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Novel concepts in Ventilator-Induced Lung Injury in preterm babies

Katinka Petronella Bach

Abstract

Respiratory distress syndrome (RDS) and its long-term associate, bronchopulmonary dysplasia (BPD), are the commonest morbidities in preterm babies. Many babies with RDS require mechanical ventilation for survival. However, mechanical ventilation contributes to the inflammation in the lung which leads to BPD. Although mortality from RDS has decreased, the incidence of BPD has not. This thesis describes a series of experiments investigating the novel concept that the bias gas flow in a ventilator circuit and, therefore, the rate at which gas enters the mechanically ventilated lung, may contribute to lung injury.

Initial experiments in an artificial lung demonstrated that bias gas flow is inversely related to inflation time. Experiments in term lambs with normal, compliant lungs verified this relationship and demonstrated that optimal efficiency of ventilation may occur with much lower bias gas flows than are currently used in the neonatal setting.

Experiments in late preterm lambs, not treated with surfactant, demonstrated that the severity of lung injury is related to bias gas flow. At higher flows there were increased mRNA levels of early response genes, known to be up-regulated in lung injury, and histological evidence of lung injury consistent with that seen in BPD.

An experiment in fetal lambs in the canalicular stage of development confirmed that ventilation at high flows alters connective tissue deposition; however, low flows led to lower relative humidity in the inspired gas. This study also investigated the interaction between bias flow and the presence of an inspiratory pressure plateau. An inspiratory plateau phase at low flow resulted in fewer alveolar septal crests and a trend towards increased levels of two early response genes.

These experimental studies demonstrate that bias gas flow affects ventilatory parameters and lung injury, with the mode of ventilation possibly also being important.
From these studies, a protocol for a pilot randomised controlled trial (RCT) in preterm, extremely low birth weight neonates has been developed to determine whether low bias gas flows result in decreased inflammatory and oxidative markers predictive for BPD. If the pilot study supports this hypothesis, a multicentre RCT would be indicated with BPD as the primary outcome.
Acknowledgements

I would like to thank my supervisor, Associate Professor Frank Bloomfield, for helping me to take the first steps into the research world. I have learned a lot from his expertise in all aspects that come with research, scientific writing and funding. He has been an outstanding mentor and I have appreciated the support, not only for PhD goals, but also towards the next step in my career, working as a senior consultant in Neonatology.

I owe a lot to Associate Professor Carl Kuschel, who came up with the hypothesis that bias gas flow affects ventilatory parameters and, therefore, possibly ventilator-induced lung injury. He was my co-supervisor until he moved to Melbourne, but has continued to play a significant role in the design of the experiments. Furthermore, I have enjoyed the joint experiences of being ‘greenies’ in the world of sheep research and I have appreciated the many times he invited me to join him and his family for dinner during the many long weeks I spent in Melbourne. And yes, you are allowed to tease me with my ‘freaky-deaky Dutch’ and initial choice of accommodation in Clayton....

I want to acknowledge Professor Jane Harding, who took over Carl’s role as co-supervisor after he left, for her advice and guidance.

A substantial amount of the analyses for my PhD was performed at the Monash University in Melbourne, under supervision of Associate Professor Stuart Hooper. Stuart, I want to thank you for taking me under your wings and for having many inspirational discussions with me.

I would like to acknowledge and thank the many people who have helped with the animal experiments described in this thesis. Shirley Peachey and Sue McKnight for the long hours and many trips to the farm, all in your spare time, for the many times you prepared a dinner for me and for cleaning up the sheep ‘drool’. Jean Bertram, Karen Anderson-Hawk and Anne Jaquiery for the help in the first animal experiments. Dr Mark Oliver and Sam Rossenrode, who taught me the basic animal handling procedures and surgical techniques. I would also
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I am very grateful to the Health Research Council of New Zealand for generously supporting me during three years of my studies, and to the Liggins Institute Doctoral Scholarship for providing support over the past year. The ADHB Charitable Trust, Auckland Medical Research Foundation provided funds towards some of my studies. Furthermore, I want to acknowledge Dräger for the loan of a Babylog 8000 plus ventilator for the experiments in preterm lambs, Fisher & Paykel Healthcare for the donation of a Neopuff and Geoff Bold and Chris Beaumont for their help during some of the experiments.

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Mam en Pap, you have always been very supportive and interested in my work. Even though I am on the other side of the world, it still doesn’t stop the two of you to surprise us with Stevers and the like. Dank jullie voor het altijd klaar staan voor me.

Mostly, I want to thank Angus for his support and ongoing encouragement, his more than fair share in cooking and household chores and his never ending optimism. I hope my sheep may safely graze and I’m looking forward to our Knapzak!
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<table>
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<th>A</th>
<th>A/C</th>
<th>assist control</th>
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<tbody>
<tr>
<td></td>
<td>AECs</td>
<td>alveolar epithelial cells</td>
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<tr>
<td>AH</td>
<td>absolute humidity</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>ANZNN</td>
<td>Australian and New Zealand Neonatal Network</td>
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<tr>
<td>αSMA</td>
<td>alpha smooth muscle actin</td>
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<tr>
<td>B</td>
<td>BAECs</td>
<td>bovine aortic endothelial cells</td>
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<td></td>
<td>BPD</td>
<td>bronchopulmonary dysplasia</td>
</tr>
<tr>
<td>C</td>
<td>°C</td>
<td>degree Celsius</td>
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<tr>
<td></td>
<td>CC</td>
<td>Clara cells</td>
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<tr>
<td></td>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CI</td>
<td>confidence interval</td>
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<tr>
<td>CLD</td>
<td>chronic lung disease</td>
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<tr>
<td>cm</td>
<td>centimetre</td>
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<tr>
<td>cmH$_2$O</td>
<td>centimetre water pressure</td>
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<tr>
<td>CPAP</td>
<td>continuous positive airway pressure</td>
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<tr>
<td>$C_T$</td>
<td>cycle threshold value</td>
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<tr>
<td>$CTGF$</td>
<td>connective tissue growth factor</td>
<td></td>
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<tr>
<td>CXCL8</td>
<td>previously known as interleukin-8</td>
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<tr>
<td>CYR61</td>
<td>cysteine rich-61</td>
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<td>D</td>
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<td></td>
<td>DAB</td>
<td>diaminobenzidine</td>
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<td></td>
<td>DNA</td>
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<td>extracellular matrix</td>
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<td></td>
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<td>ethylenediaminetetra acetate</td>
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<td>early growth response factor 1</td>
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<td></td>
<td>ELBW</td>
<td>extremely low birth weight</td>
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<td></td>
<td>ERK</td>
<td>extracellular signal-related kinase</td>
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<td></td>
<td>ERV</td>
<td>expiratory reserve volume</td>
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<tr>
<td>F</td>
<td>f</td>
<td>frequency</td>
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<td>Abbreviation</td>
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<tr>
<td>FCPLV</td>
<td>flow-cycled pressure-limited ventilation</td>
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<tr>
<td>FiO₂</td>
<td>supplemental oxygen</td>
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<tr>
<td>FRC</td>
<td>functional residual capacity</td>
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<tr>
<td>G</td>
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<tr>
<td>G</td>
<td>unit of gravitational force</td>
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<tr>
<td>HCl</td>
<td>hydrogen chloride</td>
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<tr>
<td>HFJv</td>
<td>high frequency jet ventilation</td>
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<td>HFov</td>
<td>high frequency oscillation ventilation</td>
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<tr>
<td>HFPPV</td>
<td>high frequency positive pressure ventilation</td>
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<tr>
<td>HfV</td>
<td>high frequency ventilation</td>
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<td>HUVECs</td>
<td>human umbilical vein endothelial cells</td>
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<td>Hz</td>
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<tr>
<td>ICAM</td>
<td>intercellular adhesion molecules</td>
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<td>I:E ratio</td>
<td>inspiratory:expiratory ratio</td>
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<td>Ig</td>
<td>immunoglobulin</td>
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<td>i.m.</td>
<td>intramuscular</td>
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<td>IMV</td>
<td>intermittent mandatory ventilation</td>
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<td>inspiratory reserve volume</td>
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<td>I.V.</td>
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<td>IVH</td>
<td>intraventricular haemorrhage</td>
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<tr>
<td>IQR</td>
<td>interquartile range</td>
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<td>Kg</td>
<td>kilogram</td>
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<td>L</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>LSD</td>
<td>least significant difference</td>
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<td>M</td>
<td>molar</td>
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<tr>
<td>MAP</td>
<td>mean airway pressure</td>
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<td>MAPKs</td>
<td>mitase-activated protein kinases</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>MRV</td>
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<td>N n</td>
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<td>NCPAP</td>
<td>nasal continuous positive airway pressure</td>
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<td>NEC</td>
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<td>NICU</td>
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<td>NIPPV</td>
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<td>NNTB</td>
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<td>NWH</td>
<td>National Women's Hospital</td>
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<td>O OR</td>
<td>Odds ratio</td>
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<td>P pressure</td>
<td>partial pressure of carbon dioxide in arterial blood</td>
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<td>partial pressure of oxygen in arterial blood</td>
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<td>polymerase chain reaction</td>
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<td>persistent ductus arteriosus</td>
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<td>positive end expiratory pressure</td>
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<td>pulmonary interstitial emphysema</td>
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<td>PIP</td>
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<tr>
<td>PPROM</td>
<td>preterm prelabour rupture of membranes</td>
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<td>PRVC</td>
<td>pressure-regulated volume control</td>
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<tr>
<td>PSV</td>
<td>pressure support ventilation</td>
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<tr>
<td>PTL</td>
<td>preterm labour</td>
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PVL  periventricular leucomalacia
Q  qRT-PCR  quantitative real-time polymerase chain reaction
R  R  resistance
    RCT  randomised controlled trial
    RDS  respiratory distress syndrome
    RH  relative humidity
    RNA  ribonucleic acid
    ROP  retinopathy of prematurity
    RR  relative risk
    RV  residual lung volume
S  s  seconds
    SaO₂  arterial oxygen saturation
    SEM  standard error of the mean
    SIMV  synchronised intermittent mandatory ventilation
    SIPPV  synchronised intermittent positive pressure ventilation
    SpO₂  pulse oximeter oxygen saturation
T  TA  tracheal aspirate
    TCPLV  time-cycled pressure-limited ventilation
    TGF-β  transforming growth factor-β
    Ti  inspiratory time
    TLC  total lung capacity
    TNFα  tumor necrosis factor-alpha
    TV  tidal volume
U
V  V  volume
    VAPS  volume-assured pressure support
    VC  vital capacity
    VEGF  vascular endothelial growth factor
    VEI  ventilator efficiency index
    VG  volume guarantee
    VILI  ventilator-induced lung injury
    VLBW  very low birth weight
    vs  versus
<table>
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<tr>
<th>W</th>
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Chapter 1. Introduction

Worldwide, the rates of preterm delivery are increasing, with 1-2% of babies in Australia and New Zealand being born before 28 weeks’ gestation (Australian and New Zealand Neonatal Network, 2009). All of these babies need respiratory support after birth, and about half will go on to develop bronchopulmonary dysplasia (BPD) or chronic lung disease (CLD), generally defined as still needing respiratory support or supplemental oxygen at 36 weeks’ postmenstrual age (Jobe, et al., 2001a). The consensus of an American workshop organised in 2000 by the National Institute of Child Health and Human Development and reported on by Jobe and Bancalari, is to use the term BPD and not CLD in this setting, because this is clearly distinct from the multiple chronic lung diseases of later in life (Jobe, et al., 2001a).

Many factors make preterm babies more vulnerable to bronchopulmonary dysplasia, such as the degree of prematurity, antenatal or postnatal infection, oxygen exposure and the need for mechanical ventilation (Bancalari, et al., 2003). Many improvements in therapy have been made in the last decades, such as the development of surfactant (Sinn, et al., 2002) and antenatal treatment with corticosteroids (Roberts, et al., 2006; Crowther, et al., 2006); however, respiratory disease still remains the major cause of mortality and morbidity in these preterm babies (Ambalavanan, et al., 2006).

Despite the benefits of mechanical ventilation, ventilation can also lead to ventilator-induced lung injury (VILI). Mechanisms underlying VILI include injury caused by pressure (barotrauma), volume (volutrauma), repeated opening and closing of the small airways (atelectotrauma), free radicals (oxytrauma), or infection / inflammation (biotrauma (Donn, et al., 2006). During mechanical ventilation, gas is forced into the airways to create the positive pressure ventilation. Flowing gas behaves like a fluid (Tarran, et al., 2005) and may, therefore, impart shear stress to the walls of the respiratory tree. The rate at which gas flows into the respiratory tree may affect shear stress injury and thus
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lung injury. Thus, bias ventilator gas flow might be an as yet unrecognised mechanism of VILI in the preterm lung.

This thesis will investigate the role ventilator bias gas flow plays in physiological parameters, expression levels of early response genes and development of histological lung injury in the sheep lung.

To understand the mechanisms of injury, it is first necessary to understand normal lung development.

1.1. Lung development

In the developing human embryo the respiratory diverticulum, an outgrowth of the ventral wall of the foregut, appears when the embryo is approximately four weeks old. In the fifth week it expands caudally and forms the trachea and two bronchial buds, whilst the dorsal part of the foregut forms the oesophagus (Sadler, 2006). Two longitudinal ridges fuse to form the tracheo-oesophageal septum, separating the oesophagus and laryngotracheal tube (Sadler, 2006; Moore, et al., 2003).

The endoderm of the laryngotracheal tube gives rise to the epithelium of the lower respiratory tract (larynx, trachea, bronchi and respiratory epithelium). The splanchnic mesoderm surrounding the foregut forms the connective tissue, cartilage, muscle and blood and lymphatic vessels (Moore, et al., 2003).

Lung maturation can be divided into four stages, or as the embryological phase described above is regarded by some as the first stage, into five developmental stages. The developmental stages can overlap slightly, which can result in different maturity in different areas of the lung (Pringle, 1986).
Figure 1.1: Schematic outline of the development of the human lung

The developmental stages overlap, resulting in different maturity in different regions of the lung. The yellow area represents the period of viable preterm birth.

The stage following the embryological phase is the pseudoglandular stage (5-16 weeks’ gestation), during which the bronchial bud differentiates into bronchi and primordial terminal bronchioli. The developing airways are lined with columnar cells (Froh, et al., 1994). The first differentiation of respiratory cells, into ciliated cells, goblet cells and basal cells, is seen around 13 weeks’ gestation (Fanaroff, et al., 2002). Mesenchymal cells begin to develop into cartilage and smooth muscle cells (Moore, et al., 2003; Copland, et al., 2004). Capillaries are randomly spread throughout the mesenchyme; pulmonary arteries grow in conjunction with the airways. Pulmonary veins follow a different pattern, and demarcate lung segments (Fanaroff, et al., 2002).

During the canalicular stage (16-26 weeks’ gestation) the bronchi and terminal bronchioli enlarge, and the interstitial tissue decreases, leading to a substantial
increase in potential airspace volume (Copland, et al., 2004). Furthermore, improvement in vascularisation is an important part of development during the canalicular stage. The randomly spread capillaries begin to organise themselves in close proximity to the airway epithelial cells (Fanaroff, et al., 2002). Initially, this is a double capillary network, which turns into a single layer after vascular and epithelial basement membranes have fused, thus forming the adult blood-air barrier (Burri, 2006). Towards the second half of the canalicular stage (20-24 weeks’ gestation) the alveolar epithelial cells (AECs) begin to differentiate into type I and type II epithelial cells. Type I AECs are large flattened cells with long cytoplasmatic extensions, providing the majority of the lining of the terminal saccules (Flecknoe, et al., 2004). In postnatal life, gas exchange happens via type I cells; they form the blood-air barrier (Moore, et al., 2003). Type II AECs are rounded in shape, are scattered among the type I cells, and are responsible for surfactant production (Flecknoe, et al., 2004). Surfactant is stored in the lamellar bodies of the type II AECs. The initially high glycogen pool of these cells decreases, presumably due to the glycogen being used as substrate for surfactant synthesis (Froh, et al., 1994). By 24 weeks each terminal bronchiolus has given rise to two or more respiratory bronchioli, each of which divides into three to six alveolar ducts. By the end of the canalicular stage the first terminal air saccules (primordial alveoli) have formed.

The next stage is the saccular period (26 to 36-40 weeks’ gestation), so called because the terminal airways develop into sac-like structures. In addition to a further thinning of the epithelium by continuing differentiation of AECs into type I and II cells and a further thinning of the mesenchyme, lymphatic capillaries are formed and proliferate in close proximity to the alveolar epithelium (Moore, et al., 2003; Copland, et al., 2004). During the last stage, the alveolar stage (32-36 weeks to 3-8 years of age), alveoli are formed (Moore, et al., 2003; Copland, et al., 2004). The exact age until which a human continues to develop alveoli is currently not clear. Alveoli develop at the end of respiratory bronchioli or terminal saccules. At birth in a term baby about 50 million alveoli have developed, which is about one sixth of the number present in adult lungs (Moore, et al., 2003). After birth the number of alveoli
continues to increase, mainly by subdivision of the primordial alveoli with secondary septa (Moore, et al., 2003). This development results in an exponential increase in the surface area of the blood-air barrier (Copland, et al., 2004). Thus, term babies still have structurally immature lungs, since alveoli, the gas exchange units of the lung, are only available in low numbers (Moore, et al., 2003). Preterm babies have even more immature lungs and they lack surfactant, which makes these infants prone to respiratory distress syndrome (RDS) and BPD.

1.1.1. Surfactant

The main function of surfactant in the mature lung is to decrease surface tension at the air-liquid interfaces in the alveoli and distal bronchioli. This promotes lung expansion in inspiration and prevents collapse of alveoli during expiration (Zimmermann, et al., 2005).

Surfactant is a highly organised macro-aggregate consisting of lipids (90%) and surfactant specific proteins (10%). Of these lipids, 80-90% are phospholipids, with phosphatidylcholine (PC) being the main component. Approximately 60% of PC contains two saturated fatty acids, one being dipalmitoyl phosphatidylcholine (DPPC) which is the principle surface tension-lowering component of surfactant (Zimmermann, et al., 2005). Four specific surfactant proteins (SP-A to SP-D) have been identified so far. SP-A and SP-D are hydrophilic and play a role in lung defence, since they bind pathogens and facilitate their clearance. SP-B and SP-C are both hydrophobic and are important for the stability of surfactant (Zimmermann, et al., 2005).

Surfactant is produced by type II AECs and is stored in lamellar bodies (Stoll, et al., 2000). Type II AECs can be found after week 20 (canalicular stage) and lamellar bodies are first seen at 22 to 24 weeks of gestation (Stoll, et al., 2000). Analysis of fetal lung homogenates has demonstrated the presence of surfactant after about 20 weeks' gestation, but it is not until week 28-32 that surfactant can be demonstrated in the amniotic fluid and is functionally available for a preterm baby. Mature levels of surfactant are usually present after 35 weeks (Stoll, et al.,...
Term infants have surfactant pools of about 100 mg/Kg, whereas preterm infants with RDS have pools of less than 10 mg/Kg (Zimmermann, et al., 2005).

1.1.2. Histology

The wall of the trachea consists of four layers: the mucosa (with pseudostratified ciliated columnar epithelium and a lamina propria); the submucosa; a cartilaginous smooth muscle layer, and an adventitia (Eroschenko, 2005; Ross, et al., 1989). A thin basement membrane separates the epithelial lining from the lamina propria, which contains fine connective tissue fibres and lymphatic tissue. Below the lamina propria is the connective tissue submucosa, where seromucous tracheal glands are found. The hyaline cartilage is surrounded by perichondrium, merging with the submucosa on one side and the adventitia on the other side (Eroschenko, 2005). The hyaline cartilage of the trachea consists of C-shaped rings, stacked on top of each other to prevent collapse of the trachea. The adventitia contains nerves, blood vessels and adipose tissue.

The lumen of the trachea is lined with pseudostratified ciliated columnar epithelium, which contains ciliated cells, goblet cells, basal cells and some brush cells and dense core granule cells. Ciliated cells remove inhaled particles by a coordinated sweeping motion towards the pharynx; goblet cells are interspersed among them and produce a mucous secretion. The brush and dense core granule cells seem to have a sensory function. The last type is abundant in the lungs of the human fetus and newborn, and it has been suggested that they play a role in the vascular adjustments after birth (Ross, et al., 1989).

The trachea divides into the primary bronchi, which in turn branch into the intrapulmonary bronchi, followed by subdivision into the bronchioles, terminal bronchioles, respiratory bronchioles and alveolar ducts (Figure 1.2). The respiratory tree exhibits features of a fractal tree; branching continues until 17 orders of branches are formed before birth and a further 7 orders of branches are formed after birth (Nelson, et al., 1990; Moore, et al., 2003). The intrapulmonary bronchi have a similar structure to the trachea, except that the cartilage rings are replaced by cartilage plates and there is a narrow layer of
smooth muscle between the lamina propria and the submucosa (Eroschenko, 2005).

