between the CLD and RDS groups. Neutrophils are a recognized source of NO production, although they are not the sole cellular source within the lung. Maintenance of NO production (resulting in c. 30 micromoles/litre nitrate) was seen in the CLD infants after day 10. Since it was not part of this set of experiments to establish the cellular source(s) of NO in the lung of infants with CLD, any speculative association between NO production and neutrophil count is merely speculation which my data can neither confirm nor refute.
Figure 6.1: Total cell counts from bronchoalveolar lavage fluid from infants with chronic lung disease of prematurity and respiratory distress syndrome. * p<0.05
Figure 6.2: Absolute neutrophil counts from bronchoalveolar lavage fluid from infants with chronic lung disease of prematurity and respiratory distress syndrome.
Figure 6.3: Absolute macrophage counts from bronchoalveolar lavage fluid from infants with chronic lung disease of prematurity and respiratory distress syndrome. * p<0.05
Chapter 7

Discussion
7. Discussion

7.1 Summary

This research has identified a number of novel findings. I have shown that:

i. The technique of non-bronchoscopic bronchoalveolar lavage (BAL) does not exacerbate the existing respiratory condition of the infant and did not result in increased oxygen-dependency. In addition, associated important outcomes including IVH and ROP were not increased. The lack of effect of the BAL procedure on the chest radiographs leads me to conclude that there is unlikely to be constant removal of surfactant by this technique.

ii. I noted clear differences in the production of products of NO in those infants who developed chronic respiratory failure i.e. CLD when compared to those who recovered from RDS or when compared to Control infants.

iii. I also noted differences in the BAL fluid and plasma ascorbate between the infants who developed CLD when compared to those who did not. I did, however, not find differences between the groups for glutathione, urate or total protein in BAL fluid.

In addition, I have developed several techniques to estimate NO products nitrite and nitrate in both plasma and BAL fluid. I applied the most appropriate technique to measure NO products in BAL fluid.

These findings are important contributions to research in infants and contribute further to the understanding of the pathogenesis of CLD in newborn infants. I shall discuss the importance of these findings below.
7.2 Serial Non-bronchoscopic Bronchoalveolar Lavage

Few previous studies have evaluated the adverse effects associated with the lung lavage procedure in newborn infants. Grigg et al reported transient oxygen desaturations and bradycardia during and immediately after the lavage procedure (Grigg 1992). It has been suggested that repeated BAL may “wash-out” surfactant and I proposed that if this was the case then one would note localised radiological changes in the lavaged group when compared to those infants who did not undergo any formal lavage. It was important to note that I did not demonstrate such changes on radiological grounds. There are a number of issues that need to be considered when interpreting this data. Since there was no previous data on such a study, I studied what I considered a reasonable number of infants. With the numbers that I have studied I was unable to demonstrate any differences between the groups nor did not the data suggest a trend towards adverse effects particularly on the chest radiographs. The scoring system we used was one developed for infants with CLD (Machonochie 1991), but which was not designed for comparison of specific areas of the lung. Currently there is no such scoring system that I am aware of, and I considered that the Machonochie radiographic scoring was the most suitable of those available. Development of a specific scoring system looking only at the lower lobes would have been ideal but the radiologists who were blinded as to the chest radiograph origins were unable to find any differences between the two groups.

There are a number of other factors to consider when interpreting my data. It is assumed that the catheter placement is in the right lower lobe but one can not be certain that this was the case. There is some evidence from a small number of observations that the technique I had adopted for the BAL procedure results in
repeated sampling of the right lower lobes. Mildner et al (Mildner 2001) noted that
the catheter sampled the right lower lobe on more mature newborn infants and both
Grigg (Grigg 1991) and Placzek (Placzek 1983) made similar observations that the
right lower lobe appeared to be sampled when the head is turned to the left prior to
BAL being performed. I had to use a historical group, which I recognise to be less
than ideal. Since the main aim of the study was to determine the concentration of
oxidants and anti-oxidants in BAL fluid, I opted to study a historical group that
consisted of infants born immediately prior to the group undergoing BAL. Although
my study was associated with a number of complicating factors, I believe that the data
suggests this oft-used technique should be considered safe to obtain lung lavage fluid
for research studies.

Even in an ideal world it would be difficult to assess if surfactant is “washed-out” by
performing repeated BAL. Estimation of components of surfactant e.g. SP-A or SP-D
or DPPC would not be useful as these may already be different at different gestations
or at different stages of lung disease. Even if we were able to obtain lung biopsies this
would not solve this question, as the abnormalities need to be identified in the airways
and not the lung interstitium. I believe that this question is extremely difficult to
answer definitively. My data provides some evidence to suggest that BAL is unlikely
to result in constant removal of surfactant from the lavaged areas of lung.

7.3 Products of Nitric Oxide

My findings in adapting a technique for small volumes of BALF are important. The
methodological developments allow others who with to assay nitrate and nitrite
concentration with a better understanding of the requirements to measure concentration of nitrate and nitrite. As described above (paragraph 1.9.3.2 [in amendments]) I found that commercially available kits were not sufficiently reliable to expect accurate reduction of nitrate to nitrite, and required sample volumes greater than 50 microlitres. I undertook a comparison of different methods of measuring nitrate and nitrite (the Griess reaction, and a fluorometric technique), and performed serial experiments to determine a method to minimise interference from the reduction co-factors in the final colourimetric or fluorometric assay. Of the various techniques I assessed (see Chapter 3), I concluded that the technique best suited to analysis of small volumes (<50 μl) of BALF was the modified enzymatic technique with estimation of nitrite by fluorometry. By developing these techniques I was able to gain an understanding of the basic principles that underlie a number of techniques of biochemistry including HPLC, colourimetry, fluorometry, processes of reduction (enzymatic, and cadmium catalysis).

This work is important for a number of reasons. Measurement of nitrite/nitrate has not been reported in BALF from preterm infants prior to this. A comparison of various techniques for reduction of nitrate in small BALF volumes (<50 microlitres) has not been reported before.

Application of this technique to the BAL samples revealed that the concentration of nitrate in BAL fluid was similar during the first week of life in all three groups studied. Again these findings have never been reported before. As described above, a number of studies have demonstrated beneficial effects from iNO in preterm infants. Other animal studies have shown that blockade of EDRF secretion (e.g. with nitro-L-arginine, an inhibitor of NO secretion) (Abman 1990) can prevent the normal postnatal decrease in pulmonary blood pressure in lambs. Similarly, Zellers (Zellers,
1991) showed that sodium nitroprusside, and NO (at concentrations of \(10^{-7}\) M), can cause arterial dilatation in newborn piglet pulmonary arteries. No previous work has shown evidence of NO secretion within the lung of infants. What this study did not show (and was not designed to show) was whether those infants who develop persistent pulmonary hypertension of the newborn (PPHN) have a disorder of endogenous NO production. Future research can use the techniques I have described to perform serial lavage on infants with PPHN, prior to receiving iNO therapy. This may demonstrate that nitrate/nitrite concentration is significantly lower in those who develop PPHN, than others who do not. Such an experiment would confirm the veracity of the postulated explanation for the efficacy of iNO, in these infants. For these reasons I consider the methodological developments presented for determining BALF nitrite/nitrate concentration to be of importance in allowing further understanding of the role of endogenous NO in the pathogenesis of PPHN.

The early similarity of the nitrate in BALF in the three groups is of interest. Whether the increased endogenous production of NO is due to vascular adaptation to extra-uterine life, or due to an inflammatory process can not be discerned from my studies, as I did not estimate pro-inflammatory agents. Previous evidence from Abman, and from Zellers suggest that this higher endogenous production of nitrate in the first week of life is likely to relate to mechanisms for the postnatal transition from fetal to adult circulation (Abman 1990, Zellers 1991). The presence of nitrate in BALF in the first week of life in infants who subsequently develop CLD is unlikely to be due to an intrinsic inflammatory process, as nitrate is also present in similar concentrations in BALF from those who did not develop CLD. These non-CLD infants have previously been shown to have far less inflammation within their BALF (Kotecha 1996, Kotecha 1996b, Kotecha 1995). Thus it is reasonable to speculate that the increased NO seen
in the first week of life is not due to an intrinsic inflammatory process within the lungs. An alternative source of the NO metabolites is from the uterine environment. Recent evidence suggests that NO plays a role in cervical ripening, and so nitrate and nitrite may be present in ingested/inhaled amniotic fluid (Vaisanen-Tommiska 2003). If NO does play a role in normal physiological circulatory transition, it is then possible that production of NO is deficient in infants who develop PPHN.

The later persistence of nitrate in the CLD group may be of significance, and I believe should form the basis of important future studies (see section 6.3.1 below).

I was unable to show any differences between the groups for BALF nitrite. This is likely to be due to either the instability of nitrite which readily oxidises to nitrate in the oxygen enriched environment of the infant receiving oxygen therapy (figure 7.1), or due to subsequent oxidation to nitrate even whilst in storage at -70°C. Other commonly encountered problems with studies such as ours include regular extubation of study infants as their clinical condition improves, the lack of a satisfactory marker for dilution of epithelial lining fluid and difficulty of obtaining “normal” data for preterm infants.

7.3.1 Peroxynitrite

Oxidation products of NO include nitrate and also peroxynitrite (ONOO⁻) (figure 7.1). Peroxynitrite is a very potent oxidant and in the hyperoxic pulmonary environment is likely to lead to exacerbation of existing lung injury. Peroxynitrite specifically converts tyrosine to form nitrotyrosine. This has major implications for cellular biochemistry as phosphorylation of tyrosine to form phosphotyrosine is important in
many cell signalling pathways that lead to cellular functions such as proliferation and differentiation. Cytokine binding will initiate a cascade of events that culminate in upregulation or downregulation of gene transcription (Force 1998). One such cascade is the mitogen-activated protein kinases (MAP kinases) pathway (figure 6.2). This pathway depends upon a series of enzyme activation steps; each enzyme activating the next one in the sequence. At a number of levels, activation of the enzymes is achieved by phosphorylation of specific tyrosine residues within the native protein (Force 1998). Several tyrosine pathways have been elucidated in humans: the extracellular signal-related kinase (ERK), the stress activated protein kinase/c-Jun-N-terminal kinase (SAPK/JNK), and the p38 cascade (Force 1998). If NO is converted to ONOO−, in the presence of oxygen, many tyrosine residues will undergo nitration to form nitrotyrosine. If nitration of critical tyrosine residues in cell signalling occurs, proliferation and differentiation may be adversely affected. It is equally possible that the ONOO− reacts to form other compounds. Recent interest has focussed upon the formation and role of S-nitrosothiols. It is speculated that these compounds act to ‘transport’ NO in a less volatile form from its site of secretion to sites of biological activity (Al-Sa’Doni 2001). One such S-nitrosothiol is S-nitrosoglutathione. It is unclear whether differences in the concentration of S-nitrosoglutathione, or other nitrosothiols, would explain the differences between Grigg’s observations on glutathione concentration in BALF (Grigg 1993), and those which I have presented in this thesis.