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**Figure 1.2: Branching of the respiratory tree**

A simplified model of the different generations of the respiratory tree, with an alveolar unit in the enlargement.

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At the bronchiolar level, cartilage and bronchial glands are absent and goblet cells are seen only occasionally. The terminal bronchioles are lined with columnar ciliary epithelium; goblet cells are normally not present (Ross, et al., 1989). Both the bronchioles and terminal bronchioles have mucosal folds in the lumen of the airway, as a result of smooth muscle activity.

The terminal bronchioles branch into the respiratory bronchioles, which represent a transitional zone between conducting and respiratory portions of the airways.

The respiratory bronchiole is lined with low columnar or cuboidal epithelium, which may be ciliated in the proximal portion of the respiratory bronchioles. A thin layer of connective tissue supports the smooth muscle, the elastic layers of the lamina propria and the blood vessels.
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Alveoli appear in the wall of the respiratory bronchiole as small evaginations and each respiratory bronchiole divides into several alveolar ducts (Eroschenko, 2005). These are linear air tracts which open into alveoli. Near the terminal end of an alveolar duct, clusters of alveoli, referred to as an alveolar sac, share a common opening into the alveolar duct (Ross, et al., 1989).

The alveolar wall is lined by a thin layer of squamous alveolar cells (type I AECs), covering ~95% of its area. In addition, great alveolar cells (type II AECs), brush cells and alveolar macrophages are seen. Type II cells are mainly seen at septal junctions. There are similar numbers of type I and type II cells; however, type II cells cover only 2-5% of the alveolar surface, because of their cuboidal instead of squamous shape (Ross, et al., 1989). Adjacent alveoli share a common alveolar septum, and whilst this is an extremely thin layer, it contains a rich capillary network and connective tissue elements, namely collagenous and elastic fibres, fibroblasts and macrophages.

1.1.3. Connective tissue

Connective tissue provides structural and metabolic support in tissues throughout the body. It is derived of mesenchyme; extracellular material and cells, such as fibroblasts, adipocytes and immune cells (monocyte-macrophage cells) are the two main components. The extracellular material consists of glycosaminoglycans as ground substance, and three different fibre types (collagen, elastin and reticulin). Fibroblasts synthesise and secrete the precursors of collagen (tropocollagen), elastin (tropoelastin), reticulin and glycosaminoglycans. Collagen is the principal fibre found in the matrix of all connective tissue, and can be divided into different types based on morphology, amino-acid composition and physical properties (Wheater, et al., 1984).

Lung myofibroblasts, intermediate in phenotype to smooth muscle cells and fibroblasts, express alpha smooth muscle actin (αSMA) on immunohistochemistry (Leslie, et al., 1992). This technique can been used to locate myofibroblasts during varying developmental stages. These cells first appear during the pseudoglandular stage and their numbers increase proportionally to the maturation of the terminal airways and vascular system in
human fetal tissue. Small clusters of these cells are found at the tip of secondary septal crests (Figure 1.3). They seem to play an important role in airway branching and tissue modelling during development and in tissue remodelling and tissue repair later in life (Leslie, et al., 1992; Leslie, et al., 1990).

**Figure 1.3:** Schematic representation of a secondary septal crest

A. Formation of a secondary septal crest, with elastin (black dot) and a myofibroblasts (green dot) visible at the tip. Capillaries are red. Adapted from Burri (Burri, 2006).

B. Secondary septal crests have formed an alveolus. Elastin, myofibroblasts and collagen fibres (blue lines) are located in the tip. Capillaries are red.

Elastin and collagen fibres form spirals around the larger airways, extend to encircle the alveolar openings, and then form a helix around the alveoli. This “spring coil” architecture in the larger airways and honeycomb architecture at alveolar level provides stable alveolar opening, whilst permitting reversible changes in linear and circumferential airway dimensions during respiratory movements (Young, et al., 1980).

Collagen type I-IV fibres are the most important collagen fibres in the lung. During development type I is involved in airway branching (Heine, et al., 1990). Type III collagen fibres and αSMA expressing cells, co-located in the tip of the septal crest, are thought to have a common involvement in septal crest formation (Figure 1.3) (Wright, et al., 1999). Type IV fibres maintain structure and integrity of the basement membrane (Sweet, et al., 2001). In babies who later developed
BPD, increased levels of type I and IV collagenase (MMP-8 and MMP-9) were found in broncho-alveolar lavage fluid, suggesting that these enzymes are involved in early tissue destruction in BPD, ultimately contributing to the abnormal alveolarisation (Sweet, et al., 2001). Histological analysis of lung tissue from babies with BPD who died demonstrated increased levels of parenchymal collagen. In addition, collagen fibres were thickened, tortuous and disorganised relative to tissue obtained from age-matched babies who died of a non-pulmonary cause (Thibeault, et al., 2003).

During lung development elastin deposition is also required for airway branching and alveolar development (Figure 1.3) and in postnatal life it is essential for lung recoil (McGowan, 1992). First deposition of elastin is seen during the pseudoglandular stage and elastin synthesis reaches a peak during the alveolar stage of lung development (Thibeault, et al., 2000). Elastin is deposited at the tip of the (future) secondary septal crest (Figure 1.3), and although a causal relationship between alveolarisation and elastin deposition has not been established, elastin null mice have inhibited airway branching and alveolarisation (Thibeault, et al., 2000; Wendel, et al., 2000). Prematurity and mechanical ventilation can interfere with normal elastin deposition and lung development. Lung tissue of infants who suffered from BPD showed poor alveolarisation with thickened, tortuous and abnormally distributed elastic fibres (Margraf, et al., 1991). Chronically ventilated preterm lambs and baboons with VILI had reduced secondary crest formation, poor alveolarisation and increased amounts of elastin with an abnormal distribution (Albertine, et al., 1999; Coalson, et al., 1999; Pierce, et al., 1997). It is hypothesised that reabsorption of secondary septal crests into the primary alveolar wall, rather than abnormal development, account for reduction in number of secondary crests and poor alveolarisation, since a significant lower septal crest density was found within 1 hour of intra-uterine ventilation of fetal preterm sheep (Allison, et al., 2008).
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1.1.4. Molecular pathways of lung development

Lung morphogenesis is very complex and is tightly controlled by many factors, such as transcription factors, growth factors, extracellular matrix molecules, integrins and intercellular adhesion molecules. Some of these are produced in the epithelium, such as TTF-1 (thyroid transcription factor 1) and Ssh (Sonic hedgehog growth factor), while others like FGF (fibroblast growth factor) and EGF (epidermal growth factor) are located in the mesenchyme (Copland, et al., 2004). Together, these factors influence branching, laterality and vascularisation of the developing airways and the response to mechanical stress (Copland, et al., 2004). Despite widespread interest in this area, our understanding of the mechanisms influencing lung development remains limited.

In the next section a brief overview of this development will be given, without the intention to be complete, since the complex interactions between the various growth factors and transcription factors are outside the scope of this thesis.

Molecular analyses have demonstrated that Ssh and FGF play important roles in the early development and branching of the respiratory system. Deletion of an FGF receptor (FGF2-IIIb) in the mouse resulted in an arrest of lung development after the trachea was formed and apoptosis in the strip of mesenchymal and epithelial cells that should have formed the lungs (De Moerlooze, et al., 2000). In humans, abnormalities in FGF signalling have been linked to craniosynostosis syndromes, in which tracheo-bronchial rings may also be affected (Whitsett, 2006; Haslam, 2000).

Ssh regulates many transcription factors, all ultimately influencing expression of transforming growth factor-β (TGF-β), affecting branching and laterality (Copland, et al., 2004). Whereas FGF promotes branching, TGF-β inhibits branching in cultures of lung buds from mouse embryos (Kotecha, 2000; Serra, et al., 1994). The transcription factor TGF-β decreases during gestation, allowing the branching of the respiratory system to occur (Kotecha, 2000). Mice mutants for Ssh and Gli2 (one of the transcription factors influenced by Ssh) show a variety of left-right isomerisms (Motoyama, et al., 1998). The transcription factor Pixt2 is also a powerful determinant of laterality; mice deficient in Pixt2 had
right pulmonary isomerism and altered cardiac position (Lin, et al., 1999). Disruption of other transcription factors activated by Ssh, such as Ptch1 (Patched 1) and HIP1 (hedgehog-interacting protein 1) (Copland, et al., 2004) results in altered epithelial differentiation and in various forms of malformations, e.g. tracheo-oesophageal fistula, oesophageal atresia and branching abnormalities of the airways (Chuang, et al., 2003; Miller, et al., 2001). In humans, abnormalities in Shh signalling have been linked to syndromes with respiratory tract malformations, such as Smith-Lemli-Opitz (a metabolic defect in cholesterol synthesis, leading to cleft palate and, rarely, cleft larynx plus mental retardation) and VACTERL (a mnemonic acronym for several birth defects: vertebral anomalies; anal atresia; cardiac defects; tracheo-oesophageal malformations; renal problems, and limb anomalies).

Another important factor in early lung development is the transcription factor TTF-1. Mice deficient in TTF-1 suffer from lung hypoplasia, thyroid hypoplasia and tracheo-oesophageal malformations, with the arrest of lung development occurring during the pseudoglandular stage (Yuan, et al., 2000; Minoo, et al., 1999).

A key characteristic of pulmonary development in late gestation is the thinning of interstitial tissue, causing the vessels to re-align in close proximity to the respiratory epithelium, thus forming the areas relevant for gas exchange. Apoptosis plays an important role in thinning of the interstitium, and is mediated by pro-apoptotic factors, such as TGF-β, and anti-apoptotic factors, such as insulin like growth factor 1 (Groenman, et al., 2005).

During this process secondary septal crests are formed (see Figure 1.3). Initially, these crests contain a double capillary layer separated by a sheet of connective tissue, which changes into a single capillary layer during maturation (Burri, 2006), largely under the influence of TTF-1 and vascular endothelial growth factor (VEGF).

The complex vascular development of the lung is influenced by interactions between cells, between cells and the extracellular matrix, and by different growth and transcription factors. Specifically, members of the VEGF-family have
been implicated in vascular development. VEGF is up-regulated in fetal sheep which have undergone obstruction of the left main bronchus for 36 hours at 126 days' gestation. Other up-regulated genes were cysteine rich-61 (CYR61), connective tissue growth factor (CTGF) and heat shock protein 47 (HSP47) (Sozo, et al., 2006). In embryonic mouse lungs, VEGF was initially expressed in the proximal airways; however, expression was restricted to the branching tips of the airways at later time points. Furthermore, a neovascular response was generated when VEGF-containing beads were grafted onto lung explants, thus strongly suggesting a link between airway branching, vascularisation and VEGF (Healy, et al., 2000).

1.1.5. Fetal lung liquid

Before birth the lungs are filled with a liquid, once thought to originate from inhaled amniotic fluid. However, the composition of lung fluid is quite distinct from that of amniotic fluid in osmolarity and electrolyte concentrations (Harding, 1994).

Lung fluid is secreted across the pulmonary epithelium, using Na⁺K⁺ATPase activity as the driving force (Harding, 1994; Harding, et al., 1996). The rate of secretion increases during gestation, which might be due, in part, to the large increase in epithelial surface area during the canalicular phase of lung development. In fetal sheep the secretion of lung fluid is 3-4 mL/Kg.h in the last 30 days of gestation (total length of gestation ~145 days) up to a total of approximately 40-50 mL/Kg within the lung (Harding, et al., 1996). This volume is about double the analogous volume in air-filled lungs (functional residual capacity; FRC) after delivery of the lamb (Harding, et al., 1996).

The upper airway of the fetus restricts efflux of fluid via the trachea, allowing accumulation of fluid in the future airways. The resulting distending pressure (about 1-2 mmHg) keeps the lungs expanded (Harding, 1994). Obstruction of the fetal trachea is a potent stimulus for lung growth (Harding, et al., 1996). Lung expansion provides a stretch stimulus that stimulates DNA synthesis (Harding, et al., 1996) and the expression of genes essential for lung growth and vascular development. In fetal sheep, obstruction of the trachea at 118-124 days'
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gestation stimulates DNA synthesis rates, with maximum rates two days after obstruction (Nardo, et al., 1998). The opposite is also true: reduction of lung fluid by continuous drainage of lung liquid in fetal sheep from 105 to 129 days’ gestation results in hypoplastic lungs with a lower weight and reduced DNA content (Moessinger, et al., 1990).

1.1.6. Fetal breathing movements

Fetal breathing movements (FBM) are observed as early as 10 weeks’ gestational age (Harding, 1994). FBM are intermittent and linked to activity status of the fetus, as opposed to the regular respiratory movements after birth (Harding, 1994). Mechanics of the FBM differ from postnatal breathing: in fetal sheep the ingress of lung fluid is less than 1 mL, equivalent to less than 1% of FRC, whereas the tidal volume in a newborn is approximately 20% of FRC (Harding, 1994).

Maintenance of a correct amount of lung liquid inside the lung is important for lung growth. In a sheep fetus a maximum of 2 mL will be lost to the upper airway during an episode of FBM (Harding, 1994). Lung growth is disturbed, leading to pulmonary hypoplasia, if the chest is compressed during fetal development, the lungs are not expanded (for example during oligohydramnios or diaphragmatic hernia), or when fetal breathing is abolished (by spinal cord lesions) (Haslam, 2000). Thus, even though the mechanics of FBM are different from breathing in postnatal life, they do play a role in lung development.

1.1.7. Animal paradigms of human lung development and disease

Animal paradigms have been used widely to explore normal lung development, anatomy and physiology. However, care has to be taken when the results are translated to the human. The stages of lung development might differ in length between animals and humans and end points of development might be different than in humans.

Nonetheless, animal studies have been invaluable for our understanding of the respiratory system, as long as we keep George Orwell’s “All animals are equal, but some animals are more equal than others” in mind.
The lung of the fetal lamb is a commonly used paradigm for lung development, since the developmental stages are relatively similar to those in the human. The pseudoglandular stage extends to 85-90 days, comparable to ~60% gestation (normal gestation 145-147 days); in the human lung this stage extends to ~20 weeks’ gestation, which is comparable to ~ 50% gestation (normal gestation is 40 weeks) (Pringle, 1986). The canalicular and saccular stages in a fetal lamb are more compressed than in the human lung; in the human lung the saccular stage continues beyond term (Pringle, 1986). The alveolar stage in the lamb is well established by 125 days (~86% gestation) with mature type II cells being abundant from 120 days onwards, whereas in the human the alveolar stage begins at approximately 32 weeks’ gestation (80% gestation), but is far from completed before birth and continues for another couple of years (Pringle, 1986; Moore, et al., 2003).

Rodents are also frequently used for animal experiments, since they are smaller, cheaper and have short breeding times. However, their anatomy and development is fundamentally different from the human. The rat lung has a relatively long pseudoglandular stage, extending through the first 19 days of a 22-day gestation (~86% gestation), and alveoli are only found from day 4 post-delivery and onwards (Pringle, 1986). Mice have only 6-8 generations of branching airways, compared to ~ 20 generations in the human, and they lack respiratory bronchioles. Thus, the terminal bronchioles empty straight into the alveolar ducts and alveoli (Bal, et al., 1988).

Nevertheless, studies of acute lung injury in rodents have provided a wealth of information that helps better understand the pathophysiological mechanisms of respiratory disease. The severity of the acute lung injury that can be created in rodents is limited; more severe injury would result in death of the animals or the animals would need high levels of intensive care. The level of intensive care treatment available for these animals is limited, partly as a result of their small size and subsequent limitations to care and partly for financial reasons (Ware, 2008). Furthermore, care has to be taken to translate outcomes of certain experimental paradigms to humans since the mechanisms in animals might be different from those in humans. One of the examples for this is the ability to...
regenerate alveolar tissue in rodents after drug induced pulmonary fibrosis. Bleomycin is used as a cancer drug, but a side effect is pulmonary fibrosis. This drug is, therefore, widely used in rodents to induce pulmonary fibrosis followed by investigating of the therapeutic options to reduce or mitigate the scar tissue. However, for the human lung, just the ability to halt scar tissue formation would be a major improvement in therapy (Moore, et al., 2008).

1.2. Physiology of the respiratory system

Respiration is the exchange of oxygen and carbon dioxide between blood and the atmosphere. This process involves ventilation (the movement of air in and out of the lung), gas exchange between air in the lungs and blood, oxygen and carbon dioxide transport in blood and gas exchange between blood and tissues. Air enters the lungs via the upper respiratory tract, consisting of the nasal cavity and the pharynx, and continues via the lower respiratory tract, consisting of the larynx, trachea, bronchi and the lungs. Along the way air is heated and humidified (Seeley, et al., 1992). Furthermore, inhaled particles are trapped in the mucus layer deposited on top of the ciliated cells and are removed from the airways by movement of the cilia which line the nasal cavities to the bronchioles (Wood, et al., 1990).

Air flows from an area with a higher pressure to an area with a lower pressure, following the same physics as fluid flowing through a tube. This relationship can be described as (Seeley, et al., 1992):

\[ F = \frac{(P_1-P_2)}{R}, \]

where

- \( F \) = air flow (litre per minute) in a tube
- \( P_1 \) = pressure at a point called \( P_1 \) (cmH\(_2\)O)
- \( P_2 \) = pressure at a point called \( P_2 \) (cmH\(_2\)O)
- \( R \) = resistance to air flow (cmH\(_2\)O/L.min)

The diaphragm is responsible for the majority of the respiratory workload. Depending on its initial shape, the diaphragm can generate more or less force.
Normally, the diaphragm is a dome-shaped muscle that flattens as it contracts during inspiration, thereby reducing intrapulmonary pressure below atmospheric pressure and causing air to enter the lungs (Wood, 2003). Preterm babies are at a mechanical disadvantage, as the insertion of the muscle fibres of the diaphragm is more horizontal (Wood, 2003). The average inspiratory flow at which inspiratory air enters the lung in a healthy, spontaneously breathing baby is 2.6 to 2.9 L/min as measured by a pneumotachograph (Swyer, et al., 1960; Harrison, et al., 1968). Expiration is a largely passive process based on elastic recoil of the lung tissue and surface tension forces (Wood, 2003). The intrapulmonary pressure increases above the level of the atmospheric pressure, resulting in expiratory air flow (Seeley, et al., 1992; Wood, et al., 1990) of approximately 2.2 to 2.4 L/min in a term baby (Swyer, et al., 1960; Harrison, et al., 1968). The pressure differences are small (1-2 mmHg). Nevertheless, air flows readily through the airways, since normally the resistance to airflow is small.

1.2.1. Resistance

Resistance is the result of friction. Total pulmonary resistance is made up of viscous resistance and airway resistance. The viscous resistance may account for up to 40% of the total resistance in a newborn baby, since the relative amount of pulmonary fluid, especially after a caesarean section delivery, is high. Furthermore, the ratio of lung volume to lung weight is low, resulting in more friction (Wood, 2003).

Airway resistance is the resistance that occurs between gas molecules and the walls of respiratory tract. Airway resistance is mainly determined by the diameter of the tube, its length, the viscosity of gas and the nature of the airflow (laminar or turbulent) (Wood, et al., 1990). During laminar flow, resistance is inversely proportional to the 4th power of the radius of the airway. However, resistance increases exponentially when a turbulent flow pattern is present (Wood, 2003). Because of the small diameter of the airways of an infant, even a modest narrowing will lead to an increased resistance.
Resistance is lower during inspiration compared with expiration, even though the inspiratory flow is usually faster. This results from a dilatation of the airways upon inspiration, caused by an increase in lung volume (Wood, 2003).

1.2.2. Compliance

Compliance is a measure of expansibility of the lungs and is expressed as the volume of air (in litres) by which the lungs increase for each unit of change in the intrapulmonary pressure (cmH\textsubscript{2}O) \((\text{compliance} = \Delta V/\Delta P)\).

Static compliance can be obtained by measuring the transpulmonary pressure before and after inflation of the lung with a known volume of gas. The transpulmonary pressure is the pressure difference between alveolar and pleural pressure and can be approximated by measuring the pressure at the mouth and in the oesophagus. Static compliance is the inverse of elastic recoil (Wood, 2003).

Dynamic compliance is measured during spontaneous breathing and reflects elastic recoil. The pressure-volume curve is frequently used to quantify airway resistance and lung compliance (Figure 1.4). In a healthy adult the normal compliance is 0.13 L/cmH\textsubscript{2}O (Seeley, et al., 1992); however, lung compliance is related to lung size (Wood, 2003) and for healthy babies the compliance is normally 0.005 L/cmH\textsubscript{2}O (Bancalari, 1986). A normal compliance is situated in the middle of the sigmoid curve of a lung expansion curve (Figure 1.5). A low compliance is seen when it is more difficult to expand the lungs, for example during atelectasis, surfactant deficiency in the preterm infant, pulmonary fibrosis (deposition of non-elastic fibres in the lung), or deformities in the thoracic wall (severe scoliosis) (Seeley, et al., 1992). Thus, a large change in pressure only results in a small change in volume (Figure 1.4). In lung emphysema the elastic tissue is damaged, reducing the elastic recoil, making the expansion easier and thus increasing compliance to high values (Seeley, et al., 1992). If overexpansion occurs, for example by air trapping during meconium aspiration syndrome or excessive application of distending pressure during ventilation, the compliance will decrease again as can be seen at the upper end of the lung expansion curve.
Figure 1.4: Pressure-volume curve

The left curve represents a pressure-volume curve for a healthy newborn with a normal compliance, whereas the curve on the right represents a baby with surfactant deficiency leading to respiratory distress syndrome (RDS) with a low compliance. Adapted from Wood (Wood, 2003).
Figure 1.5: Lung expansion curve

Area $a$ represents a disease state with a low compliance; area $b$ represents normal compliance and area $c$ represents an overexpanded lung, resulting in a low compliance. Adapted from Wood (Wood, 2003).

1.2.3. Normal lung volumes and capacities

During breathing, volumes of air enter and exit the lungs. Spirometry can be used to measure these pulmonary volumes. The tidal volume (TV) is the volume of air inspired or expired during a normal inspiration or expiration. On top of a normal inspiration an extra amount of air can be forcefully inspired, called the inspiratory reserve volume (IRV). After a normal expiration an extra amount of air can be forcefully expired, called the expiratory reserve volume (ERV). The volume of air that remains in the lungs and airways after this forceful expiration is called the residual volume (RV) (Wood, 2003; Seeley, et al., 1992).

Pulmonary capacities are the sum of two or more pulmonary volumes. Functional residual capacity (FRC) is the sum of ERV and RV. This is the volume of air remaining in the lung after a normal expiration. The vital capacity (VC) is
the sum of ERV + TV + IRV. This is the maximum volume a person can expire after a maximum inspiration. The total lung capacity (TLC) is sum of IRV + TV + ERV + RV.

Numbers for both the volumes and capacities change with age, sex, body size and disease status (Wood, 2003; Seeley, et al., 1992).

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**Figure 1.6: Schematic representation of the different lung volumes and capacities**

IRV, inspiratory reserve volume; TV, tidal volume; ERV, expiratory reserve volume; RV, residual lung volume; FRC, functional residual capacity; VC, vital capacity; TLC, total lung capacity. On the right hand side normal breathing in the tidal volume range, a maximum inspiration and a maximum expiration are shown.

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The tidal volume is used to calculate the minute respiratory volume (MRV) by multiplying the TV with the respiratory rate (R rate), thus MRV = TV x R rate. This is the amount of air moving in and out of the airways; however, the amount of air available for gas exchange is less than the MRV. Air not taking part in the
gas exchange is called dead space air and can be divided into anatomical dead air space, i.e. the air accumulated in upper respiratory tract, and physiological dead air space, i.e. the air in non-functional alveoli (Wood, 2003; Seeley, et al., 1992). Normally, the anatomical dead air space takes up about 30% of the tidal volume (Wood, et al., 1990).

1.2.4. Time constant

The time it takes to inflate or deflate the lungs, or the time it takes for alveolar and proximal airway pressures to equilibrate, is referred to as the time constant of a patient’s respiratory system. The time constant can change over time, if compliance or resistance changes. For example, shortly after surfactant treatment in a baby with respiratory distress syndrome, the compliance increases and subsequently the time constant will lengthen.

Exhalation is a mainly passive process, based on elastic recoil of the lung and chest wall and opposed by the airway resistance. The expiratory time constant can be calculated by multiplying lung compliance, the inverse of elastic recoil, and airway resistance (Wood, 2003).

One time constant is defined as the time it takes to discharge 63% of the tidal volume, three time constants are defined as the time it takes to discharge 95% of the tidal volume (Wood, 2003). Knowledge of these time constants becomes important during mechanical ventilation. For a healthy newborn baby the compliance is normally 0.005 L/cmH₂O and the resistance 30 cmH₂O; thus, one time constant is 0.15 seconds. The time it takes to exhale 95% of the tidal volume from the lungs will therefore take 0.45 seconds (Bancalari, 1986). If the expiratory time on the ventilator is set to be shorter than 0.45 seconds, the lungs cannot be completely emptied and air trapping will occur. Different lung conditions will result in different time constants. Infants suffering from surfactant deficiency will have a low compliance, and thus very short time constants, i.e. the lung will empty very quickly.
1.2.5. **Work of breathing**

For ventilation to occur gas must be moved into and out of the lungs and elastic and resistive forces have to be overcome. Thus, breathing requires expenditure of energy. Work of breathing is dependent on elastic forces in the lung and chest wall, pulmonary resistance, tidal volume and rate of breathing. Approximately two thirds of the work of breathing is to overcome the elastic forces, about one third is to overcome the resistive forces.

Work of breathing can be illustrated in a pressure / volume loop (Figure 1.7). If only elastic forces needed to be overcome, there would be a straight compliance line as the pressure / volume loop. However, airway resistance must also be overcome during both inspiration and expiration, thus creating the hysteresis loop. The total work of breathing is represented by the entire area ABCEA (Wood, 2003).
**Figure 1.7: Pressure-volume loop**

Pressure-volume loop showing the compliance line (AC), work done during inspiration to overcome resistance (dotted area ABCA) and work done during expiration (striped area CDAC). The total work of breathing is represented by the entire area ABCEA. Adapted from Wood (Wood, 2003).

### 1.2.6. Preterm lung

Preterm babies are faced with several disadvantages, both during birth transition and thereafter. Establishing and maintaining a normal FRC is one of the main problems. The preterm lung has a low compliance, due in part to progressive atelectasis caused by surfactant deficiency and absent or decreased numbers of alveoli. Alveoli that are present are of reduced size, making them more vulnerable to collapse as explained by the Laplace relationship (Wood, 2003). The highly compliant chest wall and muscle hypotonia offer little opposition against collapse of the lung towards end-expiration. Furthermore, the surface area for gas exchange is low, since the alveolar septation is far from complete (Davis, *et al.*, 2008; Wood, 2003).
Airway resistance in preterm babies can increase markedly after a modest narrowing of the airways, because of the small diameter of the bronchial tree (Wood, 2003).

The combination of these factors can lead to a 30% increase in work of breathing in the preterm baby (Seeley, et al., 1992). This excess workload to perform ventilation causes fatigue of the respiratory muscles and can lead to respiratory failure.

### 1.3. Respiratory distress syndrome

Respiratory distress syndrome (RDS), or hyaline membrane disease, is caused by lack of surfactant in the lungs and leads to progressive atelectasis. It has a characteristic clinical course and specific radiographic changes. RDS is a common complication after preterm birth, with an inverse relationship between gestational age and birth weight and a slightly higher incidence in male and white babies (Ramanathan, 2006; Hulsey, et al., 1993; Stoll, et al., 2000; Greenough, et al., 2005). Approximately 50% of babies born at less than 30 weeks’ gestation are affected (Ramanathan, 2006). Other common risk factors for RDS include maternal diabetes (which delays pulmonary maturation), fetal asphyxia (which inhibits surfactant production) and multiple pregnancies (Schwartz, et al., 2000). The effect of multiple pregnancy may simply be explained by the lower gestational age at which these infants are commonly delivered, although RDS is seen more often in the second twin (Rokos, et al., 1968). The increased risk of RDS in the second twin has been related to malpresentation-associated asphyxia (Shinwell, et al., 2004). However, RDS and surfactant use were independent of mode of delivery, malpresentation or birth asphyxia in an historical cohort on preterm twins born between 23 and 31 weeks’ gestation (Hacking, et al., 2001). Similar results were also seen in a population based study in 664 VLBW twin pairs, which reported an increased risk of RDS (OR 1.51, 95% CI 1.29 to 1.76) in the second twin, independent of the mode of delivery (Shinwell, et al., 2004). RDS in both twins occurs more often when they are monozygotic, which has been explained by a genetic contribution via the surfactant protein-A gene (van Sonderen, et al., 2002).
Mortality rates for preterm babies have improved over recent decades, with developments such as surfactant treatment, antenatal steroids, and artificial ventilation (Jobe, *et al.*, 2001a). Recent figures continue to report a decline in mortality rate for preterm infants: in the United States mortality rates have decreased by 19% between 1993 and 2003 (March of Dimes, 2007). Despite these advances, RDS remains the main cause of early neonatal mortality and morbidity, and contributes significantly to the health costs of neonatal intensive care (Crowther, *et al.*, 2007b).

### 1.3.1. Pathology

Lack of surfactant is one of the main problems in the progressive atelectasis of RDS. Alveolar levels of surfactant are low in preterm babies, and surfactant production can be compromised by hypothermia, acidosis and hypoxia. In addition, in preterm infants surfactant can be immature in composition, with fewer lipids and surfactant proteins (Jobe, 2006). This, combined with the highly compliant chest wall and reduced size and reduced number of alveoli, makes preterm babies prone to developing RDS.

Progressive atelectasis leads to ventilation / perfusion mismatch, decreased lung compliance and increased work of breathing. Biochemically, there is hypoxia, hypercarbia and acidosis (Stoll, *et al.*, 2000). This combination produces pulmonary arterial vasoconstriction, which may lead to right-to-left shunting and a further decline in alveolar perfusion. The ischaemic injury further impairs surfactant production and damages the vascular cells, which is followed by an effusion of proteinaceous material into the alveolar spaces (Stoll, *et al.*, 2000).

Macroscopically, the lungs of a deceased RDS patient have a liver-like appearance and, microscopically, there is extensive atelectasis and engorgement of capillaries and lymphatics (Stoll, *et al.*, 2000). The airspaces are lined with granular membranes (the hyaline membranes), although the membranes rarely occur in infants who died within 6-8 hours of birth (Stoll, *et al.*, 2000; Avery, *et al.*, 1959).
1.3.2. History

The first understanding of this process can be traced to the 18th century, when the importance of opening of alveoli, described as lung vesicles, directly after birth, combined with the start of pulmonary circulation, was acknowledged as necessary for survival (Obladen, 2005). The nomenclature of hyaline membrane disease was derived from the protein-rich, shiny, “hyaline membranes” that were observed in the alveoli and small airways of affected preterm babies at autopsy. These membranes initially were believed to consist of aspirated amniotic fluid contents and vernix, and were described later as end products of a malfunctioning coagulation (Obladen, 2005). It was not until 1959 that Mary Ellen Avery described hyaline membrane formation in preterm babies and infants of diabetic mothers, caused by a deficiency of a surface-active material responsible for the lowering of surface tension in the alveoli. Since these infants were lacking this substance, later named surfactant, they developed severe atelectasis and hyaline membranes in their lungs (Avery, et al., 1959).

1.3.3. Clinical presentation

The classical picture of RDS is a preterm baby with respiratory difficulty shortly after birth (grunting, nasal flaring, retractions of the chest wall and oxygen requirement), which becomes progressively more severe in the first hours after birth. Blood gases demonstrate an increasing respiratory acidosis and hypoxaemia. Chest radiography shows a diffuse reticulogranular pattern (“ground class appearance”) with air bronchograms (Notter, 2000).

Ventilatory support may be necessary, based on the clinical condition of the baby and on blood values for PaO₂, PaCO₂ and pH (Stoll, et al., 2000). The baby benefits from positive end-expiratory pressure, which helps to prevent the tendency of the lung to collapse and to develop progressive atelectasis. Treatment with exogenous surfactant should be considered (Stoll, et al., 2000).

In the pre-surfactant and pre-antenatal corticosteroid era, infants with RDS either deteriorated and died in a time frame of 48-72 hours or began to show an improvement in clinical condition preceded by a diuresis. All babies need to clear
fetal lung liquid after delivery, a process that is more difficult for a preterm baby compared with a term baby, since their lungs contain more fluid per unit tissue mass, the lung epithelium has fewer sodium channels, fewer sodium pumps and less Na⁺,K⁺-ATPase activity (Bland, et al., 2008). Furthermore, severe RDS can lead to hypoxia and acidosis, which can reduce renal blood flow and glomerular filtration (Bell, 2003), making it increasingly difficult to generate a sufficient diuresis to alleviate pulmonary oedema. Normally, infants have a rise in circulating atrial natriuretic peptide (ANP) concentrations, a contraction of the extra-cellular water compartment and an increased diuresis 48-72 hours after delivery, followed by an improvement in clinical condition if the infant has RDS (Bell, 2003).

RDS is still a major morbidity in preterm babies, but the clinical picture has changed. Before surfactant and antenatal steroids were widespread, severe RDS was seen in babies as big as 2 Kg or up to 34 weeks’ gestation (Northway, et al., 1967), or even later. Nowadays, preterm babies still develop RDS, but with improvements in care such as antenatal steroids and surfactant, mortality has decreased.

1.3.4. Antenatal treatment

Antenatal steroids

Liggins was the first to report the effect of antenatal corticosteroid treatment on the functional maturation of the fetal lamb lung (Liggins, 1969). He conducted the first randomised controlled clinical trial to assess the effects of antenatal corticosteroid treatment in mothers with threatened or planned preterm delivery before 37 weeks’ gestation on the lung development of their offspring (Liggins, et al., 1972). He reported a significant decrease in the incidence of RDS (25.8% to 9.0%, p=0.003), although this was confined to babies born before 32 weeks’ gestation and treated at least 24 hours before delivery (Liggins, et al., 1972). This trial was published in 1972, and the first structured Cochrane review was published in 1990 by Crowley, clearly demonstrating a favourable effect of antenatal steroids on respiratory distress syndrome, neonatal mortality and intraventricular haemorrhage (Crowley, 2006).
Since then a meta-analysis of a total of 21 trials comparing antenatal treatment with betamethasone, dexamethasone or hydrocortisone (corticosteroids which are able to cross the placenta) with placebo or no treatment has been published, including 3,885 women and their babies. This meta-analysis confirmed that antenatal corticosteroids result in a 30% reduction in neonatal death and a 35% reduction in RDS, which was more pronounced for severe RDS, but no effect on bronchopulmonary dysplasia (Roberts, et al.). These benefits were apparent even in the presence of prolonged rupture of the membranes, if the membranes had not been ruptured for more than 48 hours. Secondary outcomes were consistent with these primary outcomes, in that less respiratory support was needed after antenatal exposure to steroids (described as either a reduction in need or duration of mechanical ventilation / CPAP, days on supplemental oxygen, or a trend towards reduction in need for surfactant) (Roberts, et al., 2006). Reduction of RDS was reported when glucocorticoids are administered between 26 and 34 6/7 weeks' gestation (Roberts, et al., 2006). Antenatal corticosteroids have, however, also been demonstrated to be beneficial at a later gestational age, prior to elective caesarian section at 37 weeks' gestation or beyond. Steroid treatment resulted in a reduction of admission to a special care baby unit for respiratory distress (relative risk (RR) 0.46, 95% CI 0.23 to 0.93), an effect that persisted until 39 weeks' gestation (Stutchfield, et al., 2005).

Reduction in RDS (RR 0.66, 95% CI 0.59 to 0.73) is seen up to 7 days after the first dose of antenatal steroids (Roberts, et al.), but not thereafter (McLaughlin, et al., 2003). Liggins and Howie were the first to suggest that repeated doses of steroids at weekly intervals may be beneficial in those women who had not delivered but remained at risk of preterm birth, but that further work was needed before this could be implemented as routine clinical care (Liggins, et al., 1972). A preterm lamb paradigm using weekly maternal injections of betamethasone starting at 104 days of gestation (normal gestation 147 days) showed improvement in lung function in the offspring (delivered at 125 days): there was an improvement in pressure-volume curves combined with lower PaCO₂ values and better ventilator efficiency indices (an integrated measurement of gas exchange) (Willet, et al., 2001). It was suggested that the
improved lung function in preterm lambs was due to a relatively rapid thinning of the alveolar wall and a slower, longer lasting effect on the surfactant system (Willet, *et al*., 2001). This study also evaluated alveolar formation, but did not find any inhibition of alveolarisation following repeated courses of steroids, in contrast to previous studies in rhesus macaques and rats. The macaques had delayed alveolarisation, and the rats had impaired septation, which persisted to term equivalent age after the cessation of treatment with steroids (Massaro, *et al*., 1986; Bunton, *et al*., 1984). However, the changes in lung structure seen in the preterm lambs following repeated doses of antenatal glucocorticoid were deemed to be reversible, since the changes were not seen when lambs were delivered at 145 days (term 147 days). Repeated doses of antenatal corticosteroids in sheep are clearly beneficial for pulmonary function; however, preterm lambs were growth restricted following antenatal steroids (Ikegami, *et al*., 1997).

A Cochrane review of the effect of repeat doses of antenatal corticosteroids in the clinical setting was first published in 2000 (Crowther, *et al*., 2000). In the latest update, five studies were included, involving 2,028 women, between 23 and 33 weeks’ gestation, at continued risk of preterm birth after their first dose of corticosteroids (Crowther, *et al*., 2007b). Significantly fewer infants had RDS (RR 0.82, 95% CI 0.72 to 0.93), surfactant treatment (RR 0.71, 95% CI 0.61 to 0.83) or serious morbidity (RR 0.79, 95% CI 0.67 to 0.93). Although there was a reduction in severe lung disease (RR 0.60, 95% CI 0.48 to 0.75), no significant difference was seen for the need of mechanical ventilation (RR 0.72, 95% CI 0.51 to 1.02) or the development of BPD (RR 0.95, 95% CI 0.75 to 1.21). Mean birth weights were not different between treatment groups; however, one trial demonstrated a reduction in birth weight Z-score (weighted mean difference (WMD) -0.13, 95% CI -0.26 to 0.00) and two trials an increase in babies born small for gestational age (RR 1.63, 95% CI 1.12 to 2.37) (Crowther, *et al*., 2007b). Since then the results from the multiple courses of antenatal corticosteroids for preterm birth trial, the MACS trial, have become available (Murphy, *et al*., 2008). The results of this trial, performed in 1,858 women between 25 and 32 weeks’ gestation, who remained undelivered 14-21 days after the initial course of
antenatal steroids, demonstrated no advantage on the composite primary outcome of perinatal or neonatal mortality, severe RDS, BPD, intraventricular haemorrhage, periventricular leucomalacia or necrotizing enterocolitis (mean difference 1.04, 95% CI 0.77 to 1.39), nor on individual outcomes for RDS (mean difference 1.14, 95% CI 0.80 to 1.58) or BPD (mean difference 1.50, 95% CI 0.68 to 2.95) (Murphy, et al., 2008). However, there was a significant reduction in body weight, length and head circumference (all p<0.01) in the babies exposed to multiple courses of steroids (Murphy, et al., 2008). Preliminary data on an update for the Cochrane review (Crowther, et al., 2007b) included a total of 9 studies and 5,585 babies and demonstrated that repeat doses decreased neonatal lung disease (RR 0.84, 95% CI 0.76 to 0.92) and composite neonatal morbidity (RR 0.84, 95% CI 0.75 to 0.94) (McKinlay, et al., 2010). Birth weight was significantly reduced (mean difference -77 g, 95% CI -119 to -34) without any effect on gestational age at birth (McKinlay, et al., 2010). Thus, repeated courses of antenatal steroids improve the short-term neonatal outcomes; however, this is at a cost to birth weight.