It has been shown that decreased alveolisation is a major feature of infants who develop CLD (Margraf 1991, Thibeault 2000, Hussain 1998). My data raises the possibility that the impaired lung growth that is noted in CLD may result from
interference with cell signalling cascades in the lung by the persistence of NO and its oxidation products including peroxynitrite. Clearly further work is required to address some of the questions that are raised by my observations.

There are a number of experiments that can be performed to address these questions. Our group has used the product tetranitromethane (TNM), which specifically converts tyrosine to nitrotyrosine to further elucidate this interference. We have noted that the Platelet Derived Growth Factor BB (PDGF BB) stimulated phosphorylation of specific tyrosine residues in the cell-signalling MAPK pathway is prevented by the presence of TNM (Pandya 1999). Similar experiments can be devised in vitro to determine if the formation of nitrotyrosine interferes with cell signalling pathways. Ultimately animal models can be used to determine if treatment with NO or induction of endogenous NO results in dysregulation of lung growth in newborn, preterm animals. My data when taken into context of existing NO biology suggests that careful evaluation of NO toxicology is required prior to extending the use of NO in clinical practice.

Our data do not allow the source of the NO to be determined. There are thought to be as many as forty different cell types within the lung (Nunn, 1993). Cell types which have previously been shown to contain NOS I (neuronal cells), NOS II (see Table 6.1) or NOS III (endothelial cells) are present within the lung. Many cell types have been shown to increase NOS II expression after LPS stimulation NO has been observed to be produced by many different cells found within the lungs. We cannot determine which isoform of nitric oxide synthase was responsible for the production observed. Xue (Xue 1996) demonstrated the presence of the different isoforms in fetal and postnatal rat lung. He described that type I, and type II NOS were also found in
the epithelium, and type III NOS was only present in lung endothelium. Shaul et al (Shaul 1994) demonstrated type III NOS in human bronchial epithelial cell culture. Similarly, Asano demonstrated ‘constitutive’ and ‘inducible’ NOS in human lung epithelial culture, although this group did not distinguish between type I and type III NOS (Asano 1994). There is no direct evidence of NOS in human airway smooth muscle. However Boota (Boota 1996) has shown, in vitro, that rat pulmonary vascular smooth muscle cells do produce NO in response to ‘cytomix’ (a cocktail of IL-1β, TNF-α, and IFN-β) (a response previously observed to be via type II NOS). There may be more than one cellular, or enzymatic, source of the NO accounting for the similar nitrate concentration in the first week, and the subsequent different nitrate concentrations in infants who develop CLD, and those who do not. Analysis of cellular constituents of BAL fluid for presence of NOS isoforms, may provide some evidence as to the source of the NO observed.

7.4 Antioxidants

The most significant finding was the delayed increase in ascorbate in BAL fluid from infants who developed CLD. An earlier increase was seen in the RDS and Control infants. Of particular interest is the increase in the ratio of ascorbate in BAL fluid to plasma at day 4. Since ascorbate is not produced within the mammalian body, any increase in ascorbate in a particular area of the body (e.g. BALF) must be due to some process of preferential sequestration of the ascorbate into that particular organ. This could be by either an active or passive process. It is unclear how physiological immaturity could hinder a passive (diffusional) process to account for the observed differences in BALF:plasma ratio. Rybakowski (Rybakowski 1995) suggested an ascorbate pump may be present within the placenta. If his suggestion is correct, it is
plausible that the differences I have observed are also due to a difference in function of a similar pump within the lung. The relative immaturity of infants who develop CLD may account for a functional immaturity of such a speculative ascorbate concentrating mechanism within the lung. This would increase the oxidative burden on the lung at risk of CLD.

Furthermore, one might speculate that enhancement of a pump proposed by Rybakowski (Rybakowski 1995) by (currently unknown) therapeutic means may enhance the anti-oxidant defences of newborn infants who develop respiratory illness. It is unlikely that added ascorbate to the diet will enhance the concentration across the membrane pump but clearly adequate ascorbate must be available in the plasma for it to be transported to the lung interstitium. Cell culture systems may provide one model to study the transport of ascorbate across the cell membranes and can be used to study various therapeutic agents (e.g. corticosteroids) to determine if maturation can be advances at least in vitro. Similarly, animal models can be used to further study the effects of therapeutic manoeuvres to mature the pump especially in preterm animals and the results compared to more mature term animals.

The oxidant-antioxidant system is a balance between the oxidant effects of both reactive oxygen species and other oxidants such peroxynitrite and the anti-oxidant defences such as the anti-oxidant enzymes (e.g. superoxide dismutase) and non-enzymatic means (e.g. glutathione, ascorbate and urate). Thus the ratio between the oxidised and reduced agent is likely to be more important than the simple measurement of an individual agent alone. In this study it would perhaps have been more useful to have measured both the total and reduced glutathione and ratios of
Figure 7.1: Diagram showing the products of oxidation of nitric oxide (NO). Note that nitrate is a stable end product.
Figure 7.2: Diagram showing several cell signalling pathways which rely on phosphorylation of specific tyrosine residues (P). The formation of nitrotyrosine by peroxynitrite may interfere with the normal phosphorylation of critical tyrosine residues thereby affecting cellular functions such as cell proliferation and differentiation.
<table>
<thead>
<tr>
<th><strong>Immune system cells</strong></th>
<th><strong>Non-immune cells</strong></th>
</tr>
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<tbody>
<tr>
<td>Macrophage/monocyte system cells</td>
<td>Epithelial cells (gastric mucosa, lung*, intestine, kidney)</td>
</tr>
<tr>
<td>Blood*, bone marrow, lung, peritoneum*, Kupffer cells, mesangial cells*</td>
<td>Vascular smooth muscle*</td>
</tr>
<tr>
<td>Microglia*</td>
<td>Cardiac myocytes</td>
</tr>
<tr>
<td>Splenocytes</td>
<td>Connective tissue cells (Chondrocytes*, osteoblasts*, fibroblasts)</td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>Keratinocytes*</td>
</tr>
<tr>
<td>Natural killer cells*, T helper cells (T&lt;sub&gt;H1&lt;/sub&gt; but NOT T&lt;sub&gt;H2&lt;/sub&gt; cells)</td>
<td>Hepatocytes*</td>
</tr>
<tr>
<td>Neutrophils*</td>
<td>Pancreatic β cells</td>
</tr>
<tr>
<td></td>
<td>Astrocytes*</td>
</tr>
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<td></td>
<td>Neuronal cells</td>
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</tbody>
</table>