Concerns about the long-term effects of single or repeat doses of antenatal corticosteroids on areas such as neurodevelopment, cerebral palsy, childhood behaviour and endocrinological development have been raised in many studies (non-randomised / observational). An Australasian RCT on repeat doses of antenatal corticosteroids reported outcome data on 1,085 children at 2 years of age. Compared with placebo treatment, no difference was seen in the rate of survival free of major disability (RR 1.04, 95% CI 0.98 to 1.10), child behaviour scores, blood pressure or body size, although more infants needed assessment for attention problems (p = 0.04) following treatment with repeat doses of antenatal steroids (Crowther, et al., 2007a). The 7 year follow-up of this study has just been completed and the results are awaited. A 2 to 3 year follow-up study comparing single dose to repeat doses of antenatal corticosteroids showed no difference in physical or neurocognitive measures (Wapner, et al., 2007). The 18 to 24 month follow-up of the MACS trial reported similar rates of death or neurological impairment and similar length or head circumference in children exposed to repeat doses of corticosteroids compared with a single dose. Weight
was slightly lower (WMD -0.19, 95% CI -0.38 to 0.01; with a reported significance value of \( p = 0.04 \)) following repeat doses of steroids (Asztalos, et al., 2000). Furthermore, links between corticosteroid exposure and adult cardiovascular disease and diabetes have been suggested in observational studies following a single dose of steroids (Doyle, et al., 2000) and in animals following repeated doses of steroids (Benediktsson, et al., 1993; Nyirenda, et al., 2001). In 30-year old human offspring of the original and largest RCT of a single dose of antenatal glucocorticoids, no differences in cardiovascular disease risk factors were identified, although increased insulin concentrations 30 minutes after a glucose bolus were recorded in offspring treated with a single dose of antenatal steroids compared with placebo (Dalziel, et al., 2005). Thus, treatments with single and repeat doses of antenatal corticosteroids have been shown to reduce the risk of RDS and neonatal morbidity. However, caution is warranted in the use of repeat doses of steroids, since the long-term cardiovascular, endocrine and neurodevelopmental effects remain uncertain as yet.

**Antenatal antibiotic treatment**

Routine use of antenatal antibiotic therapy has been recommended in women with preterm prelabour rupture of membranes (PPROM) (Kenyon, et al., 2003), but not in women with preterm labour (PTL) (King, et al., 2002). The Cochrane review by Kenyon et al reported on the use of antibiotic treatment in over 6,000 women with PPROM before 37 weeks’ gestation, which resulted in a reduced risk of delivery within 48 hours (RR 0.71, 95% CI 0.58 to 0.87) or even 7 days (RR 0.80, 95% CI 0.71 to 0.90) (Kenyon, et al., 2003). Secondary outcomes also improved, with fewer babies needing surfactant treatment (RR 0.83, 95% CI 0.72 to 0.96) or any oxygen treatment (RR 0.88, 95% CI 0.81 to 0.96), but the number of babies needing oxygen treatment at 36 weeks’ corrected gestation (BPD) did not change. However, only one trial contributed to these respiratory data (ORACLE I); this study included 4,809 babies. The main outcomes for the ORACLE I study (composite measure of death; BPD, defined as supplementary oxygen treatment at 36 weeks; or major cerebral abnormality at discharge) did
not significantly differ between antibiotics and placebo treatment (Kenyon, et al., 2001).

The Cochrane review by King et al reported on the use of antibiotic treatment in 7,428 women with PTL with intact membranes before 36 weeks’ gestation, which reduced maternal infection, but did not change the prespecified neonatal outcomes (King, et al., 2002). There was a trend, however, towards increased infant mortality in the treated group (RR 1.52, 95% CI 0.99 to 2.34), which led to the recommendation to not use routine antenatal antibiotics for PTL (King, et al., 2002).

These outcomes were also found for lower gestations (less than 34 weeks). A meta-analysis performed on 21 studies, including 9,896 women presenting with either PPROM or PTL at 22 to 34 weeks’ gestation, concluded that antenatal antibiotic treatment prolongs pregnancy in PPROM (p<0.01), but not PTL, as measured by average latency in days, or delivery within 48 h or 7 days (Hutzal, et al., 2008). The potential advantage of delaying the delivery, even for 1-2 days, may be greatest for babies born before 34 weeks’ gestation, since this may facilitate administration of antenatal corticosteroids. In contrast with the Cochrane review on PPROM, this study did not find a significant difference in RDS or BPD between treatment groups, which possibly can be explained by the relatively low use of antenatal corticosteroids (only 9% of women) (Hutzal, et al., 2008) compared with 76 % in ORACLE I (Kenyon, et al., 2001) and 30 to over 90 % in the Cochrane meta-analysis on PTL (King, et al., 2002).

The ORACLE I study (on PPROM) collected follow-up data in 75% of eligible children at 7 years of age (a total of 3,298 children were tested). No significant difference was found in functional impairment or educational outcomes after prescription of antenatal antibiotics (Kenyon, et al., 2008a). The ORACLE II study (on PTL) collected data in 71% of eligible children at 7 years of age (a total of 3,196 children were tested). The prescription of erythromycin, with or without amoxicillin-clavulanate, increased functional impairment compared with no erythromycin (OR 1.18, 95% CI 1.02 to 1.37). However, the prescription of amoxicillin-clavulanate, with or without erythromycin, did not lead to significant
differences in functional impairment compared with no amoxicillin-clavulanate. Educational outcomes and behaviour were not different, but there was a slightly increased risk of cerebral palsy with any antibiotic treatment when compared with no treatment (Kenyon, et al., 2008b). Therefore, the follow-up data support the current recommendations to treat women with PPROM with erythromycin, but not to prescribe antibiotics for women with PTL without signs of infection.

1.3.5. Postnatal treatment with surfactant

As mentioned previously, surfactant has surface tension lowering qualities and is an efficacious treatment in babies suffering from RDS who have lungs with a tendency to collapse.

The first clinically successful treatment with surfactant of 10 neonates suffering from RDS was reported by Fujiwara in 1980 (Fujiwara, et al., 1980). In a multicentre trial including 146 neonates with RDS (BW 700-2,000 g), mortality rate was reduced from 51% to 31% and the incidence of pneumothorax was reduced from 35% to 18% with one single large dose of poractant alfa, a natural surfactant (Collaborative European multicenter study group, 1988). Since then, treatment of preterm babies with surfactant has become standard care.

Trials have studied a wide variety of surfactant treatment, either used to prevent (prophylactic or delivery room administration) or to treat (selective or rescue administration) RDS. Significant reductions in the incidence of pneumothorax and improvement in survival have been described with either treatment (Soll, et al., 2001b). A Cochrane review, based on 8 randomised controlled trials, compared prophylactic with selective administration of surfactant in babies of less than 30-32 weeks’ gestation with RDS and demonstrated a reduced rate of pneumothorax (RR 0.62, 95% CI 0.42 to 0.89), mortality (RR 0.61, 95% CI 0.48 to 0.77), and of a composite outcome of BPD and mortality (RR 0.85, 95% CI 0.76 to 0.95), but not of BPD alone (RR 0.96, 95% CI 0.82 to 1.12) (Soll, et al., 2001b). The reduced mortality with surfactant treatment is not at the cost of increased morbidity, as shown in meta-analyses at 1 and 2 years of age (Sinn, et al., 2002).
Treatment responses to surfactant in infants with RDS can be divided into acute and longer-term responses. The acute treatment response is dependent on a rapid distribution of surfactant throughout the lung. Distribution is influenced by surface activity, gravitational forces, volume and rate of administration, ventilator settings and amount of lung fluid (Jobe, 2006). Larger volumes, rapid administration and ventilatory support to clear the airways more quickly all improve distribution (Jobe, 2006). The lowered surface tension permits the lung to inflate to a larger volume from a lower pressure, and it reduces the tendency to collapse when pressure is decreased (Rider, et al., 1993).

A likely longer-term treatment response is the improvement in the quality of endogenous surfactant production. When exogenous surfactant is given, the preterm lung continues its endogenous production. When preterm infants are given exogenous surfactant (without SP-A) a subsequent increase in SP-A in tracheal aspirate can be measured, indicating increased endogenous SP-A release and thus ongoing endogenous surfactant production (Gerdes, et al., 1992; Chida, et al., 1988). In the preterm lung, both endogenous and exogenous alveolar surfactant are recycled and transported back to the type II AECs, where the lipids are, in part, stored in the lamellar bodies for resecretion (Jobe, 2006; Jobe, et al., 2001b). Thus, exogenous surfactant is recycled and used as substrate for new surfactant production. Furthermore, an improvement in quality of surfactant was observed in the preterm rabbit lung after administration of an exogenous dose (Higuchi, et al., 1992). Since the half life of surfactant is up to 3 days in patients with RDS, the treatment effect can last for days, until endogenous surfactant production is sufficient (Jobe, 2006). Even so, some babies require a second dose of surfactant, especially when they have significant lung injury with oedema and inflammatory products in the airspaces, which can reduce their surfactant production and function (Jobe, 2006).

Many surfactant preparations have been developed, both natural and synthetic. Natural surfactants are expensive to produce, which is why there is a need to develop synthetic surfactants. Natural surfactant has a very complex structure; the new synthetic surfactants have a simplified phospholipid structure and only small amounts of peptides in place of the surfactant proteins (Curstedt, et al.,
2006). So far, trials have shown the natural surfactants to be superior to the synthetic surfactants, mainly in reducing mortality and pneumothorax (Soll, et al., 2001a).

1.4. **Bronchopulmonary dysplasia**

Prevention and treatment of RDS has improved over the last decades, but development of neonatal chronic lung disease, or bronchopulmonary dysplasia (BPD) (Jobe, et al., 2001a), remains a major complication in preterm infants. Although the survival of preterm babies has improved significantly over the last decades, the number of babies developing BPD has increased, partly because some of the babies who previously would have died can now survive (Donn, et al., 2003).

1.4.1. **“Old” BPD**

The terms BPD and chronic lung disease have been used interchangeably to describe the pulmonary sequelae after preterm birth or mechanical ventilation. Northway introduced the term BPD when he reported on a group of 32 babies with severe RDS (birth weight 900 to 3,204 g, gestational age 28 to 39 weeks), who received mechanical ventilation and supplemental oxygen treatment. He described a progression through 4 stages of disease, with stage 4 being chronic lung disease with persistent respiratory failure, hypoxaemia, hypercapnia and with specific radiographic changes (areas of increased density, due to fibrosis, alternating with areas of hyperinflation). Nineteen babies died and pathognomonic findings at post-mortem were terminal pulmonary air spaces with areas of overdistension next to areas of atelectasis, interstitial fibrosis, smooth muscle hyperplasia and squamous metaplasia (Northway, et al., 1967). He implicated oxygen toxicity and intermittent positive pressure ventilation as aetiological factors in the development of BPD (Northway, et al., 1967).

Since then, understanding of factors involved in ventilator-induced lung injury has improved; for example, it was demonstrated that ventilation with large volumes is more damaging to the lung than ventilation at high pressures (Dreyfuss, et al., 1992; Dreyfuss, et al., 1988). This improved knowledge,
combined with technological advances, has led to modifications in ventilatory strategies. Furthermore, antenatal steroids and surfactant treatment were introduced to the neonatal care. The combination of the above resulted in improved outcomes and in a milder form of BPD, also called “new” BPD or chronic lung disease (CLD) (Jobe, 1999).

### 1.4.2. “New” BPD

This disorder occurs mostly in extremely low birth weight babies (ELBW), often requiring mechanical ventilation and oxygen treatment for mild RDS, pneumonia, apnoea or poor respiratory effort. After the initiation of ventilation, these babies often experience a ‘honeymoon period’ for a few days, where they need no or only limited mechanical ventilation and oxygen treatment (Bancalari, et al., 2003). After a few days to weeks their lung function deteriorates, characterised by increased requirements for respiratory support and supplemental oxygen. Radiographic changes are very different from the changes described by Northway, with diffuse haziness extending to areas of hyperinflation and a fine, lacy pattern extending to the periphery (Donn, et al., 2006). Although this disorder is described as the “new BPD” (Jobe, 1999), Hodgman argued that this disorder was originally described as the Wilson-Mikity syndrome in 1960 and might, therefore, be not so new (Hodgman, 2003). However, the Wilson-Mikity syndrome was described before it was possible to ventilate preterm babies; therefore, some, but not all of the babies currently diagnosed with “new BPD” could fall under this description. In 2000, a consensus was reached that “new BPD” should be called BPD and not chronic lung disease, since this differentiates the disease from the multiple chronic lung diseases of later life (Jobe, et al., 2001a).

Further definitions have been developed which serve to clarify the diagnosis of BPD as an outcome measure of clinical care and research trials.

Instead of the 4 stages described by Northway to describe BPD, Bancalari proposed a definition based on the need for oxygen treatment during the first 28 days of life and a radiograph consistent with BPD (Bancalari, et al., 1979). The use of radiographic changes, however, did not improve sensitivity or specificity
of the diagnosis (Fletcher, et al., 1993). Shennan et al demonstrated that using oxygen treatment at 36 weeks’ postmenstrual age as the criterion for the definition of BPD improved the positive predictive value for later abnormal pulmonary function from 38% to 63% and the sensitivity from 79% to 83% compared with supplemental oxygen at 28 days (Shennan, et al., 1988). It is not clear if their criterion of supplemental oxygen at 28 days was used for babies on oxygen at day 28, or for babies who had been on oxygen for 28 days; the former could potentially include infants who needed supplemental oxygen on that day, for example caused by sepsis, but who were not on oxygen for 28 days in total. Besides being a risk factor for later abnormal pulmonary function, the diagnosis of BPD at 36 weeks’ postmenstrual age is also recognised to be a risk factor for later neurodevelopmental impairment (Walsh, 2008).

To address concerns that some babies might not be classified as suffering from BPD when the 36 weeks’ gestational age limit was used for supplemental oxygen, the NIH consensus definition of BPD was defined (Jobe, et al., 2001a). Babies born before 32 weeks’ gestation are assessed at 36 weeks’ gestational age or when discharged home, whereas babies born at or after 32 weeks’ gestation are assessed at > 28 days, but < 56 days’ postnatal age or when discharged home, whichever comes first. In every case, the definition requires treatment with supplemental oxygen (>21%) for at least 28 days. Mild, moderate or severe BPD is based on no, <30%≥30% supplemental oxygen and / or mechanical ventilation or continuous positive airway pressure (CPAP) at 36 weeks’ gestation or discharge home, when born before 32 weeks, or 56 days or discharge home, when born at or after 32 weeks’ gestation.

1.4.3. Incidence of BPD

The reported incidence of BPD varies widely between 2 and nearly 70%, depending on the definition that is used, and also on the gestational age at birth and birth weight (Bancalari, et al., 2003). The use of the NIH consensus definition has increased the number of babies with BPD by adding the group of babies who were on oxygen for at least 28 days of life, but in room air at 36 weeks’ gestational age, to those diagnosed as having mild BPD, resulting in the overall
rate of BPD increasing from 46% to 77% (Ehrenkranz, et al., 2005). The Australian and New Zealand Neonatal Network (ANZNN) uses a different definition and, therefore, reports a different incidence of BPD. The ANZNN definition of BPD is babies born at less than 32 weeks’ gestation, who receive any form of respiratory support (supplemental oxygen and / or assisted ventilation) for their initial respiratory disease and who continue to require respiratory support at 36 weeks’ postmenstrual age (Australian and New Zealand Neonatal Network, 2009). Their overall rate of BPD for all babies born before 32 weeks’ gestation is 13.7% (Australian and New Zealand Neonatal Network, 2009). Furthermore, the ANZNN reports that 24.5% of babies received supplemental oxygen on day 28, calculated as the need for at least 4 consecutive hours of supplemental oxygen on day 28. This result, therefore, does not reflect the total number of days a baby needed supplemental oxygen and cannot be used to compare Australiasian data with the latest American data using the NIH consensus definition.

Apart from the definition used for BPD, it is also important to consider the oxygen saturation levels which are targeted. As there is currently no consensus on the correct oxygen saturation target, accepted levels vary as widely as 84 to 98% (Walsh, 2008). Understandably, this affects the amount and duration of supplemental oxygen, and therefore the incidence of BPD. Using the physiological definition for BPD, described by Walsh et al, clearly illustrated this (Walsh, et al., 2003; Walsh, et al., 2004). At 36 weeks’ postmenstrual age, infants without supplemental oxygen were regarded as not having BPD, whereas infants with >30% supplemental oxygen or on mechanical ventilation or CPAP were regarded as having BPD. Infants receiving <30% supplemental oxygen were subjected to a 30 minute challenge in room air, after supplemental oxygen was weaned to room air in small 5 minute increments. A total of 1,598 babies were assessed in 17 centres, 227 babies were eligible for the challenge in room air and of these, 101 babies were able to maintain oxygen saturations above 90% and were not diagnosed with BPD. Using the physiological definition reduced the overall rate of BPD by 10% and reduced the variation among centres compared
with the clinical definition of BPD by supplemental oxygen alone (Walsh, et al., 2004).

1.4.4. Oxygen treatment and saturation targeting

The STOP-ROP trial (supplemental therapeutic oxygen for pre-threshold retinopathy of prematurity) hypothesised that less supplemental oxygen, achieved by accepting lower saturations, could decrease the proportion of infants progressing to threshold retinopathy of prematurity (ROP) requiring surgery (The STOP-ROP multicenter study group, 2000). Preterm infants with confirmed pre-threshold ROP were assigned to saturation ranges on pulse oximetry of 89 to 94% or of 96 to 99%. No differences in ROP were reported. However, in the group randomised to the higher oxygen saturation range, oxygen requirement increased by 5-9% in the first 24 hours after enrolment. At three months corrected age the combined outcomes of pneumonia and / or BPD were 1.8 times more likely to have occurred in the group with the higher oxygen saturation range (NNT 13.7). However, the two outcomes were not assessed separately, and the definition of BPD was not well described. More infants in the higher oxygen saturation range group were still receiving supplemental oxygen at examination at 3 months of age (RR 1.25, 46.8% versus 37.0% in the low saturation range group, p 0.02), but this may simply reflect the effect of aiming for higher saturation levels. Furthermore, the infants in the high oxygen saturation range group had a higher rate of (re)hospitalisation (The STOP-ROP multicenter study group, 2000). Higher saturation levels thus seemed to lengthen the duration of supplemental oxygen, and might increase the length of hospitalisation in preterm babies.

The multicentre Benefits of Oxygen Saturation Targeting (BOOST) trial compared targeting saturation ranges of 91-94% with 95-98% in babies born before 30 weeks’ gestation, who were still dependent on supplemental oxygen at 32 weeks’ postmenstrual age. No significant benefit was reported with respect to growth or neurological development at the higher saturation targets; however, these babies had a longer duration of supplemental oxygen (40 vs. 18 days, median difference 17, 95% CI 12 to 23 d, p<0.001) and more babies were still
dependent on supplemental oxygen at 36 weeks’ post-menstrual age (RR 1.40, 95% CI 1.15 to 1.70, p<0.001) (Askie, et al., 2003). A meta-analysis of five trials could not identify the optimal target for saturation levels, although it was demonstrated that restriction of oxygen reduced ROP (Askie, et al., 2009).

To evaluate the effect of saturation targeting in infants with a younger gestational age, 5 randomised control trials are currently recruiting over 5,000 babies in total, born at less than 28 weeks’ gestation and less than 24 hours of age at time of enrolment. These studies assess the effect of a targeted saturation of 91-95% versus 85-89% on death or major disability as the primary outcome and BPD as one of the secondary outcomes. The trials are the Australian BOOST II trial (ACTRN012605000055606), the New Zealand BOOST NZ trial (ACTRN12605000253606), the BOOST II UK trial in the United Kingdom (ISRCTN00842661), the COT trial in Canada (NCT00637169) and the SUPPORT trial in America (NCT01124331). Data from these 5 studies will be used in a prospective meta-analysis, performed by the Neonatal Oxygenation Prospective Meta-analysis Collaboration. The results from one of the trials, the Surfactant, Positive Pressure, and Pulse Oximetry Randomised Trial (SUPPORT) have recently been published (SUPPORT Study Group of the Eunice Kennedy Shriver NICHD Neonatal Research Network, 2010b). A total of 1,316 infants who were born between 24 weeks and 27 weeks and 6 days of gestation were enrolled. This trial not only randomly assigned targeted saturation, but also randomly assigned babies to intubation in the delivery room and surfactant treatment within the first hour or nasal CPAP started in the delivery room. The primary outcome for the saturation targeting was the composite outcome of severe ROP or death before discharge, which was not significantly different. Severe retinopathy occurred less often in the low saturation group (RR 0.52, 95% CI 0.37 to 0.73, p<0.001), whereas death occurred more often (RR 1.27, 95% CI 1.01 to 1.60, p=0.04) compared with the higher saturation group (SUPPORT Study Group of the Eunice Kennedy Shriver NICHD Neonatal Research Network, 2010b). The latter is a major concern, since a lower target for oxygen saturation is applied increasingly in the neonatal intensive care setting. The incidence of BPD was reduced compared with the higher saturation group (RR 0.82, 95% CI
0.72 to 0.93); however, this result was not significant using the physiological definition of BPD (RR 0.92, 95% CI 0.81 to 1.05).

The results of the other trials are awaited with much anticipation; depending on the optimal target value for oxygen saturations clinical treatment with supplemental oxygen might change, which potentially could alter the incidence of BPD.