*Table 7.1: Cells which have been shown to increase expression of type II nitric oxide synthase, after stimulation by lipopolysaccharide or cytomix. * denotes reports in human cell lines. (Lincoln 1997)*
ascorbate to dehydroascorbate, and urate to allantoin and should be considered in any future studies.

Nutrition is likely to play an important part in providing the substrate for the antioxidant defences including ascorbate and other vitamins such as vitamin A and E. In my study there was a difference in the time when full enteral feeding was established between the infants who developed CLD (median 21 days), and those who did not (RDS: 7 days, Control: 7 days). However, the CLD infants were given intravenous nutrition from an earlier age. Since ascorbate is not manufactured endogenously within mammals, its source in newborn infants must be either transplacental or nutritional. If the source is solely transplacental, then it is to be expected that the maximal serum concentration would be immediately after delivery. In fact the maximal serum concentration in the RDS group was seen at approximately two weeks of age. This would either suggest that transplacental transfer is not the exclusive source of ascorbate in infants, or that there is a reservoir of ascorbate within the infant, and which releases ascorbate into the blood. The existence of such a reservoir has not been demonstrated in mammals. Thus, it may be assumed that the increase in serum ascorbate concentration observed is determined by the infant’s nutrition. Future studies should recognise the possible influence of dietary intake on ascorbate concentration, and if possible should control for this. This may not be such an easy task especially as the amounts administered to the infants may vary vastly (e.g. variability in breast milk due to maternal intake) and may be difficult to control for in human studies. Berger (Berger 1998) used supplemental ascorbate and tocopherol but did not find reduced concentration of conjugate dienes (markers of lipid peroxidation) in baboon tracheal aspirate. This would suggest that to simply increase the nutritional
intake of ascorbate does not automatically result in an increase in ELF ascorbate concentration.

We did not find any differences between the groups for either BAL fluid urate or glutathione. The results are in variance to those reported by Grigg et al (Grigg 1993) who found decreased BAL fluid glutathione in a small number of preterm infants. The likely reasons for this may be due to improved nutrition and milder respiratory disease (their group was from a pre-surfactant era). As described above it is likely that the ratio of oxidised vs. reduced glutathione is more important than simple measurements of individual agents involved in the anti-oxidant defences of the body.

7.5 Comparison of ascorbate and nitrate concentrations

The interaction between ascorbate and nitric oxide has been examined quite extensively. A search in Medline for “ascorbate” and “nitric oxide” produced 256 results. Most of these “hits” refer to the relationship between ascorbate and NO in vasculature, gut, nervous system, skeletal muscle, and the kidney. Only two directly relate to NO secretion in the lung. Chambers (Chambers 2000) reported that ascorbate supplementation in healthy volunteers did not alter eNO concentration. Schrammel (Schrammel 2000) observed that in vitro, addition of ascorbate resulted in reduced concentration of cyclic GMP, which was in part due to scavenging of NO by the ascorbate. Although these two studies suggest there is a limited interaction between ascorbate and NO in the lung, other, in vitro studies on specific cell lines that may occur in the lung (polymorphonuclear leukocytes, macrophages, endothelium) show other effects.
Sharma has shown that ascorbate will potentiate synthesis of NO from polymorphonuclear leukocytes (Sharma 2004). There are no data of this effect from ascorbate on any other cell lines typically found in the lung. Borutaite has shown that ascorbate can prevent NO induced apoptosis in a specific (J774) macrophage cell line (Borutaite 2003). These macrophages are from a murine sarcoma clone, and therefore possibly not directly comparable with the various macrophage lines found in the lung.

Ascorbate has also been shown to enhance iNOS activity in a mouse macrophage cell line (RAW 264.7), but only when co-stimulated with interferon-gamma, and lipopolysaccharide (Nakai 2003). Interestingly Mo found that ascorbate could prevent TNFα induced NO secretion in endothelium (Mo 2003). The previously cited studies which demonstrate an association between ascorbate concentration and NO production refer to specific cell lines, and are in vitro data. It may be that the overall relationship between ascorbate and NO is complex, and is influenced by the relative numbers of migratory cells (neutrophils, macrophages) present in the lung, as well as non-migratory cells (endothelium). Certain, specific effects from ascorbate on particular cell lines within the lung, may very likely go unrecognised if the only assessment of this effect was via the final concentration of nitrate/nitrite found in BALF, or eNO in exhalate.

The data from the studies in this thesis do not suggest any degree of relationship between BALF ascorbate concentration (corrected for plasma concentration), and BALF nitrate concentration (data not shown). However, these studies were not designed to elucidate any such association within BALF of infants who develop CLD. It is unknown whether the absence of a relationship between ascorbate and NO, is because studies designed to test different hypotheses resulted in an association being missed, or because no such relationship does in fact exist.
The slides made of recovered cellular components of BALF at the same time as the ascorbate samples were taken could be stained for iNOS mRNA (to assess iNOS enzymatic activity) in those cell lines present (neutrophils, macrophages, epithelia). This method has its limitations since any effect from cell lines capable of secreting NO, but not present on the slides (e.g. endothelial cells) would be absent. Such analysis was not performed during the project, but could be subsequently undertaken.