1.4.5. Persistent ductus arteriosus

A strong association between the presence and duration of a persistent ductus arteriosus (PDA) and the risk of development of BPD has been reported (Gonzalez, et al., 1996). Excess fluid, whether related to excessive fluid administration or lack of diuresis during the first 10 days of life has been demonstrated to be associated with an increased incidence of PDA and development of BPD in a retrospective analysis of over 1,000 babies with a birth weight of 400 to 1,000 g (Oh, et al., 2005). However, a meta-analysis in four studies, including 526 babies with a birth weight of less than 2,000 g, did not report a significant effect of fluid intake on BPD development (RR 0.85, 95% CI 0.63 to 1.14) (Bell, et al., 2008). Furthermore, treatment of a PDA, either with prophylactic or symptomatic treatment with indomethacin, or surgical ligation of a PDA, did not lead to a reduction in BPD (Cooke, et al., 2003; Knight, 2001).

1.4.6. Pathology

Macroscopically, the lungs of a deceased infant with “old BPD” had alternating areas of overinflation and atelectasis, interstitial fibrosis, smooth muscle hyperplasia and squamous metaplasia as reported by Northway (Northway, et al., 1967). Ventilation of preterm baboons (delivered at 140 days, term 180 days) for 21 days using hyperoxia, but not using surfactant, documented similar patterns of alternating atelectasis and overinflation, although the hyperplasia and metaplasia could not be reproduced (Coalson, et al., 1992).

In contrast, treatment with antenatal corticosteroids and / or surfactant changed pathological findings. In babies who died of the “new BPD”, arrested lung development with impaired alveolarisation, vascular development and airway
growth, but minimal fibrosis have been described (van Marter, 2006; Jobe, 1999). This arrest in development is reproduced in several animal studies. Coalson et al studied extremely immature baboons (125 days’ gestation, term 185 days), which were delivered after antenatal corticosteroid treatment. Postnatally, the baboons were treated with surfactant and maintained on appropriate oxygen and positive pressure ventilation for at least 1 to 2 months after delivery. Supplemental oxygen and ventilation were adjusted to target PaCO₂ values of 45-55 mmHg and PaO₂ values of 55-70 mmHg. Alveolar hypoplasia, decreased and dysmorphic capillary vasculature, and abnormal deposition of elastin were observed (Coalson, et al., 1999). Albertine et al described a complete arrest of alveolar septation, resulting in fewer and larger alveoli, an arrest in vascular development and an abundance of elastin deposition in preterm lambs (125 days’ gestation, term 147 days) ventilated for 3-4 weeks compared with age-matched controls (Albertine, et al., 1999).

Various hypotheses have been prepared to explain the different pathological findings between “old” and “new” BPD. Willet et al have investigated the effect of antenatal corticosteroids on the normal pattern of lung development. They administered antenatal corticosteroids to pregnant ewes at 104 days’ gestation (term 146 days) and repeated this weekly in some of the ewes. Fetal lung tissue was collected at 125, 135 and 146 days of gestation (Willet, et al., 2001). Changes in lung structure of the fetuses were more pronounced after multiple doses of antenatal corticosteroids compared with a single dose. Pathological findings in lung tissue of fetuses treated with antenatal corticosteroids included increased alveolar volumes and decreased septal volumes compared with control animals. Fetuses allowed to proceed to term showed no structural differences in alveolar development from controls, indicating that the corticosteroid effect might be reversible over time (Willet, et al., 2001).

Another explanation might be found in the inflammatory response of a preterm baby. Chorioamnionitis and postnatal infections likely are major triggers for lung inflammation that plays a role in the development of BPD (Sosenko, et al., 2008). In addition, mechanical ventilation and oxygen treatment can also lead to an inflammatory response as will be described below. Following an initial triggering
event, all of the factors described above can amplify and perpetuate an injurious inflammatory response. This is also referred to as the pulmonary injury sequence, where each step will trigger the next step in the inflammatory process, if not contained by an anti-inflammatory mediator (Attar, et al., 2002; Speer, 2006b). The extremely preterm infant has an increased vulnerability to lung injury that might relate to the immature state of their lung development (Sosenko, et al., 2008). The inflammatory response, possibly aggravated by therapeutic interventions, such as mechanical ventilation or oxygen treatment, could disrupt the normal development of the lung, leading to the pathognomic changes in lung architecture as described in the “new BPD”, with reduced number of alveoli and abnormal vascular development (Husain, et al., 1998; Coalson, 2003).

The next sections review the inflammatory response in the preterm baby, chorioamnionitis, ventilator-induced lung injury and ventilatory strategies in more detail; all of these contribute to the multifactorial origin of BPD.

1.4.7. Inflammatory response

The immune system normally initiates an inflammatory response to protect individuals from infectious pathogens or to repair damaged tissue. The inflammatory process can be divided into acute and chronic responses. The acute inflammatory response is rapid in onset, lasting for minutes up to a few days, and leads to increased vascular permeability, oedema and migration of phagocytic cells (Kumar, et al., 2004). The chronic inflammatory response is of longer duration, and is characterised by infiltration with mononuclear cells and tissue repair by angiogenesis and fibrosis (Kumar, et al., 2004).

The mediators involved in the inflammatory response are either plasma-derived or cell-derived. Plasma-derived mediators are complement proteins and coagulation/fibrinolysis products; cell-derived mediators are sequestered in granules and released upon stimulation (histamine, serotonin, lysosomal enzymes) or newly synthesised (cytokines, oxygen radicals, nitric oxide, platelet activating factors, leukotrienes and prostaglandins) (Kumar, et al., 2004). Cytokines, in particular, play an important role in ventilator-induced lung injury.
Chapter 1

**Cytokines**

Cytokines are proteins that are produced by lymphocytes, macrophages, and also endothelial, epithelial and connective tissue cells. Cytokine production follows stimulation by endotoxin or other microbial products, tissue injury or other inflammatory stimuli such as oxygen free radicals (Kumar, et al., 2004). Large numbers of cytokines exist with some playing a role in activation and recruitment of inflammatory cells, others regulating lymphocyte growth, activation and differentiation or stimulating haematopoiesis (Charo, et al., 2006). The cytokines involved in the early inflammatory response, the early response cytokines, can be divided into pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6, CXCL8 (previously known as IL-8) and IFN-γ (Kumar, et al., 2004), or anti-inflammatory cytokines, such as IL-4, IL-10, IL-12 or IL-13 (Mulligan, et al., 1997).

Pro-inflammatory cytokines mediate the acute inflammatory response by affecting endothelial cells, fibroblasts and leukocytes and initiating acute phase reactions.

Endothelial activation leads to up-regulation of intercellular adhesion molecules (such as ICAM-1), synthesis of more cytokines (such as IL-1, IL-6, CXCL8), and activation of the clotting and fibrinolytic system, all supporting neutrophil recruitment (Kumar, et al., 2004). Fibroblasts are activated to support fibrosis; leukocytes are activated and produce more IL-1 and IL-6, thus enhancing the inflammatory response. Furthermore, TNF-α and IL-6 stimulate acute phase reactions that involve recruitment of neutrophils to the circulation and systemic effects such as fever, decreased appetite and septic shock (decreased peripheral vascular resistance, hypotension, tachycardia) (Kumar, et al., 2004).

The main biological function of anti-inflammatory cytokines, such as IL-10, is to limit and terminate the inflammatory response, probably achieved by suppression of the production of CXCL8, TNF-α and IL-1 by neutrophils and macrophages (Asadullah, et al., 2003; Jones, et al., 1996).
Normally, the balance between pro-inflammatory and anti-inflammatory cytokines is well contained; however, if the inflammatory cascade is not contained, it may ultimately lead to progressive organ dysfunction (Ward, et al., 1999). In preterm infants the balance between pro-inflammatory and anti-inflammatory cytokine responses is likely to be in favour of a pro-inflammatory response (Speer, 2006a), since the preterm baby has lower expression and lower levels of IL-10 compared with older babies (Jones, et al., 1996; Le, et al., 1997).

*Production of early response cytokines*

Both the fetus and newborn baby can produce early response cytokines. It has been demonstrated convincingly that TNF-α, IL-1β and IL-6 do not cross the term placenta. Therefore, when raised concentrations of cytokines are found in amniotic fluid these must be of fetal origin (Aaltonen, et al., 2005). Early response cytokines can rise quickly in adults and both term and preterm babies in response to a trigger, such as hyperoxia or hypoxia, infection or mechanical ventilation (Speer, 2006a). In *in vitro* experiments in rat lungs, TNF-α concentrations were increased in tracheal aspirate within 30 min (Tremblay, et al., 2002), TNF-α and IL-6 concentrations were increased in perfusate within 30 to 60 min (von Bethmann, et al., 1998) and expression of TNF-α and IL-6 in pulmonary alveolar and airway epithelium occurred within 30 min (Tremblay, et al., 2002) in response to injurious ventilation. In *in vivo* experiments in rats, concentrations of TNF-α and MIP-2 (the cytokine equivalent for CXCL8 in the rat) in tracheal aspirate and blood samples were increased within 2 hours in response to ventilation (Chiumello, et al., 1999), implicating a local and systemic role for these early response cytokines. In all three of these studies, the quantum of the cytokine response was dependent on the ventilatory strategy, with higher concentrations of cytokines following more injurious ventilation (Chiumello, et al., 1999; Tremblay, et al., 2002; von Bethmann, et al., 1998).

Clinical studies support the role of these cytokines in the initiation of lung injury and the recruitment of inflammatory cells to the lung. The elevation of early response cytokines in preterm babies is correlated to later development of BPD. CXCL8 is probably the most important chemotactic factor in the lung (Speer,
Concentrations of CXCL8 in tracheal aspirate were elevated as early as day 1 in preterm babies less than 33 weeks’ gestation, with a 5 fold increase in the preterm babies who later developed BPD compared to those who did not. Furthermore, the CXCL8 concentration doubled in the first week of life in patients who later developed BPD (Huang, et al., 2005). Other early response cytokines, such as TNF-α, IL-1β and IL-6 were also elevated in the first 5 days of life in tracheal aspirate of preterm babies (less than 32 weeks' gestation) who later developed BPD (Speer, 2006a; Lista, et al., 2006; Huang, et al., 2005; Kakkera, et al., 2005; Munshi, et al., 1997).

**Neutrophil recruitment**

To facilitate influx of neutrophils to the site of injury or into lung tissue, a complex process of attachment, rolling and transmigration is necessary. In response to injury, early response cytokines are formed leading to production and release of selectins, such as E-selectin and P-selectin, by endothelial cells (Kumar, et al., 2004). These selectins generate low-affinity binding between leucocytes and endothelial cells, which results in a continuous binding and detaching process of neutrophils along the endothelial surface, also called rolling. The next step is a firm binding of these leucocytes to the endothelium, mediated by intercellular adhesion molecules, such as ICAM-1. ICAM-1 expression is quickly (within 60 minutes) up-regulated by TNF-α in *in vitro* experiments in rat pulmonary artery endothelial cells and human umbilical vein endothelial cells (Mulligan, et al., 1993). *In vivo* studies in rats with alveolitis, induced by deposition of IgG immune complexes in the lung, demonstrated up-regulation of ICAM-1 in the lung over an 8 hour period, whereas animals pre-treated with antibody to TNF-α had significantly lower expression of ICAM-1 (Mulligan, et al., 1993). Preterm infants are also able to increase ICAM-1 expression following chorioamnionitis, as seen by an increased expression in cord endothelial cells and increased concentrations in serum (D’Alquen, et al., 2005).
When the leucocytes are firmly attached via these processes, their cytoskeleton is re-organised and they spread out on the surface; this is followed by transmigration of the leucocytes through the endothelium (Kumar, *et al.*, 2004).

**Neutrophil influx**

The normal preterm fetal (human and lamb) lung contains very few macrophages or neutrophils (Stahlman, *et al.*, 2002; Carlton, *et al.*, 1997). Recruitment of these cells to the site of inflammation, following activation by early response cytokines, is part of the acute inflammatory response in order to eliminate the injurious agent or repair the damaged tissue. An influx of neutrophils into the airspace of preterm ventilated lambs (127 ± 1 days) occurred as early as 2 hours after preterm delivery, and occurred in parallel with a decrease in the number of circulating neutrophils (Carlton, *et al.*, 1997). This decline in circulating neutrophils was reported as early as 5 minutes after birth in another study in which preterm lambs were born at 132 days' gestation (Jaarsma, *et al.*, 2004). In preterm babies (< 32 weeks' gestation), maximum levels of neutrophils were seen in airway secretions on days 5 and 7 of life, preceded by increased concentrations of CXCL8 and IL-6 on days 1 and 3 in airway secretions (Munshi, *et al.*, 1997). Another study in preterm babies described high levels of neutrophils in tracheal aspirate at only one hour of age (Ferreira, *et al.*, 2000) with a concurrent decrease in circulating neutrophil number (Ferreira, *et al.*, 2000; Speer, 2006a). This is compatible with the theory that neutrophils are recruited from the circulation into lung tissue. Preterm babies who develop BPD have higher numbers of neutrophils and macrophages in their tracheal aspirate compared with babies who recovered from RDS (Munshi, *et al.*, 1997; Kakker, *et al.*, 2005; Groneck, *et al.*, 1994). In babies who died of RDS, TNF-immuno reactive cells, neutrophils and macrophages were seen in pulmonary interstitial tissue as early as 5 hours after birth, with a peak when death occurred after 3 days of life. A strong correlation was found between increased numbers of infiltrated cells and more extensive damage to endothelial and basement membrane and damage to extracellular matrix, again maximal at 3 days (Murch, *et al.*, 1996).
Chapter 1

Phagocytosis and proteolytic enzymes

After migration to the site of infection or tissue damage, a process called phagocytosis takes place, during which pathogens or other particles are ingested by neutrophils. Neutrophils contain several granules; the two main types are filled either with collagenase and lysozyme, or with myeloperoxidase, lysozyme, elastase and collagenase. Myeloperoxidase catalyses a reaction to form oxygen free radicals (Strayer, 2008). Release of these enzymes and oxygen free radicals result in destruction of the ingested particle by the neutrophil. During phagocytosis some of the enzymes and oxygen free radicals are released into the extra-cellular space, resulting in local tissue destruction (Kumar, et al., 2004). When recruitment of phagocytic cells is persistent and not contained this may amplify the initial tissue injury.

One of the enzymes, elastase, is possibly important in the development of lung injury in the preterm infant. Elastase is normally contained by its inhibitor α1-proteinase inhibitor (α1-PI), but α1-PI can be functionally inactivated by oxygen free radicals (Merritt, et al., 1981). Under these conditions free elastase may affect pulmonary elastin, a protein in the extracellular matrix of the lung, important in the morphological development (Speer, 2006a; Pierce, et al., 1997) and expansion and recoil of the normal lung (Joyce, et al., 2003). During normal development of the fetal lung, the absolute amount of parenchymal elastic tissue increases slowly between 22 and 30 weeks of gestation, and increases rapidly thereafter. One of the findings in preterm babies at risk of BPD who died between 5 and 59 days after birth was an increased amount of elastic tissue located in aberrant places, mainly at saccular-ductal junctions, compared with preterm babies dying from a non-respiratory cause (Thibeault, et al., 2000). As yet, it is not clear if elastolytic damage by elastase plays a more important role in the aberrant location of elastin in infants with BPD or if mechanical ventilation combined with immaturity and inflammation are more important. The fact that these infants also have increased total amount of elastic tissue suggest a more multifactorial mechanism.
Chapter 1

Apoptosis of neutrophils

Normally, neutrophils attracted to the site of inflammation are removed quickly by apoptosis, but this can take longer in certain infants. Preterm babies at risk of BPD (defined as supplemental oxygen $\geq 0.3$ and/or peak inspiratory pressure $\geq 16$ cmH$_2$O at day 10 postnatal age in babies with a birth weight less than 1,200 g) had increased numbers of neutrophils in tracheal aspirates, which persisted for the first 15 days during which tracheal aspirates were evaluated ($P<0.01$) (Groneck, et al., 1994). In control infants, not in the at-risk group, neutrophil counts started to decrease on day 3 and were significantly lower from day 7 onwards compared with counts in the at-risk group (Groneck, et al., 1994).

Persistence of neutrophils in tracheal aspirates of infants with BPD might reflect inappropriate suppression of apoptosis (Kotecha, et al., 2003). Neutrophils normally have a maximum life span of 24 hours in the circulation and are extremely sensitive to apoptosis (Whyte, et al., 1999). In the normal resolution phase of inflammation, apoptosis leads to a functional downregulation of inflammation and clearance of apoptotic cells by macrophages. In tracheal aspirates taken over the first two weeks of life in infants requiring mechanical ventilation for RDS or respiratory insufficiency, the infants who fully recovered showed significantly more apoptotic neutrophils (on day 7) and apoptotic activity (on days 1 and 2) ($p<0.05$) compared with infants later developing BPD. Furthermore, children with later BPD had significantly higher total cell count on day 10 ($p<0.05$) compared with infants who recovered (Kotecha, et al., 2003), both supporting the hypothesis that these infants have reduced apoptosis. In addition, the decreased rate of neutrophil apoptosis in preterm infants may be explained by a decreased expression of the Fas receptor compared with adult neutrophils. The Fas ligand, a member of the TNF family, exhibits both pro- and anti-inflammatory activity. During the initial phase of inflammation, the Fas ligand promotes neutrophil influx, whereas during downregulation of the inflammatory response binding of the Fas ligand to the Fas receptor is necessary to initiate apoptosis (Hanna, et al., 2005).
Thus, both the increased number and prolonged survival of the neutrophils might contribute to lung injury and subsequent BPD.

**Treatment options**

If the inflammatory response is not contained properly, this can lead to more severe local tissue injury as well as to progressive organ dysfunction (Ward, *et al.*, 1999). Absence of the anti-inflammatory IL-10 may lead to a disturbance in the balance of pro-inflammatory and anti-inflammatory cytokines, thus favouring inflammation. IL-10−/− mice developed progressive chronic inflammatory bowel disease (similar in process to ulcerative colitis in human), which was reversed with exogenous IL-10 (Asadullah, *et al.*, 2003). Administration of anti-IL-10 to rats with IgG-immune complex lung injury resulted in increased vascular permeability, increased TNF-α levels and increased neutrophils counts in the lung (Shanley, *et al.*, 1995). When these rats were treated with 4 anti-inflammatory cytokines (IL-4, IL-10, IL-12 and IL-13), IL-10 was demonstrated to be the most powerful anti-inflammatory cytokine (Mulligan, *et al.*, 1997). IL-10 suppressed the production of CXCL8, TNF-α and IL-1 by neutrophils and macrophages (Asadullah, *et al.*, 2003; Jones, *et al.*, 1996).

Recombinant cytokines have been used to enhance immunity against microbial infections (Kumar, *et al.*, 2004), and exogenous treatment with the anti-inflammatory IL-10 in humans has proven to be effective in the treatment of ulcerative colitis and many other auto-immune and inflammatory diseases (Asadullah, *et al.*, 2003). Furthermore, TNF antagonists given to patients with rheumatoid arthritis have shown very positive results in joint protection (Charo, *et al.*, 2006; Feldmann, *et al.*, 2010). Since preterm babies have lower levels of the anti-inflammatory cytokine IL-10 (Jones, *et al.*, 1996; Le, *et al.*, 1997), either substitution with recombinant IL-10 or inhibitory therapy directed at the pro-inflammatory cytokines might have potentially beneficial effects on lung injury in preterm babies.

Other regulatory factors described in the lung are IL-1 receptor agonist (IL-1ra) and Clara cell (CC) 10. IL-1ra is produced by stimulated macrophages and prevents IL-1 from binding to its natural receptor. Blockage of IL-1ra in an IgG-
immune complex lung injury model in rats resulted in significant increases in vascular permeability and the number of neutrophils in lung fluid (Shanley, et al., 1996); thus, reducing the availability of IL-1ra increased the inflammatory response. It is possible to produce human IL-1ra protein and treatment with exogenous IL-1ra might have the potential to reduce lung injury. However, contradictory results are described in ventilated, preterm infants less than 30 weeks’ gestation; infants developing BPD had higher levels of IL-1ra in tracheal aspirates in the first week of life (p<0.001 on day 1, p<0.01 on day 3, p<0.05 on day 5) compared to infants who recovered completely from their respiratory disease (Kakkera, et al., 2005).

CC10 is a small protein (10 kDa) produced mainly by Clara cells in the lung. Normally CC10 is abundant in the respiratory tract; in preterm infants, CC10 is found only in low concentrations in tracheal fluid and the limited amounts are oxidised and are less bioactive (Ramsay, et al., 2001). In a recent pilot study in 22 preterm babies who were ventilated for RDS (birth weight 700 – 1,300 g, gestational age >24 weeks) a single dose of CC10 was administered in the first day of life, which resulted in significant reduction in neutrophils, total cell count and total protein (p<0.01) and a trend towards a reduction of IL-6 (p<0.07) in tracheal aspirates over the first 3 days of life (Levine, et al., 2005). There also was a trend towards shorter duration of hospitalisation and oxygen treatment, although numbers were too small to be reliable.