7.6 Summary

In these series of studies, I have made some important contributions to the study of respiratory disease in newborn infants. Since the observations I have made have been in infants there is a greater relevance of my findings to the bedside, than in animal or cellular models.

The observation that serial BAL does not cause long term radiological changes is important as it demonstrates a degree of long term safety with this procedure (BAL) that has not been shown ever before.

I have compared several methods to estimate both nitrate and nitrite in BAL fluid, and determined, and then applied the most appropriate method to the small volumes of BAL. This series of methodological studies is valuable for future research projects to help them not to suffer the difficulties from reagent interference I found.

The observations I made with the developed methodology are novel, and significant in furthering our understanding of the pathogenesis of CLD in preterm infants. For the first time, I have presented data to show the pattern of NO production in the lungs of
preterm infants. No previous studies have done this. I noted similar concentrations of
nitrate in BAL fluid from all three groups studied but that the concentration decreased
in the RDS and Control infants but appeared to persist in the CLD infants possibly
due to the inflammatory phase that has been seen in these infants at days 7 – 10 of
age.

The observations that both ascorbate concentration, and macrophage concentration
have a brief, but statistically significant, rise at day 4 in those children who recover
from their RDS is novel, and possibly of importance. The suggestion from these two
observations is that development of CLD may not be due to an “excess” of pro-
inflammatory events, as is suggested by all the data published up to the time when I
started my research. Instead, the problem may be due to a failure of resolution of
inflammation in those children who develop CLD. This inference from my data has
now been superceded by published data demonstrating failures in mechanisms of
downregulation of inflammation (e.g. apoptosis [Kotecha 2003, Oei 2003], low II-10
concentration [Oei 2003], or a different patterns of IL-10 concentration changes
[Beresford 2002]. This observation was especially important for me as it altered my
own thinking into the aetiological mechanisms of CLD, and other chronic
inflammatory lung diseases.

The fact that the results come from infants, and not from an animal model of preterm
lung disease, or a cellular model of increased NO exposure gives my data much
pertinence and importance in furthering our understanding of the pathogenesis of
CLD.
7.6.1 Suggested Further Studies

My results have opened several further lines of investigation which if followed should provide further understanding of the anti-oxidant systems in preterm infants. The method described by Tsiakis (Tsikas, 1997) may be able to be used with my developed technique for enzymatic reduction of nitrate of small (<50 microlitre) volumes of sample. Use of smaller volumes of BALF would allow for other assays to be performed on a single sample. This would make any direct comparisons between different constituents (e.g. NO and ascorbate) to be performed from one sample.

The enzymatic reduction technique (in conjunction with the previously described fluorometric assay for nitrite) could be applied to a study of infants who develop PPHN after delivery. Such a study would be able to confirm or refute the widely held opinion that it is indeed a defect of NO synthesis that causes PPHN.

The exact cellular source of the NO seen in all three clinical groups on the first week, and which persists in the CLD group in week two is unknown. Indeed, the source may be different in week one and week two, or between the different diagnostic groups. Staining the cytospin slides for iNOS could identify whether the neutrophils, macrophages or even epithelial cells recovered were contributing to the NO secretion seen in the BALF analysis. Such a study could not be definitive in identifying all the NO sources since many other pulmonary cell lines would be missing from the slides.

However, given that much evidence of the cellular events in CLD suggest that the influx of macrophages and neutrophils plays an important role in the pathogenesis of CLD, clarification of whether these cells significantly contribute to the overall NO secretion would still be a very important finding.
The sustained concentration of nitrite/nitrate in BALF, in infants who develop CLD also raises the question of whether peroxynitrite exerts an effect on subsequent lung growth.

The findings that, at day 4, both ascorbate concentration in BALF, and macrophage count are raised also warrants further investigation. This speculation that wound repair may be defective in those who develop CLD suggests that further research should examine whether intra-tracheal administration of ascorbate (initially in an animal model) has a beneficial effect on incidence of CLD. Alternatively, one could harvest macrophages from preterm infants, and observe the effects of adding ascorbate \textit{(in vitro)} to their production of pro-inflammatory and anti-inflammatory mediators.

It is for these reasons that I consider this work to have been important in furthering our understanding of neonatal lung disease, and in suggesting additional research questions which, if tested, will add even more to our knowledge of the pathogenesis of CLD.

This thesis, and the findings described within it, are submitted for the postgraduate degree of Doctor of Medicine at the University of Leicester.
Chapter 8

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Chapter 9

Appendices
COMMITTEE ON THE ETHICS OF CLINICAL RESEARCH
INVESTIGATION
APPLICATION FOR RESEARCH ETHICS APPROVAL

The following application form is for submission to the Ethics Committee for approval of proposed medical research involving human subjects*. Please complete the form in typescript and return to the address given at the bottom of this page for approval.
The following must also be submitted with the protocol:

- Detailed Protocol
- Questionnaires Used
- Proposed Consent Form
- Patient Information Leaflet

N.B. The Ethics Committee in considering an application for ethical approval, bears in mind the Royal College of Physicians guideline that "badly planned, poorly designed research that causes inconvenience to subjects and may carry risk without producing useful or valid results, is unethical."

Notes for completing application.

Please complete every section of the form as fully as possible. The shaded areas are only for office use, so please do not fill these in. Respond to all Yes/No questions by circling the appropriate answer.