In ventilated rabbits, neutrophil depletion by pre-treatment with nitrogen mustard resulted in reduced protein leak into the alveoli, an absence of hyaline membrane formation and improved gas exchange compared with rabbits with a normal neutrophil complement (Kawano, et al., 1987). These data suggest that neutrophil influx into the alveoli might per se result in lung injury. Although neutrophil depletion may not be a clinical possibility, these data suggest that anti-inflammatory treatment might be a promising route towards protecting against lung injury and vulnerability to BPD.
1.4.8. Chorioamnionitis

The chorio-amnion is a membrane of fetal origin and there is direct contact between the amniotic fluid surrounding the baby and the fetal lung and fetal gut. It is often assumed that the fetus, amniotic fluid and membranes (the fetal compartment) are sterile in utero; however, colonisation with bacteria can occur during pregnancy, thereby creating a non-sterile fetal compartment (Kramer, et al., 2005). Chorioamnionitis, an inflammation of the chorio-amnion, is frequently associated with preterm labour and delivery (Goldenberg, et al., 2000), and also with development of BPD (Jobe, et al., 2008). Chorioamnionitis can be diagnosed clinically, based on findings such as maternal fever, a tender uterus, often, but not necessarily, preterm or prolonged rupture of membranes and an elevated white cell count in the mother (Jobe, et al., 2008). Chorioamnionitis can also be diagnosed by histological examination of the fetal membranes, umbilical cord and amniotic fluid to detect inflammation or organisms. The diagnosis of clinical chorioamnionitis does not correlate well, however, with histological chorioamnionitis, and an amniotic fluid diagnosis of infection may or may not predict chorioamnionitis with preterm delivery (Jobe, et al., 2008).

The organisms most commonly associated with chorioamnionitis are primarily vaginal commensal or low pathogenic organisms such as *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Gardnerella vaginalis*, peptostreptococci and bacteroides species (Jobe, et al., 2008; Goldenberg, et al., 2000). Group B streptococci and *Escherichia coli* are most often associated with ruptured membranes (Goldenberg, et al., 2000). Infants delivered before 30 weeks’ gestation had a high rate of histological chorioamnionitis, or amniotic fluid positive for these bacteria, even if the membranes were intact until shortly before delivery (Goldenberg, et al., 2000). Intrauterine infection can occur quite early in pregnancy but remain undetected for months (Goldenberg, et al., 2000). Specific fluorescence in situ hybridisation techniques (FISH) have demonstrated that organisms can be found deep within the fetal membranes of nearly all preterm and many term deliveries. Samples taken from the fetal membranes on delivery by caesarean section at term, in women who were not in labour and had intact membranes had organisms present in 19 out of 26 cases (Steel, et al.,...
In a cohort of amniotic fluid samples collected at 15-17 weeks’ gestation for genetic testing 11% were positive for *Ureaplasma* (29 out of 254 samples). Preterm labour and birth happened more often in these women (p<0.0001) (Gerber, *et al.*, 2003). Furthermore, identification of organisms, using the FISH-technique, occurred as often in membranes collected after preterm delivery by caesarean section with intact membranes (10 out of 12), preterm labour (12 out of 13) or prolonged rupture of membranes (18 out of 22) (Steel, *et al.*, 2005). Thus, exposure of the fetal compartment to bacteria is more common during pregnancy than often assumed and can lead to colonisation of the fetal membranes and a low-grade infection. Many of these pregnancies still continue to term; however, colonisation seems to increase the risk of preterm delivery.

**Chorioamnionitis and the fetal lung**

The effect of exposure of the fetal lung to chorioamnionitis and inflammation has been examined in several animal studies, often using intra-amniotic endotoxin to induce chorioamnionitis in sheep. Caution needs to be taken in analysing these data, since this creates a sterile inflammatory response which might not reflect the inflammatory response caused by a chorioamnionitis as a result of a bacterial infection.

Sheep paradigms have investigated single versus multiple doses of *E. coli* endotoxin and the dose-response relationship between the resulting inflammatory mediators, lung histology and lung maturation. Intra-amniotic injections resulted in increased concentrations of the interleukins IL-1β, IL-6 and CXCL8 in amniotic fluid and the chorio-amnion, and in an influx of neutrophils in the fetal lung. In the lung, expression of the pro-inflammatory cytokines TNF-α, IL-1β and CXCL8 was increased, whereas expression of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) was decreased (Kramer, *et al.*, 2002; Kallapur, *et al.*, 2001). One single dose of endotoxin, given 5 hours to 25 days before preterm delivery of the lamb at 119-125 days’ gestation (term 150 days) induced biochemical and physiological lung maturation starting at 4-7 days and persisting until 25 days after endotoxin. At post-mortem there were increased levels of saturated phosphatidylcholine, one
of the main components of surfactant, and improved lung gas volume upon insufflation of air at 40 cmH₂O (Kallapur, et al., 2001). Thus, the inflammatory response accelerated maturation of the fetal lung, perhaps to prepare the system for preterm delivery; however, this seems to happen at the expense of normal lung development. Histological analysis demonstrated vascular remodelling in pulmonary arterioles 4-7 days after endotoxin with an increase in arteriolar smooth muscle wall thickness and collagen deposition in the adventitia of the vessels (Kallapur, et al., 2004). Furthermore, one single dose of endotoxin at 119 days’ gestation, followed by delivery at 125 days and ventilation for 40 min, resulted in abnormal alveolarisation with a 20% increase in alveolar volume and 30% decrease in total alveolar number in animals treated with endotoxin versus controls (p<0.05) (Willet, et al., 2000). Surprisingly, these developmental changes were no longer present when lambs were delivered close to term (no marked vascular injury and no significant decrease in alveolar numbers) (Kallapur, et al., 2005).

Another interesting finding was the fact that the changes in alveolar development following endotoxin were similar to the changes observed after one dose of betamethasone at the same time. Since no increase in fetal plasma cortisol concentration or cord plasma cortisol concentration were seen in animals exposed to endotoxin, it was suggested that the abnormal alveolarisation was caused by up-regulation of the pro-inflammatory markers, and not by up-regulation of endogenous corticosteroids in these animals (Willet, et al., 2000). The response to inflammation, with abnormal alveolarisation, but also with enhanced biochemical maturation, might explain why preterm babies exposed to chorio-amnionitis are at a decreased risk of RDS development (Watterberg, et al., 1996) and clinically behave similar to babies who have been exposed to antenatal corticosteroids. Repeated doses or high doses of endotoxin did not alter the biochemical, physiological or maturational effects compared with one low dose (Jobe, et al., 2000); thus, the fetus appears to be able to adapt to inflammation and modulate the inflammatory response (Kramer, et al., 2005).

In clinical studies, raised concentrations of inflammatory markers are seen in babies at an increased risk of later BPD development (Watterberg, et al., 1996;
Yoon, et al., 1997). Raised concentrations of IL-1β, IL-6 and CXCL8 in amniotic fluid and raised concentrations of IL-6 in cord plasma were found in babies of mothers with chorioamnionitis who delivered preterm (<33 weeks’ gestational age) within 5 days of amniotic fluid collection and whose babies developed BPD compared with babies who did not develop BPD (p ≤ 0.05) (Yoon, et al., 1997). Raised concentrations of IL-1β were found in tracheal aspirates within 48 hours after birth in children (birth weight < 2 Kg) exposed to chorioamnionitis compared with aspirates taken from children not exposed to chorioamnionitis. Furthermore, the children who later developed BPD had higher concentrations of IL-1β compared with children who did not develop BPD (Watterberg, et al., 1996). Other studies found similar increases of IL-1β and also of IL-6, CXCL8 and TNF-α in tracheal aspirates of babies of less than 34 weeks’ gestational age who subsequently developed BPD. Unfortunately, however, these studies did not report if these babies had been exposed to chorioamnionitis (Jonsson, et al., 1997).

Other aspects of the inflammatory cascade have also been examined. Endothelial adhesion molecules play an important role in the migration of leucocytes to the site of infection. A marked up-regulation of one of the adhesion molecules, ICAM-1, has been reported in cord endothelial cells of preterm infants following exposure to chorioamnionitis, especially if there was also funisitis. Simultaneously, there was an increase in serum concentrations of ICAM-1 in these infants (D’Alquen, et al., 2005). Since the concentrations of other pro-inflammatory markers (IL-1β, IL-6, CXCL-8 and E-selectin) were also increased in serum, chorioamnionitis is associated with a local and systemic inflammatory response. Involvement of the lung with chorioamnionitis becomes apparent from the increased numbers of phagocytic cells in broncho-alveolar lavage and on histological examination of lung tissue at post-mortem (Schmidt, et al., 2001).

The role of chorioamnionitis in the development of BPD remains controversial. In a study from the pre-surfactant era that excluded mothers who were treated with antenatal steroids, chorioamnionitis, defined as the presence of inflammatory cells in the placenta and fetal membranes or polymorphonuclear cells in amniotic fluid, decreased the risk of development of RDS (probably via
enhanced lung maturation), while the risk of BPD was increased (Watterberg, et al., 1996). A recent meta-analysis including 2,216 infants reported an association between *Ureaplasma* colonisation or infection (assessed by tracheal or nasopharyngeal aspirate or PCR positive for *Ureaplasma*) and an increased risk of BPD; however, the greatest effects were found in the smaller studies published between 1988 and 1998 (Schelonka, et al., 2005). Plausible explanations for this might be that clinical care had improved during the later studies and the risk of BPD development was greater in studies not reporting on the use of surfactant compared with studies who did report on surfactant use (Schelonka, et al., 2005). The use of antenatal corticosteroids was not reported on; however, later studies are more likely to have used corticosteroids following a review on the use of antenatal corticosteroids in 1990 (Crowley, et al., 1990), which could have affected the incidence of BPD in these studies. Furthermore, the fact that the smaller studies reported a higher incidence of BPD might be a publication bias.

A recent retrospective study (105 infants) classified preterm infants with BPD (defined as the need for supplemental oxygen at day 28 and abnormal chest radiograph), based on the presence or absence of intra-uterine infection. Intra-uterine infection was defined by histological and bacteriological examination of the placenta and elevation of *Ureaplasma* IgM-levels in cord blood. Placental colonisation with *Ureaplasma* was significantly related to chorioamnionitis, and neonatal colonisation with *Ureaplasma* (demonstrated by positive cultures of gastric aspirates, nasopharyngeal swabs and/or tracheal aspirates) was an independent risk factor for BPD in infants with chorioamnionitis (Honma, et al., 2007). The association between BPD and *Ureaplasma* might therefore be dependent on colonisation of the neonate, and chorioamnionitis should be taken into account when analysing the effect on BPD development.

1.5. Ventilator-Induced Lung Injury

Ventilator-induced lung injury (VILI) is a significant component in the development of BPD. During mechanical ventilation positive pressure builds up in the ventilator circuit, resulting in a forcible injection of gas into the lungs enabling gas exchange to occur.
Several mechanisms may contribute to the lung injury inflicted by mechanical ventilation. Although several terms are used to describe the individual components, they are all inter-related and may even work synergistically.

1.5.1. Barotrauma

For years barotrauma, injury caused by pressure, has been held responsible for ventilator-induced lung injury. An association between high airway pressures during positive pressure ventilation and alveolar rupture (air leak syndromes) has been described in several studies (Moylan, et al., 1978; Slutsky, 1999; Petersen, et al., 1983). Moylan et al. recognised the importance of air leak syndrome, reporting an increased incidence of BPD in babies suffering from RDS combined with alveolar rupture (Moylan, et al., 1978). Alveolar rupture was a strong predictor of BPD, independent of its effect on duration of mechanical ventilation or oxygen treatment (Moylan, et al., 1978).

Pulmonary barotrauma may cause BPD by causing rupture of alveolar walls resulting in emphysema and damage to the connective tissue fibres, which in turn results in loss of elastic recoil. Ventilation will expand the most compliant alveoli; however, the lack of elastic recoil might result in overinflation in these areas and atelectasis in neighbouring areas, the latter simply on the basis of lack of space (Moylan, et al., 1978). In addition to air leak syndrome, pulmonary oedema has been extensively studied and used as measure of severity of barotrauma (Dreyfuss, et al., 1998). Pulmonary oedema is caused by altered microvascular permeability (Dreyfuss, et al., 1998). Airway pressures monitored during ventilation are peak pressure, mean airway pressure and positive end-expiratory pressure. Webb and Tierney described a study in mechanically ventilated rats, using peak airway pressures of 14, 30 and 45 cmH\textsubscript{2}O. Higher pressures led to more rapid and more severe formation of pulmonary oedema and death in the majority of the animals (Webb, et al., 1974), suggesting an important role for peak airway pressure on development of lung injury.

In contrast, studies using low volume ventilation with high airway pressures, achieved by strapping the chest and abdomen of rats to restrict thoracoabdominal expansions, reported no pulmonary oedema and the
ultrastructure of these lungs on electron microscopy appeared normal. However, rats exposed to high volume ventilation with similar high airway pressures, or rats exposed to high volume ventilation with negative airway pressure using an iron lung, had severe pulmonary oedema and electron microscopy of the lung was abnormal with diffuse damage of the alveolar epithelium and endothelium (Dreyfuss, et al., 1988). Thus, these studies demonstrate that high volume and not high pressure *per se* lead to lung injury; therefore, the term ‘volutrauma’ was introduced as the more appropriate term for lung injury provoked by mechanical ventilation (Dreyfuss, *et al*., 1988; Dreyfuss, *et al*., 1992).

1.5.2. Volutrauma

Hernandez *et al*.

also confirmed the importance of volume distension rather than high inspiratory pressures in the development of pulmonary oedema (Hernandez, *et al*., 1989). In a 1 hour ventilation study in immature rabbits, one group had a full body cast (to achieve limitation of inspiratory inflation), one group had a normal closed-chest, and one group consisted of isolated excised lungs (Hernandez, *et al*., 1989). Each group was ventilated for 1 hour: the first two groups had animals ventilated at 15, 30 and 45 cmH$_2$O; the isolated lungs were ventilated with an inspiratory pressure of either 15 or 30 cmH$_2$O. The capillary filtration coefficient was used as a marker of microvascular permeability and thus formation of pulmonary oedema. The results clearly demonstrated an increase in capillary filtration coefficient in the isolated lung, where distension was unlimited, and also in the closed-chest animals, whereas the rabbits with a full body cast did not develop an increased capillary filtration coefficient. Thus, volume distension, rather than just high inspiratory pressures, produced the microvascular damage in this study.

Volutrauma can be achieved quickly. Even manual ventilation with only a few large breaths (35-40 ml/Kg, which equalled the inspiratory capacity in surfactant treated controls) after birth, can be sufficient to generate lung injury in surfactant deficient preterm lambs, as measured by compliance and histology (greater neutrophil influx, hyaline membranes and necrosis) (Bjorklund, *et al*., 1997). More severe lung injury (increased vascular permeability) was
documented after 20 minutes of high volume ventilation (25, 33 or 45 mL/Kg) in rats, which had previously been exposed to alpha-naphthylthiourea to create lung injury, compared with normal tidal volume ventilation (7 mL/Kg) in rats with non-injured lungs (Dreyfuss, et al., 1995), thus suggesting a synergistic effect between volutrauma and pre-existing lung injury. Preterm babies face several risk factors for volutrauma, such as manual ventilation at birth, highly compliant chest walls and high incidence of ante- or postnatal infection, making them extremely vulnerable to lung injury.

It is assumed that volutrauma creates lung injury during mechanical ventilation by overdistension of the lungs, leading to epithelial damage and increased vascular permeability. However, ventilation with insufficient lung volume can also lead to lung injury, since this will lead to repetitive collapse and re-opening of alveoli (atelectotrauma).

1.5.3. Atelectotrauma

Acute lung injury, as in infants suffering from RDS, is a non-uniform process and can result in an unequal distribution of ventilation (Attar, et al., 2002). A study in patients with acute respiratory failure (mean age 39.8 years, range 11 to 74 years) using computerised tomography proposed three zones of aeration; 1) normal aerated lung tissue, 2) poorly aerated lung tissue, recruitable during inspiration but followed by collapse during expiration, 3) collapsed and non-ventilated lung tissue (Gattinoni, et al., 1987). During ventilation, the distal airways in zone 2 will have repetitive opening and collapse, which can happen more than 50,000 times per day given a rate of 40 breaths per minute for an infant. This repetitive re-opening of atelectatic distal airways might lead to epithelial damage and protein leakage into the airways, which is described as atelectotrauma (Slutsky, 1999).

As RDS is a non-uniform process, atelectatic and open alveoli exist next to each other in a diseased lung. In normal healthy lungs the radial stress on the alveolar wall is similar in all alveoli, whereas in diseased lungs the walls of the collapsed alveoli are stretched inwards.
The walls surrounding these collapsed alveoli generate counteracting stretch forces, in an attempt to open up these alveoli and reduce the non-uniformity. The forces in these areas can far exceed the transpulmonary pressures and are suspected to lead to structural damage to the alveolar unit (Mead, et al., 1970).

In the diseased lung, application of positive end-expiratory pressure (PEEP) appears to have a stabilising effect. In patients with respiratory failure, mechanical ventilation combined with PEEP markedly decreases the fraction of lung tissue undergoing tidal reopening and collapse (Gattinoni, et al., 1987). Muscedere et al. studied the effect of different PEEP levels in isolated, non-perfused injured rat lungs, in an attempt to exclude the interference of pulmonary blood flow or oxygen treatment (Muscedere, et al., 1994). PEEP levels of 0 and 4 cmH\textsubscript{2}O resulted in less compliant lungs (a right shift of the pressure-volume curve), more hyaline membrane formation (even in the non-perfused lung) and a higher lung injury score at histology. PEEP above the inflection point (the critical pressure needed to open the lungs), or a continuous distending pressure without mechanical ventilation in the control group, resulted in more compliant lungs (a left shift of pressure-volume curve), no hyaline membrane formation and less lung injury (Muscedere, et al., 1994).

This stabilising effect of PEEP in atelectotrauma can be explained via multiple mechanisms. There is recruitment of a larger number of alveoli to a normal opened state, thus resulting in decreased repetitive opening of alveoli (Falke, et al., 1972). When more alveoli are opened, the non-uniformity in the lung decreases, and stretch forces in the individual alveolar units will decrease, likely resulting in less injury to the endothelium and less microvascular permeability. Furthermore, recruitment of alveoli improves oxygenation by decreasing the ventilation / perfusion mismatch (Falke, et al., 1972). For the preterm baby, the improvement in pulmonary compliance also might be explained by a more efficient surfactant system. If pulmonary oedema is less severe, the influx of proteins inhibiting surfactant synthesis is minimalised (Jobe, 2006), thus leaving more surfactant available for surface lowering activity in the alveoli.
1.5.4. Oxygen

A human being is dependent on oxygen for energy production to occur. The most important role for the respiratory system is gas exchange; oxygen is transferred from the outside air into the lung, followed by diffusion of oxygen to the blood and subsequent delivery of oxygen to the tissues (Wood, 2003). Treatment with supplemental oxygen is a well known therapy; however, oxygen is known to be toxic in excess, either by concentration, duration of treatment or susceptibility of the patient. Hyperoxia, and also inflammation, result in formation of oxygen free radicals, such as superoxide, hydrogen peroxide and hydroxyl radicals, produced by macrophages and neutrophils (Speer, 2006a; Strayer, 2008). These radicals can lead to membrane damage, via lipid peroxidation and protein damage, and to DNA damage, ultimately leading to increased vascular permeability and chemotaxis, thus enhancing the inflammatory response (Saugstad, 2003). Damage to cells resulting from oxygen free radicals has been implicated in diseases of many organs (e.g. joints, heart, kidneys as well as the lungs (Strayer, 2008).

Preterm babies are more vulnerable to free oxygen radicals, as they have an immature anti-oxidant defence system. Superoxide is normally scavenged and converted into hydrogen peroxide and oxygen by superoxide dismutase (SOD). Hydrogen peroxide is subsequently reduced into water via glutathione and catalase (Strayer, 2008). However, preterm babies have a relative glutathione deficiency and an immature enzymatic anti-oxidant system, especially of catalase (Saugstad, 2005; Thomas, et al.). In addition, transferrin capacity and ferroxidase activity are low in preterm babies, which are necessary to eliminate hydroxyl radicals (Saugstad, 2005).

Oxygen toxicity

In neonatology, the detrimental effects of hyperoxia became evident when the Cooperative Study of Retrolental Fibroplasia demonstrated that prolonged administration of 50% oxygen (4 weeks) to preterm babies caused increased rates of retinopathy of prematurity (ROP) and vision loss (Kinsey, 1956). Northway et al. suggested that hyperoxia induced lesions in the respiratory
mucosa, alveoli and capillaries and might augment hyaline membrane formation plus delay their disappearance (Northway, et al., 1967).

Experimental animal studies confirm the toxic effects of hyperoxia. In preterm rats exposed to prolonged hyperoxia (2 weeks), an inflammatory response and an inhibitory effect on lung development was documented. Influx of phagocytic cells was seen into the lungs and several pro-inflammatory mediators were up-regulated (genes involved in inflammation, extracellular matrix turnover and coagulation) in hyperoxic rats compared to rats kept in room air (Wagenaar, et al., 2004). Histology revealed enlarged alveolar spaces, a decrease in secondary septation, an increase in septal thickness, and loss of lung capillaries together with down regulation of vascular endothelial growth factor (VEGF) (Wagenaar, et al., 2004; Thebaud, et al., 2005). VEGF is involved in vascular development and expression of VEGF and VEGF receptor-2 appears to be down-regulated by hyperoxia (Maniscalco, et al., 2005; Wagenaar, et al., 2004). Postnatal treatment with intra-tracheal VEGF in one of these hyperoxic rat models improved survival, promoted vascular development and resulted in normal alveoli (Thebaud, et al., 2005). In addition to its effect on inflammation and maturation, hyperoxia also results in inhibition of surfactant synthesis and inactivation of surfactant (Holm, et al., 1988; Holm, et al., 1985). Exposure for 64 hours to 100% oxygen in rabbits led to decreased phospholipid levels in broncho-alveolar lavage fluid and to decreased surfactant synthesis in isolated type II cells (Holm, et al., 1988).

Studies ventilating newborn piglets with 21 or 100% O₂ whilst aiming for low or normal arterial CO₂ levels demonstrated that hyperoxic piglets (independent of ventilatory strategy) had significantly reduced pulmonary compliance, increased pulmonary oedema and increased inflammation (increased neutrophil count and elastase activity in tracheal aspirates) compared with control piglets ventilated with 21% O₂, thus supporting the role for oxygen toxicity in ventilator-induced lung injury (Davis, et al., 1991).

Oxygen free radicals can be formed as a result of supplemental oxygen therapy and subsequent hyperoxia, but can also be formed by neutrophils and macrophages as part of an inflammatory response. Neutrophils, attracted to the
lung upon injury, generate oxygen free radicals and release myeloperoxidase, which in turn catalyses a reaction that produces hypochlorous acid (Speer, 2006a; Buss, et al., 2003). This acid can react with tyrosil residues in proteins to produce 3-chlorotyrosine, a minor reaction, but nevertheless the only physiological source of 3-chlorotyrosine (Buss, et al., 2003; Kettle, 1996). Therefore, 3-chlorotyrosine can be used as a marker of oxytrauma and / or biotrauma. Significantly higher levels were found in preterm babies compared with term babies without respiratory disease (median 83 (interquartile range (IQR) 44-189) compared with 13 (range 0-13; only range and no IQR was given) μmol/mol tyrosine; p<0.001) (Buss, et al., 2003). Furthermore, increased levels of 3-chlorotyrosine have been demonstrated in tracheal aspirates of preterm babies < 1,500 g who developed BPD compared with babies who did not (values for IQR were taken from a box plot with IQR and are therefore an approximate value; median 88, IQR 50-180 vs 49, IQR 25-80 μmol/mol tyrosine) (Buss, et al., 2003).

In addition to the role of oxidative stress in neonatal lung disease and ROP, it can also play a role in necrotising enterocolitis, periventricular leucomalacia and the persistent ductus arteriosus, which was coined by Saugstad as ‘oxygen radical disease of the neonate’ (Saugstad, 2005). The ability to treat with supplemental oxygen is an important and essential part of neonatal care (Finer, et al., 2009), both during treatment on the neonatal unit and resuscitation; therefore, to avoid or reduce oxidative stress more attention has been paid in recent years to careful targeting of oxygen levels.

**Oxygen treatment during resuscitation**

The use of 100% oxygen during resuscitation has long been perceived as “best practice”; however, even brief exposure to oxygen has the potential to be toxic and this has led to a more critical review of the use of 100% oxygen as part of the newborn resuscitation (Fowlie, et al., 2008).

A meta-analysis in newborn infants requiring resuscitation at birth compared resuscitation with 100% oxygen with resuscitation with air (5 studies; 1,302 infants, of which 76% were term and 24% preterm babies with a birth weight >
1 Kg) and demonstrated decreased mortality (RR 0.71, 95% CI 0.54 to 0.94) with air (Tan, et al., 2007). Therefore, even short exposure to oxygen during resuscitation can influence the outcome in newborn infants. However, over 500 infants were born in India and the overall mortality in the meta-analysis was 15%. Mortality for babies born at <32 weeks’ gestation, with a birth weight <1,500 g or who needed assisted ventilation or major surgery is only 7% in Australasia, as reported by the ANZNN (Australian and New Zealand Neonatal Network, 2009). Therefore, it is likely that the number needed to treat will be higher in the Australasian setting. A more recent review, which describes the data of 10 studies and 2,133 infants, separately identifies data from all studies and strictly randomised trials, to overcome the criticism that the initial trials included in the meta-analysis were not methodologically rigorous. This review reported a decrease in mortality with the use of room air, both in all studies (RR 0.69, 95% CI 0.54 to 0.88) and in strictly randomised studies (RR 0.32, 95% CI 0.12 to 0.84), the latter all performed in Europe (Saugstad, et al., 2008). Therefore, since hypoxia and the need for resuscitation do occur in both poor and rich countries, a change away from resuscitation with 100% oxygen has the potential to save many lives across the world.

However, in the assessment of these data, not only mortality, but also morbidity needs to be evaluated. Disability-free survival should be one of the more important outcomes on which clinical practice should be based. Saugstad reported on long-term neurodevelopmental outcome, and although these data were secondary outcomes of the original Resair 2 trial and did not reach significance, they do need to be considered seriously. There was an increase in both cerebral palsy (OR 1.38, 95% CI 0.52 to 3.62) and in abnormal development as assessed by the paediatrician (OR 1.67, 95% CI 0.73 to 3.80) at 18 to 24 months of age when resuscitation with 100% oxygen was compared with room air (Saugstad, et al., 2003). Currently, research is performed that will initiate resuscitation at 21% compared with 90 or 100% and that will increase or decrease supplemental oxygen according to pre-defined target SpO2 levels (Finer, et al., 2010), thereby attempting to resemble physiological SpO2 levels post-delivery (Finer, et al., 2009; Finer, et al., 2010; Kamlin, et al., 2006; Dawson,
et al., 2009). These studies are powered to detect a difference in disability-free survival at 2 years of age (Finer, et al., 2010).

1.6. Ventilatory support in the neonate

When an infant has severe RDS or fails to establish or maintain adequate spontaneous breathing for other reasons, respiratory support can assist in gas exchange. Mechanical respiratory support utilises supra-atmospheric pressures to generate an airflow into the lungs during inspiration, but still relies on the recoil of the lung for expiration (intrapulmonary pressure is higher than the atmospheric pressure) (Bhutani, 2002).

Ventilatory support in neonates can be provided through non-invasive or invasive forms. Non-invasive ventilatory support includes modalities such as low- or high-flow nasal oxygen, continuous positive airway pressure (CPAP) and nasal intermittent positive pressure ventilation (NIPPV). Invasive forms of ventilation include CPAP, conventional ventilation, and high frequency techniques administered through an endotracheal tube. All share the same aims – namely, (a) to provide adequate gas exchange, (b) to minimise ventilator-induced lung injury, (c) to reduce work of breathing and (d) to optimise patient comfort (Gupta, et al., 2007).

1.6.1. Basic principles of mechanical ventilation

Positive pressure ventilators are designed to deliver a gas to the lungs by creating a pressure gradient along which gas can enter the lungs. To achieve this, a continuous or variable bias gas flow runs through the ventilator circuit. Neonatal ventilators commonly use an Ayre’s T-piece, which was originally designed for anaesthetic procedures (Ayre, 1956, 1937). If the expiratory end is (partially) occluded, pressure in the ventilatory circuit will rise (Rees, 1950; Goldsmith, et al., 2003). If this pressure exceeds pulmonary pressure, the gas will be diverted into the patient’s lung (Wood, 2003).

Peak inspiratory pressure (PIP) and positive end expiratory pressure (PEEP) are determined by the clinician. Some ventilators allow the clinician to alter the bias gas flow. Furthermore, inspiratory and expiratory times, and thus ventilator
rates, are either set by the clinician or determined by lung mechanics, if the ventilation mode allows this. Different ventilation modes either provide mandatory breaths, regardless of the patient's own breathing pattern, or allow the patient to trigger the onset of inspiration, and in some cases expiration. A final variable in ventilation is the gas flowing through the circuit, which can be purely air, a mixture of air and oxygen, or air, oxygen and nitrogen. This gas is mostly humidified and heated using a humidifier.

1.6.2. Modes of mechanical ventilation

Several modes of ventilation are currently available to the neonatal population. To better understand these different modes, ventilation can be classified into variables that are controlled and cannot change, or “phase” variables that are changeable and can be used to create different breath types to suit specific conditions (Sinha, et al., 2008; Gupta, et al., 2007).

Control (parent mode) variables:

At any one time, the ventilator can only be controlled by pressure, volume or flow. Since volume is the integral (area under the curve) of flow and time, volume and flow- controlled ventilators are basically the same. So-called parent modes of ventilation are pressure-controlled or volume controlled ventilation.

Phase (daughter mode) variables:

Each breath has four phases: initiation of a new breath; limitation to further inflation of the lung; end of inspiration / start of expiration, and end of expiration (Figure 1.8). During mechanical ventilation, pressure, volume, time or flow are phase variables used to trigger, limit or cycle a breath. Thus, for example, in time-cycled pressure-limited ventilation, pressure limits the inspiratory flow once the set inspiratory pressure has been reached, to maintain, but not further increase, the attained pressure, whereas time defines when inspiration cycles into expiration. If this inspiratory time is long enough, an inspiratory pressure plateau will be reached.
Figure 1.8: Phase variables of a mechanical breath

A: trigger mechanism, i.e. the stimulus for the breath to start (pressure, volume, flow or time);
B: limit, i.e. the factor limiting gas flow during inspiration (pressure, volume or flow);
C: cycle, i.e. the factor ending each breath (time or flow);
D: end of expiration, lung has completed its recoil. Adapted from Gupta et al (Gupta, et al., 2007).

Pressure-targeted ventilation

Pressure-targeted modes of ventilation apply a pressure limit, determined by the clinician, that the ventilator will not exceed. Examples of pressure-targeted ventilation are time-cycled pressure-limited ventilation (TCPLV) and flow-cycled pressure-limited ventilation (FCPLV) (Gupta, et al., 2007).

Mechanical ventilation of the neonate has traditionally been performed using time-cycled pressure-limited ventilation (TCPLV). A preset PIP and defined inspiratory and expiratory times are used to deliver a breath to the lungs. However, because of the relationship between pressure and volume, the size of the tidal volume delivered depends primarily on compliance of the lungs (Figure 1.4).
Thus, at a given pressure, the tidal volume will be lower when lungs are non-compliant, possibly leading to collapse of the airways (atelectotrauma). When compliance improves, the same pressure will deliver a much larger tidal volume, which may lead to overdistension (volutrauma) (Singh, et al., 2006; Sinha, et al., 2008; Dreyfuss, et al., 1992; Clark, et al., 2001). Theoretically, the neonate is at even greater risk of volutrauma than an older child or adult, as the chest wall is more compliant (Clark, et al., 2001). In infants with RDS compliance can change rapidly, particularly shortly after surfactant treatment (Gupta, et al., 2007), leading to rapid increases in the tidal volume delivered if pressure is not decreased. In addition, ventilator breaths combined with spontaneous breathing movements in the neonate may result in excessive tidal volumes (McCallion, et al., 2005). Therefore, the clinician needs to be aware of the rapidly changing lung mechanics and measured tidal volumes and adjust the ventilator settings accordingly.

In addition to time-cycled pressure-limited ventilation, there are also forms of flow-cycled pressure-limited ventilation (FCPLV) available for neonatal mechanical ventilation. Examples of neonatal ventilators with FCPLV are the Babylog 8000plus (Dräger, Lübeck, Germany) in pressure support ventilation mode (PSV) and the Bear Cub 750PSV equipped with VGM Bear Graphics (Viasys Healthcare, Conshohocken, Pennsylvania, USA). PSV in the Babylog differs from PSV in adult ventilation. Both are flow-cycled; however, a back-up rate is guaranteed with the Babylog, meaning that a reliable spontaneous respiratory effort is not an absolute requirement (Keszler, 2009; De Luca, et al., 2009).

FCPLV has a fixed bias flow running through the ventilator circuit (Gupta, et al., 2007), not to be confused with the inspiratory flow. The inspiratory flow entering the patient’s lung, measured in the flow sensor in the Wye piece just before entering the endotracheal tube, is variable and depends upon lung mechanics. At the beginning of inflation inspiratory flow will be high, whereas towards the end of inflation the inspiratory flow will have decreased. In a flow-cycled ventilator, mechanical inspiration is terminated once the measurement of the inspiratory flow falls below a certain percentage of the peak inspiratory flow (Gupta, et al., 2007), usually 10 to 20% of peak flow (Keszler, 2009).
Consequently, inspiratory time will vary based on the lung mechanics, but will be limited by a set maximum inspiratory time. The preset inspiratory and expiratory pressures will be delivered, unless the set maximum inspiratory time is not long enough for the inspiratory pressure to be reached.

An advantage of FCPLV over TCPLV is that the mechanical inspiration terminates once the lung is nearly full, whereas during TCPLV inspiration is maintained for the duration of the set inspiratory time (Gupta, et al., 2007). This can lead to an inspiratory plateau phase (De Luca, et al., 2009) during which active expiration can occur (Greenough, 1988). Long inspiratory times have been associated with an increased risk of pneumothorax (Kamlin, et al., 2003). A short term cross-over trial in 10 infants of less than 32 weeks’ gestation compared TCPLV with FCPLV. A total of 1,840 breaths were analysed. FCPLV resulted in significantly shorter inspiratory time, lower respiratory rate and mean airway pressure and higher tidal volume (p<0.001). TCPLV tended to result in inspiratory pressure plateaus, which were not seen with FCPLV. Furthermore, infants on FCPLV had lower heart rates and higher SpO2, which might be an indication that this type of ventilation is more comfortable and efficient for them than TCPLV (De Luca, et al., 2009).

**Volume-targeted ventilation**

To overcome the problem of variable tidal volume delivery seen with pressure-targeted ventilation, volume-targeted ventilation has been developed using modern microprocessor techniques and sensitive flow sensors that can measure small tidal volumes accurately, even in the preterm infant. The clinician can determine a "set" tidal volume; the ventilator can measure inspiratory or expiratory tidal volume and make adjustments to PIP or inspiratory time on a breath-to-breath basis to try to deliver this volume.

Volume-targeted ventilation can be subdivided into volume controlled ventilation, volume assured pressure support and volume limited ventilation. The first is a volume-cycled mode; inflation ends as soon as the set volume has been delivered. Flow, PIP and inspiratory time can vary between breaths. Inspiratory tidal volume is used, measured at the ventilator rather than the Wye
piece. Because tidal volume is measured at the ventilator, disadvantages of this technique largely relate to variation in the volume delivered to the infant because of the large dead space in the humidifier and ventilator circuit. In addition, substantial leaks around the endotracheal tube (a relatively common problem, since most neonatal tubes are uncuffed) are not compensated for (McCallion, et al., 2008; Keszler, 2006). Volume control modes are available on the VIP Bird (Viasis Healthcare) and Servo 300 (Maquet Inc., Rastatt, Germany). The Servo 300 also features a modified mode of volume controlled ventilation (pressure-regulated volume control ventilation, PRVC), in which an upper limit for PIP can be set.

Viasis has since developed two new ventilators, the VIP Bird Gold and the Avea, which can ensure tidal volume delivery (VAPS; volume assured pressure support). These ventilators combine flow-cycling and volume-targeted ventilation and are also referred to as variable flow ventilators. The VIP Bird Gold is a hybrid mode of ventilation, starting a breath in pressure support ventilation, but ending it in volume-targeted ventilation, if necessary. If the set tidal volume is not reached at the set minimum inspiratory flow (a percentage of peak inspiratory flow), the ventilator prolongs the inspiratory time by continuation of this set minimum inspiratory flow. This flow continues until the targeted volume has been reached and results in a passive increase in PIP (Keszler, 2006; Sinha, et al., 2003). A disadvantage of lengthening the inspiratory times is that this can lead to an inversed inspiratory:expiratory (I:E) ratio. Inverted I:E ratios have been associated with gas trapping, reduced compliance and an increased risk of air leaks (Kamlin, et al., 2003). In this mode, inspiratory tidal volume is measured, which can lead to underventilation if a significant endotracheal tube leak exists. Furthermore, there is no provision for avoidance of excessive tidal volume delivery or facilitation of automatic weaning of PIP (Keszler, 2006). For example, when compliance improves (after surfactant treatment) with a subsequent increase in tidal volume, the PIP is not reduced automatically. The Avea ventilator shares the basic concept of VAPS, but the algorithm has been refined to respond earlier in the respiratory cycle, therefore avoiding the excessively long inspiratory times (Keszler, 2009). Furthermore, the
Avea has a volume limit that terminates inspiration if the set upper limit for volume is exceeded and thus reduces the risk of volutrauma. However, this ventilator also uses inspiratory tidal volume measurements and does not provide automatic weaning of PIP (Keszler, 2006).

Volume limited ventilation is time-cycled pressure-limited ventilation, in which inflation is aborted if the inspiratory tidal volume exceeds a pre-set limit. However, PIP values are not adjusted if tidal volume is too small, and thus tidal volume delivery is not guaranteed. This mode is available on the Bearcub 750 PSV (Viasis) and SLE 5000 (Specialised Laboratory Equipment, South Croydon, UK).

The Babylog 8000 plus ventilator has an option to guarantee the delivery of the set tidal volume, called volume guarantee ventilation, which is a form of time-cycled pressure-limited ventilation (McCallion, et al., 2005). Expiratory tidal volume – measured at the Wye piece and thereby compensating for leaks up to 60% around the endotracheal tube – is used by the ventilator algorithm to increase or decrease PIP in a stepwise fashion in subsequent breaths (maximum increase 3 cmH2O per breath), if tidal volume is not reached or is exceeded. Another advantage of measuring flow at the endotracheal tube is that dead space in the ventilator circuit does not play a role. Inspiratory tidal volumes are also measured, and are used to stop an inflation if inspired tidal volume exceeds >130% of set tidal volume.

**Volume-targeted versus pressure-limited ventilation**

A recent update of the Cochrane review on volume-targeted versus pressure-limited ventilation analysed 9 parallel trials (629 infants) and 3 cross-over trials (64 infants), all in infants of less than 28 days of age (Wheeler, et al., 2010). The primary outcome of death or BPD was significantly reduced in infants supported with volume-targeted ventilation compared with pressure-limited ventilation (RR 0.73, 95% CI 0.57-0.93; NNT 8) based on 439 infants in 5 trials. For infants less than 1,000 g this result was of borderline significance (RR 0.79, 95% CI 0.62-1.01; p = 0.06) based on 224 infants and 4 studies. The sole outcome of BPD (defined as the need for supplemental oxygen at 36 weeks) had a strong trend
towards favouring volume-targeted ventilation based on 5 studies including 413 infants (RR 0.73, 95% CI 0.53-1.00) and in infants of less than 1,000 g based on 4 studies and 202 infants (RR 0.81, 95% CI 0.59-1.12). Furthermore, volume-targeted ventilation resulted in reductions of the rate of pneumothorax (RR 0.46, 95% CI 0.25-0.84; NNT17), duration of ventilation (WMD -2.36, 95% CI -3.9 to -0.8), hypocarbia (RR 0.56, 95% CI 0.33-0.96; NNT 4) and the combined outcome of periventricular leucomalacia or grade III-IV intraventricular haemorrhage (RR 0.48, 95% CI 0.28-0.84; NNT 11). No difference was found in neurodevelopmental impairment (RR 0.86, 95% CI 0.47-1.59) at follow-up in two studies; however, this was a secondary outcome and, therefore, not powered to detect a difference (Wheeler, et al., 2010). One of these studies reported at the two year follow-up that fewer children following volume-controlled ventilation used inhaled corticosteroids (OR 0.3, 95% CI 0.1 to 0.9, p = 0.04) and reported a trend for both reduction in readmission to hospital (p = 0.07) and the combined outcome of death or disability (p = 0.07) (Singh, et al., 2009).