Address: Director of Public Health
Leicestershire Health
Gwendolen Road
Leicester
LE5 4QF

(* This application form is available on computer floppy disc (3.5in) as a template in WORD for Windows 2.0 format.)
1. Title Of Project

The role of Cytokines and Anti-oxidants in the pathogenesis of Chronic Lung Disease of Prematurity.

2. Miscellaneous Details

2.1 Where will the research be done? (tick one as appropriate) Hospital [X] GP Other

If other please state where:

2.2 Starting Date (DD-MON-YY): 01 10 1996
2.3 Duration (in months): 36

3. Responsible Investigator (Supervisor of Project)

N.B. This is the individual with overall responsibility of the proposed study, not necessarily the individual who will be carrying out the study.

3.1 Name: Dr Sailesh Kotecha Title (Dr, Prof etc.): Dr

Address: Department of Child Health
Leicester Royal Infirmary

Position/post held (Consultant, Senior Nurse etc.): Lecturer in Child Health
Qualification: MA, MRCP, DCH

3.2 List the individual(s) who will be carrying out the study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Position/Post Held</th>
<th>Qualification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr David Field</td>
<td>Consultant in Child Health</td>
<td>DM, FRCP</td>
</tr>
<tr>
<td>Dr Andrew Currie</td>
<td>Research Fellow</td>
<td>BM, MRCP</td>
</tr>
<tr>
<td>Dr Julian Vyas</td>
<td>Research Fellow</td>
<td>BS, MRCP</td>
</tr>
</tbody>
</table>

4. Purpose of Research

4.1 Please state the objectives of the research and briefly and simply describe the scientific background.

Preterm infants often need to be mechanically ventilated due to immature lungs and a condition called Respiratory Distress Syndrome. Many of these will develop Chronic lung disease of Prematurity (CLD), with long-term respiratory problems and oxygen dependency lasting months or years.
The pathogenesis remains unclear. Our previous work has demonstrated that pulmonary inflammation in babies who develop CLD is maximal at 10 days of age. But, in earlier work by our group, the fibrosis-promoting growth factor TGF-B was increased in bronchoalveolar lavage fluid at 4 days of life, thus challenging the widely accepted view that fibrosis follows inflammation.

The aims of our current studies are a) to determine if other profibrotic agents are increased early in the development of CLD and b) to determine if perinatal factors are important in triggering the pulmonary inflammation seen in babies who develop CLD.

5. Design of Study

5.1 Please describe what will be done, what results you expect and how you will analyse the results. Remember to attach a full copy of the protocol. Please identify potential dangers, discomfort or inconvenience to subjects of any of the techniques involved.

Maternal blood, a small placental sample, amniotic fluid and cord blood will be collected at the time of delivery. The maternal blood will be taken at the same time as clinically indicated venepuncture after the delivery.

From those infants who go on to need ventilation, regular bronchoalveolar lavage samples and blood samples will be collected. In the first week this will be on alternative days, and subsequently twice a week until day 28, or extubation, whichever is earlier.

Bronchoalveolar lavage is performed using a 5F gauge suction catheter which is passed through the endotracheal tube into the right lung. Two aliquots of 1 ml/kg of saline are then separately instilled into the lung via this catheter and immediately sucked back; the lavage fluid is collected for further analysis. We have previously obtained ethics approval (see Ref. No. 3871) for this, and used it without ill-effect. The technique replaces the routine tracheo-bronchial lavage these ventilated babies undergo. Blood samples (0.5-1.0 ml) will be collected at the same time as clinically indicated blood-taking times.

We will analyse all samples collected from mother and baby for proinflammatory and profibrotic cytokines, as well as antioxidants, and relate these to the clinical development of CLD.

As samples will be taken at a time when there are clinical reasons for taking samples we do not anticipate any additional discomfort or inconvenience for our patients. As is conventional with studies of acute clinical care, formal consent will be delayed until after delivery of the infant. We anticipate that samples will be collected non-invasively at the time of delivery and will only be used following formal consent, which will be obtained within 6 hours of delivery.

6. Specific Details on Purpose and Design of Study

6.1 Has this work been carried out before? No
   If yes, why is there a need to repeat?

6.2 Is this a multi-centre study? No

6.3 What type of a study is this (tick one as appropriate):
   Pilot
   Definitive
Follow on of a previous study  
Modification of a previous study

If a follow on or modification of a previous study, please give reference number of previous application and approval date:

Previous study reference number: 3871  
Approval date: 07/07/95

NB: The study is an extension of previous work conducted by our group in the Hammersmith and Leicestershire hospitals. In particular we have set up the technique of BAL in ventilated newborn infants.

N.B. For the next two sections circle one as appropriate.

6.4 Is this research of direct benefit to the subject: No

6.5 Is this research related to: Diagnosis or Therapy or Neither

If yes to therapy then what type (tick any as appropriate):
- Drugs
- Surgery
- Other

7. New Chemical Entity/Therapeutic Agent or Established Agent

7.1 Is this an investigation of an established agent? No
If yes, is the established agent being used for a new/unlicensed indication? Yes/No

7.2 Is this a study of a new chemical entity/therapeutic agent? No
If yes, What stage is this in its evaluation? (tick one as appropriate)
- Phase II
- Phase III
- Phase IV

7.3 If this drug is being supplied by a pharmaceutical company as part of sponsored research, has a clinical trial certificate/exemption certificate been provided? Yes/No
Not applicable
### 8. Substances to be Administered

<table>
<thead>
<tr>
<th>Route</th>
<th>Amount/Frequency</th>
<th>Risks</th>
<th>Precautions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drugs*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-saline</td>
<td>Endotracheal</td>
<td>Two aliquots of 1 ml/kg of N/Saline</td>
<td>The lavage procedure will replace the usual tracheal suctioning that these babies would have as part of their routine care</td>
</tr>
<tr>
<td>Isotopes</td>
<td></td>
<td></td>
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<tr>
<td>Fluids &amp; Diets</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
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</tbody>
</table>

* On completion of a clinical trial the investigator should notify the pharmacist who will destroy any chemical trial material still being held.
9. Financial Arrangements

9.1 Will you be receiving any financial contributions towards your research?

If yes, from whom and how much?

N.B. Tick main source only.

Whom: Pharmaceutical Company
Research Grant (MRC, BHF, RHA e.t.c)
Other

How much? (Please state approx. total amount): £96,000.00 over 2 years.

9.2 How will these funds be spent?

(tick any as appropriate)
Running Costs
Other

If 'other' briefly describe:

10. Recruitment of Subjects

Please say how you will recruit subjects, with rules of inclusion and exclusion and any proposals to deny and delay treatment and any other relevant details e.g. age, sex, type of patient.

N.B. Investigators are reminded of the need to notify General Practitioners when patients under their immediate care are to be included in a study. Investigators should ensure that any patients or healthy volunteers involved in a particular project are not included in another study which also involves drugs or isotopes.

10.1 Subjects?

We will recruit 30 patients with CLD (defined as oxygen dependency at 28 days of age) and 30 patients with acute respiratory distress syndrome who recover. Infants will be recruited from the Leicester maternity units.

10.2 Controls?

30 infants who require mechanical ventilation for non-respiratory reasons will be recruited as controls (e.g. Surgery, muscle disorders, birth asphyxia), as well as infants at Glenfield Hospital requiring cardiac catheterisation or surgery.

10.3 Number of subjects to be recruited: see above.

10.4 Type of subjects to be recruited? Patients

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Volunteers

If volunteers, then please tick any of the options from the list below to specify the type:
- Staff
- Student
- Other

10.5 Any financial inducements offered to subjects or relatives? No
If yes, what?

10.6 Informed Consent
N.B. Written informed consent is preferred, *a copy of the consent form and information leaflet to be used should be supplied.*
Please note that special consideration must be given to children, mentally ill and handicapped.

Will informed consent be obtained? Yes
If yes, then tick any of the options from the list below to specify the method used:
- Written: X
- Oral

NB: As is conventional with studies of acute clinical care, samples will be obtained noninvasively at the time of delivery and only used following formal, written consent, which will be obtained within six hours of delivery. If not, why not?

10.7 Compensation
What kind of arrangements for compensation/indemnity for subjects are in place for the study, please tick any of the options from the list below:
- ABPI Guidelines on compensation for medicine induced injury X
- Crown Indemnity
- Other X

If yes to 'Other', please give details:

10.8 Investigations of Subjects/Controls:
Venous samples Yes

1) 10 mls of blood will be collected from the indicated infants delivery.

2) Blood (0.5-1.0 ml) will be collected at the time of clinical investigation from ventilated bronchoalveolar lavage.
Arterial samples see above.
If yes, then Where From:
Frequency: As above
Amount: As above

N.B. If yes to any of the following questions, please give details below.
X-rays No
Radiation No
Ultrasonics No

Biopsies No
If yes, then Site:
Method:
Size: c.
Number:

Anaesthesia No
If yes, then Local or General

Other invasions Cannulae/ Probes/ Catheters/ Endoscopes/ Lumbar Punctures

Any non-invasive tests? EEG/EMG/ECG NONE

Psychological tests? No

Questionnaires? No
If yes, please include copy.

Other Activities Yes

Additional Details: Placental samples, amniotic fluid, amniotic membranes, and umbilical cord blood will be collected following delivery. Bronchoalveolar lavage will be performed at the time of clinically indicated tracheal suctioning.

11. Likely Benefits Of Study
Chronic lung disease of prematurity is a common disorder of preterm infants. The aim of the study is to enhance our understanding of the pathophysiology of this disease. By understanding the pathogenesis of CLD at a molecular and cellular level our studies may identify potential therapeutic sites in the prevention of this disabling disorder.

12. Documents Enclosed

Please remember to enclose the following documents where appropriate:
• Detailed Protocol  Yes
• Questionnaire  Yes
• Proposed Consent Form  Yes
• Patient Information Leaflet  Yes

13. Applicant(s) Signature

Signature(s) of Applicant(s)  Date:

14. Countersignature of Consultant

Countersignature of Consultant (in the case of junior medical and dental staff), Head of Department, Nurse Tutor, Director of Nursing Services etc.

I have discussed the research proposal with the investigator, who is in my department, and I support his/her application to the Ethical Committee.

Signature(s)  Date:
9.2 Appendix 2
INFORMATION SHEET FOR PARENTS

You are being asked to participate in a research project. This will involve both mother and child. The statement below explains in ordinary language what will happen if you agree to take part; it describes any risks or discomfort that you or your baby may experience, and it also explains what we hope to learn as a result of taking part.

You should not take part if you do not wish to do so. If you decide not to take part, the treatment of you and your baby will not be affected by your decision.

THE ROLE OF INFLAMMATION AND FIBROSIS IN CHRONIC LUNG DISEASE OF PREMATURITY

Babies born prematurely have immature lungs, and often require a ventilator (breathing machine) and oxygen for breathing problems. Some babies need ventilating for long periods and often need oxygen for several weeks or months - a condition called Chronic Lung Disease (CLD). We are trying to find out why some babies become oxygen dependant and why others do not.

Part A below, sets out what we aim to do during this project. It explains about the samples that we need. Part B, on the next sheet, answers some common questions you may have.

Part A.

We are trying to determine why some babies develop oxygen dependency and why some don't. To do this we wish to estimate if chemicals which promote inflammation are increased in: 1) the mother's blood taken at delivery, 2) the placenta ("After birth"), 3) amniotic fluid ("womb fluid"), and 4) in the babies blood and lung fluid.

In order to do this we are asking if we can collect an extra 10 ml (2 teaspoonfuls) of your blood when the midwife or doctor takes your blood for tests. We would also like to use a small part of your placenta, some womb fluid and a sample of umbilical cord blood after your baby is delivered. These would normally be thrown away.

All babies who need help with a ventilator have their breathing tubes sucked out regularly to stop them from blocking with phlegm. We wish to collect this fluid for our studies. Finally, only when your baby has blood tests taken we would like to collect an extra 0.5-1.0 ml (1/5 th of a teaspoon) at the same time for our research.

We will measure agents which cause inflammation and fibrosis in the samples collected and from this try to identify why only some babies develop oxygen-dependency or Chronic Lung Disease.

Dr. Andrew Currie  
Paediatric Research Fellow

Dr Julian Vyas  
Paediatric Research Fellow
PART B.

Premature babies who require ventilation for a long time can get inflamed and scarred lungs which causes chronic lung disease (CLD). We are trying to find out which chemical agents cause the inflammation or scarring in CLD.

All babies who are ventilated need their airways clearing regularly to stop them blocking with phlegm. This is done by squirting a small amount of saline (salt water) into the windpipe, through the breathing tube, to loosen the phlegm, and then removing it by sucking. Normally the fluid would be thrown away. We would like to use this fluid for our studies to determine why some babies develop CLD.

To do this, rather than put the saline straight into the breathing tube, we would pass a very thin tube (called a suction catheter) into the breathing tube and then squirt the saline through this. It is then sucked up straight away into a special collection pot attached to this catheter.

On the same day as collecting the lung fluid we would also collect a very small sample of blood (1 ml). This will be done at the same time as blood is taken for clinically indicated tests.

A Few Questions and Answers:

Why does my baby need sucking out?

Babies on ventilators get lots of secretions in their airways. If they are not sucked out regularly it can become thick and sticky then there is a risk the breathing tube will block.

What is the difference in the two suctioning methods?

The difference between the methods is how the saline gets into the lungs. Normally it is squirted straight into the breathing tube. In our method it is squirted into the lungs through a thin tube that goes into the breathing tube. For the research this is important because it means we can collect it much better.

How often will this be done?

In the first week we would collect samples on alternate days. After this we will collect samples twice per week whilst your baby is on a ventilator. Samples will only be collected whilst the baby is on a breathing machine.

Are there any risks?

Sometimes when babies are sucked out their heart rate and oxygen needs can go up temporarily. This happens with both methods and there is no difference between the two.

Will entering my baby in this project mean lots of extra procedures being performed?

No. We simply wish to collect the lung secretions that would normally be thrown away. There will be no need for any extra needles or other unpleasant procedures.

Will I be allowed to watch?

Yes. You are more than welcome to watch what we do.

What about the blood samples?

Blood will only be collected when doctors are collecting blood to assess the babies condition. We would like to collect 0.5-1.0 ml of blood at this time. It will not involve any extra needles.

What if I do not agree to the research project?

You should only agree if you feel comfortable about it. If you decide not to take part the treatment of your baby will not be affected in any way.
What if I agree, and then change my mind later on?
If you decide at any time that you do not want your child to take part any more, please let us
know and your child will not have further lavage performed for our research. Again, this will not
affect in any way the care your baby receives.

If you have any questions, please feel free to ask..

Dr. Andrew Currie
Paediatric Research Fellow

Dr. Julian Vyas.
Paediatric Research Fellow
9.3 Appendix 3

CONSENT FORM FOR STUDIES ON CHRONIC LUNG DISEASE OF PREMATURITY

Dr __________________ has explained to me the nature of the research project. I/we understand that the research involves collection of samples at delivery which will include amniotic fluid, placental sample, umbilical cord blood, and my blood. My/our baby may have blood and lung fluid taken for the research. I understand that neither I nor the baby will have blood taken purely for research purposes.

I/we are happy to participate in the research.

Signed_________________________ Date ___________________
Parent’s name________________________

Doctor’s signature __________________ Date __________________
Doctor’s name __________________________

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9.4 Appendix 4: Papers arising from this research


IV. **Vyas JR.** Currie AE, Dunster C, Kelly FJ, Kotecha S. Ascorbate acid concentration in airway lining fluid from infants who develop chronic lung disease of prematurity. Eur J Ped 2001;160(3);177-184


Other papers authored during the research period:

I. Currie AE, **Vyas JR.** MacDonald J, Field D, Kotecha S. Epidermal growth factor in the lungs of infants developing chronic lung disease of prematurity. Eur Respir J 2001;18:796-800

II. Mildner R, Taub NA, **Vyas JR.** Killer H, Firmin R, Field D, Kotecha S. Repeatability of cellular constituents and cytokine concentration in fluid obtained by non-bronchosopic bronchoalveolar lavage of infants receiving ECMO. Thorax 2001;56:924-931

IV. Kotecha S, Mildner RJ, Usher LR, Vyas JR, Currie AE, Lawson RA, Whyte MKB. The Role Of Neutrophil Apoptosis In The Resolution Of Acute Lung Injury In Newborn Infants. Accepted by Thorax 2003

9.5 Appendix 5: Presentations of work arising from this study