A recent survey in European and Australasian neonatal intensive care units demonstrated that volume-targeted ventilation was routinely used in only 50% of the units (Klingenberg, et al., 2010) despite this evidence demonstrating clinically important benefits over pressure-limited ventilation.

**Patient triggered (synchronised) ventilation**

Traditionally, intermittent mandatory ventilation (IMV) was used for neonatal ventilation. In IMV, a TCPLV, the ventilator cycles at a programmed rate and if the patient breathes spontaneously, this can be in or out of synchrony with the mechanical breaths (Ramanathan, 2005; Jarreau, et al., 1996). Asynchrony has been shown to result in adverse physiological respiratory consequences, such as inconsistent tidal volume delivery, insufficient gas exchange, air leaks and increased work of breathing (Sinha, et al., 2008; Gupta, et al., 2007). An association with IVH has also been described (Sinha, et al., 2008). Synchronisation results in more uniform tidal volume delivery (Gupta, et al., 2007; Jarreau, et al., 1996), better oxygenation (Cleary, et al., 1995) and a
reduction in blood pressure fluctuations which potentially could aid in reducing the number of (severe) IVH (Hummler, et al., 1996).

Synchronised ventilation uses a patient derived trigger to initiate a mechanical breath. This signal can be a change in pressure or flow, abdominal movement or thoracic impedance. Inspiratory flow signals can also be used to terminate a mechanical breath, which is referred to as “flow-cycled ventilation”. This way both inspiration and expiration are triggered by the neonate. A short response time to the trigger is essential for effective patient triggered ventilation (Gupta, et al., 2007; Sinha, et al., 2008). Response time varies from 6 to 100 milliseconds, with most ventilators having a response time of ~50 milliseconds (Ramanathan, 2005).

Synchronised intermittent mechanical ventilation (SIMV), assist / control (A/C) or synchronised intermittent positive pressure ventilation (SIPPV) and pressure support ventilation (PSV) are modes of patient triggered ventilation. In the time-cycled SIMV mode, spontaneous breaths are mechanically supported up to the set rate, if a trigger threshold is met. If the set rate is not met, the ventilator delivers additional mechanical breaths to achieve this rate. The patient can breathe spontaneously at a rate above the set ventilator rate; however, these breaths are only supported by the baseline pressure (PEEP), and are thus not fully supported.

In the time-cycled A/C or SIPPV mode, all spontaneously initiated breaths – including those above the set rate – are fully supported by the ventilator if the trigger threshold is met. In case of insufficient respiratory drive and a low rate of spontaneous ventilatory effort or even apnoea, the ventilator delivers the set rate of mechanical breaths. Both SIMV and time-cycled A/C modes only provide synchronisation of triggered inspiration; the inspiratory pressure is maintained for the duration of the inspiratory time as set by the clinician.

The flow-cycled A/C and PSV modes terminate inspiration before the set maximum inspiratory time, once the inspiratory flow decreases to a certain percentage of peak inspiratory flow, usually 10-20% (Keszler, 2009), thus providing full synchronisation between patient and ventilator (Sinha, et al.,
2008; Gupta, et al., 2007). For example, the Babylog8000 plus in PSV mode will cease the inflation when the inspiratory flow reduces to 15% of the peak flow seen during the inflation (Figure 1.9). These flow-cycled modes can therefore be considered as “triggered expiration”, allowing deflation when gas is no longer entering the lungs.

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**Figure 1.9: Pressure and inspiratory flow wave forms during FCPLV and TCPLV**

Pressure wave-form and inspiratory flow wave-form during flow-cycled ventilation on the left (PSV-mode) and during time-cycled ventilation on the right (SIPPV-mode). Peak inspiratory pressure is indicated by the blue arrow. During flow-cycling, expiration is initiated once inspiratory flow is less than 15% of peak inspiratory flow (red arrow). During time-cycling, the inspiratory time is maintained for the set inspiratory time, here resulting in an inspiratory pressure plateau. During this time there is no or minimal inspiratory flow recorded (red two-sided arrow). Waveforms were recorded using Ventview whilst ventilating with a Babylog 8000 plus.
Chapter 1

Intermittent mandatory versus synchronised ventilation

Many studies have been performed comparing conventional IMV to synchronised modes of ventilation. The first publication appeared in 1992; 10 VLBW babies were successfully ventilated for 1 hour on a flow-cycled mode, which improved oxygenation compared with IMV (Servant, et al., 1992).

The first multicentre trial of synchronised ventilation compared IMV with SIMV (synchronisation based on abdominal impedance) in 327 infants. This trial reported that babies receiving SIMV had lower mean airway pressures, improved oxygenation and a shorter duration of mechanical ventilation (Bernstein, et al., 1996).

Another international randomised-controlled trial of 924 infants (<32 weeks’ gestation) compared synchronised ventilation with IMV in infants needing ventilation within 72 hours after delivery. They used the SLE 2000 (SLE Ltd, South Croydon, UK) or Babylog 8000 ventilators. The mode of ventilation was not reported. The settings were different between the two groups; inspiratory time was set at 0.2 to 0.25 s and back-up rate was initially set at 35 breaths per minute in synchronised ventilation, whereas inspiratory time was 0.2 to 0.6 s and ventilator rate 40 to 65 breaths per minute in infants receiving IMV. There were no significant differences in the incidences of death or oxygen dependency at 36 weeks’ postmenstrual age, IVH or periventricular leucomalacia (PVL) at 6 weeks’ postnatal age, duration of ventilation, or rate of pneumothorax (OR 1.35, 95% CI 0.90 to 2.02). However, in a post-hoc analysis, infants of less than 28 weeks’ gestational age had a trend towards a higher risk of developing a pneumothorax when on synchronised ventilation (OR 1.72, 95% CI 0.98 to 3.02) (Baumer, 2000). Most babies in this study were ventilated with the SLE2000, which is a pressure-triggered ventilator and seems to synchronise less well than the flow-triggered Babylog in babies less than 28 weeks’ gestation as observed in a small study in 10 babies (Dimitriou, et al., 1998). Furthermore, the SLE2000 might be more prone to autotriggering at the highest sensitivity setting (Baumer, 2000). Both may have led to asynchrony between the ventilator and the patient,
possibly explaining the higher pneumothorax rates in the subgroup of babies less than 28 weeks’ gestation.

A Cochrane review has evaluated short- and long-term benefits of synchronised modes of ventilation in neonates less than four weeks old. The review included 12 studies, including the 2 above, and made the following comparisons: high frequency positive pressure ventilation (HFPPV) versus IMV (HFPPV defined as a ventilator rate of 60 breaths per minute, resembling more closely the respiratory pattern (and rate) of a preterm baby, and thus more likely to result in synchronous ventilation); SIMV or A/C versus IMV; A/C or PRVC versus SIMV, and SIMV plus pressure support versus SIMV. HFPPV resulted in a reduction of pneumothorax rate (RR 0.69, 95% CI 0.51 to 0.93) and A/C or SIMV resulted in a shorter duration of ventilation (WMD -34.8 hours, 95% CI -62.1 to -7.4) compared with IMV. No significant difference was seen for incidence of death or BPD amongst HFPPV, A/C and SIMV. Both PRVC and SIMV plus VG were each only tested in a single RCT with no significant advantages in important outcomes (Greenough, et al., 2008).

Although evidence for long term benefits of synchronised modes of ventilation is currently lacking (Keszler, 2009), it seems advisable, with the current knowledge, to ventilate neonates using synchronised modes of ventilation whenever possible, preferably combined with volume-targeted ventilation based on the expiratory tidal volumes.

**High frequency ventilation**

High frequency ventilation (HFV) uses extremely small tidal volumes (less than the anatomical dead space) at high rates (~200-900 breaths per minute, or 3.3-15 Hertz) to provide gas exchange. Ventilation can normally be accomplished at lower pressure during HFV (Donn, et al., 2006). Since volutrauma, atelectotrauma and to a lesser extent barotrauma are seen as main determinants in the development of VILI, HFV could be considered as a lung protective mode of ventilation (van Kaam, et al., 2007).
Two primary forms of HFV exist: high frequency jet ventilation (HFJV) and high frequency oscillation ventilation (HFOV), as well as other hybrid forms (Donn, et al., 2003; Cools, et al., 2009). HFJV uses high velocity pulsations (rate 240-660 breaths per minute) in tandem with a conventional ventilator, the latter providing PEEP and optional “sigh” breaths (Donn, et al., 2003). Expiration is passive and relies on lung recoil (Krishnan, et al., 2000). During HFOV a mean airway pressure (also called continuous distending pressure, CDP) is applied to inflate the lung to a static volume and oscillations around this point are used to effect gas exchange. The frequency is normally set at 8-15 Hertz. HFOV uses reciprocating diaphragms, leading to active inspiration and expiration (Donn, et al., 2003; Krishnan, et al., 2000).

A number of mechanisms have been identified as having a contributory role in gas exchange during HFOV, the most important being bulk convection, asymmetrical velocity profiles, Taylor-type dispersion, pendelluft, cardiogenic mixing and molecular mixing (Pillow, 2005; Chang, 1984). Unlike in conventional ventilation, bulk convection plays a relatively small role, mainly occurring in the proximal tracheo-bronchial tree. At airway bifurcations, inspiratory gas streams along the inner wall (in a skewed shape) towards the alveoli, whereas alveolar expiratory gas is streamed away along the outer wall (in a more square shape). This asymmetrical profile promotes gas mixing and thus gas exchange between inspiratory and expiratory gas flow (Figure 1.10) (Pillow, 2005). Taylor-type dispersion, also known as augmented diffusion, is an important mechanism of gas exchange during HFOV. In a straight tube a parabolic velocity profile exists, with gas near the centre moving more quickly than gas near the tube wall. With repeated respiratory cycles, gas in the centre will advance further into the lung, and this gas will mix with gas along the outer walls. When turbulent flow occurs (for example at branching points), this mixing between central core and periphery is much faster (Chang, 1984).
Figure 1.10: Gas transport during HFOV

At branching points inspiratory gas streams into the lung via the inner wall in a skewed shape (arrow), and leaves the lung in a more square shape (arrowhead). Pendelluft is demonstrated on the left side of the drawing at the end of expiration (curved arrow). Drawing adapted from Pillow (Pillow, 2005).

Pendelluft is dependent on differences in time constants between parallel lung units. Gas will flow from a unit with a short time constant into a unit with a long time constant at the end of inspiration, whereas the airflow will be reversed at the end of expiration, thus leading to recirculation of air between neighbouring units (Figure 1.10) (Chang, 1984).

Cardiogenic mixing, the superimposition of the cardiac contractions, may further promote the peripheral gas mixing by generation of flow within neighbouring lung units (Pillow, 2005). At more distal regions of the lung, molecular diffusion overcomes the Taylor dispersion as the principle mechanism for gas exchange (Pillow, 2005).

The clinically important variables are the distending pressure, which recruits and maintains lung volume if sufficient pressure is given, and the oscillatory wave form which promotes diffusion and gas exchange by the mechanisms
described (Donn, et al., 2003). This wave form can be changed by altering the amplitude, frequency or inspiratory-expiratory ratio of ventilation.

HFV is used either as an elective mode of ventilation or as rescue mode in infants with severe respiratory failure (Thome, et al., 2008). To optimise gas exchange and to prevent or diminish VILI during HFV, a high volume strategy, also referred to as the open lung concept, can be applied. This strategy aims to recruit all alveoli and thus prevent alveolar overdistension (volutrauma) and repetitive opening of alveoli (atelectotrauma) (van Kaam, et al., 2003).

In the Cochrane review comparing elective HFOV with conventional ventilation, high volume strategy is defined as treatment with at least two of the following: initial use of higher mean airway pressure than on conventional ventilation, initial weaning of supplemental oxygen before weaning of mean airway pressure, or use of alveolar recruitment manoeuvres (Cools, et al., 2009). In surfactant depleted piglets, comparison of 5 hours of ventilation with HFOV or conventional ventilation, used with or without an open lung strategy, demonstrated an improvement in oxygenation, compliance, lung injury scores and alveolar protein influx when the open lung concept was used (van Kaam, et al., 2003).

High frequency ventilation versus conventional ventilation

Most studies of HFV have focussed on HFOV versus conventional ventilation.

A Cochrane review on elective HFOV versus conventional ventilation evaluated 17 trials involving 3,652 patients. Patients were <35 weeks' gestation, <2,000 g at birth and ≤12 hours old at randomisation. No reduction was seen in mortality, and reduction of BPD was of borderline significance during HFOV (RR 0.89, 95% CI 0.81 to 0.99) (Cools, et al., 2009).

This Cochrane review includes studies performed over the last 20 years; therefore, changes in practice, for example the introduction of surfactant, will be reflected in the outcomes. Furthermore, lung protective strategies during conventional ventilation have been introduced as standard practice for most neonatal units. These strategies were defined in the Cochrane review as short
inspiratory times, rate of 60 breaths/min, PEEP of 4-6 cmH₂O, limiting tidal volume, patient triggering or permissive hypercapnia (Cools, et al., 2009).

Performing subgroup analysis revealed that HFOV did not result in a reduction of BPD, when routine surfactant was used (RR 0.91, 95% CI 0.82 to 1.01) or when HFOV was compared with conventional ventilation using lung protective strategies (RR 0.86, 95% CI 0.71 to 1.03), probably using lung protective strategies (RR 0.96, 95% CI 0.84 to 1.10), or probably not using lung protective strategies (RR 1.09, 95% CI 0.79 to 1.49) (Cools, et al., 2009). On the other hand, comparing HFOV with conventional ventilation without lung protective strategies resulted in a significant reduction in BPD (RR 0.48, 95% CI 0.31 to 0.75) (Cools, et al., 2009), which most likely reflects an advantage for HFOV in prevention of BPD when less modern strategies of conventional ventilation are used.

Other subgroup analysis demonstrated that HFOV reduced the incidence of BPD when piston oscillators were used (RR 0.82, 95% CI 0.70 to 0.95) and when randomisation occurred early (2-6 hours of age) (RR 0.72, 95% CI 0.59 to 0.87) (Cools, et al., 2009). Secondary outcomes of this Cochrane review showed a small, but significant increase in incidence of pulmonary air leaks (RR 1.19, 95% CI 1.05 to 1.34) on HFOV. An increase in severe IVH (RR 1.45, 95% CI 1.09 to 1.93) and periventricular leucomalacia (RR 1.64, 95% CI 1.02 to 2.64) was observed when HFOV without a high volume strategy was used. However, neither IVH nor periventricular leucomalacia reached significance, when HFOV with high volume strategy was compared with CV, or when HFOV was compared with CV using lung protective strategies (Cools, et al., 2009), both strategies more representative for the current standard of care.

Another Cochrane review analysed 3 trials using HFJV versus conventional ventilation (248 infants, <35 weeks’ gestation, <2,000 g). HFJV was associated with a decrease in BPD (RR 0.59, 95% CI 0.35 to 0.99) and a decrease in home oxygen (RR 0.24, 95% CI 0.07 to 0.79); the latter was only reported in one study by Keszler (Bhuta, et al., 1998; Keszler, et al., 1997). There was no overall increase in PVL with HFJV, although one study described a significant increase in
the incidence of PVL. The method of conventional ventilation was not described for two studies, for the third studies ventilatory rates were ≤ 60 breaths/min (Bhuta, et al., 1998). Thus, the benefit of HFJV over conventional ventilation in these studies published between 1990 and 1997 most likely reflects the fact the lung protective strategies were not used.

HFV is also used as rescue therapy in infants with severe respiratory failure. Two Cochrane reviews have focussed on rescue therapy; however, the number of eligible studies was small. Only one study was included in the comparison between HFJV and conventional ventilation and this was performed in the pre-surfactant era. This trial in 144 infants (< 35 weeks, < 2,000 g) reported no significant difference in mortality, BPD or adverse outcomes (Joshi, et al., 2006; Keszler, et al., 1991). For the comparison of HFOV and conventional ventilation only 1 trial of 81 patients was included (>34 weeks, <14 days old and >2,000 g). No difference was found in mortality, number of patients requiring extracorporeal membrane oxygenation, number of ventilated days, oxygen days or hospital days (Bhuta, et al., 2001; Clark, et al., 1994).

Overall, HFV can be used as initial and as rescue ventilatory strategy in preterm infants; however, no clear benefits of HFV have been demonstrated compared with conventional ventilation. It seems more important to use the optimal ventilatory strategy with the chosen mode: either high volume strategy during HFV or lung protective strategies during conventional ventilation.

1.6.3. Inspiratory time

As described above, different variables can be altered during conventional ventilation including pressures, tidal volume, ventilator rate, inspiratory and expiratory times and, on some ventilators, the bias gas flow.

Depending on the mode of ventilation, the length of inspiratory time can be set by the clinician or is determined by the infant’s lung mechanics. The time necessary to inflate the lung is dictated by the time constant (compliance x resistance) of the respiratory system (Gerhardt, et al., 2008). From this it follows that in infants with non-compliant lungs (such as is seen with RDS), the time
constant – and, therefore, the time needed to inflate the lung – will be short (Spitzer, et al., 2003). The time constant will be longer in term infants with normally compliant lungs, following treatment with surfactant in infants with RDS, and in infants with increased resistance, for example caused by airway obstruction.

Data on the normal inspiratory time for infants are limited. Spontaneously breathing healthy term babies had a mean inspiratory time of 0.51 seconds and preterm infants with RDS had an inspiratory time of 0.30 seconds measured using a nasal pneumotachograph (Harrison, et al., 1968). In a study of newborn infants ventilated with IMV for RDS, the median spontaneous inspiratory time was also approximately 0.30 (range 0.26-0.34) seconds measured using a Graseby capsule (Ahluwalia, et al., 1994). No data are available on ventilator bias gas flow or the use of PEEP for this study; the latter can affect the compliance and, therefore, the time constant.

A longer inspiratory time during TCPLV is associated with a higher mean airway pressure and subsequently often better oxygenation. A long inspiratory time has been a preferred ventilator setting for many years, even though this strategy is mostly based on two small studies (Kamlin, et al., 2003; Herman, et al., 1973; Reynolds, 1971). The first study measured the effect of altering the I:E ratio on PaO₂ in 6 infants with RDS (Reynolds, 1971). Since the rate was maintained at 30 breaths/min, altering the I:E ratio affected the inspiratory time. An inspiratory time of 1.3 s led to PaO₂ values that were 69.2 ± 14.6 mmHg higher and an inspiratory time of 1.6 s led to PaO₂ values that were 97.3 ± 21.9 mmHg higher compared with an inspiratory time of 1.0 s (p < 0.005) (Reynolds, 1971). The second study studied both the effect of altering the I:E ratio from 1:2 to 2:1 and the application of PEEP on PaO₂ and alveolar-arterial oxygen difference (A-aDO₂) in 9 infants with RDS. The ventilator rate was maintained at 30 breaths/min. An inspiratory time of 1.3 s doubled PaO₂ and decreased A-aDO₂ values compared with an inspiratory time of 0.7 s, with even higher values for PaO₂ and lower values for A-aDO₂ following PEEP of 5 cmH₂O compared with no PEEP (all p < 0.05) (Herman, et al., 1973). A long inspiratory time, however, can lead to active expiration by the baby against the inspiratory pressures of the
ventilator ("fighting the ventilator"), if the set inspiratory time is long enough to create an inspiratory pressure plateau (Greenough, 1988; Gerhardt, et al., 2008). Ultimately, a long inspiratory time leads to an increased risk of pneumothorax in babies with RDS (RR 1.56, 95% CI 1.25 to 1.94, NNT 8, 95% CI 5 to 14) (Greenough, 1988; Kamlin, et al., 2003), and interstitial emphysema (Gerhardt, et al., 2008). Newer modalities of mechanical ventilation, such as flow cycled A/C mode and PSV mode, offer patient triggered expiration, where inspiration will end based on lung mechanics of the baby. It has to be determined if these modes of ventilation will result in a lower pneumothorax rate and less BPD than modes of ventilation with a fixed inspiratory time.

1.6.4. Bias gas flow

In many neonatal ventilators, a continuous bias gas flow, also called circuit flow, circulates in the ventilator and ventilator circuit. Pressures in the ventilator circuit can be regulated by the opening or closing of a valve, placed in the circuit, which results in the cycling of the ventilator (Spitzer, et al., 2003). During the time the valve is closed, pressure will build up in the circuit and this pressure will rise faster if the velocity of the bias gas flow is greater (Gerhardt, et al., 2008). On the other hand, if the bias gas flow is too low, the set inspiratory pressure may not be achieved within the set inspiratory time, which could result in inadequate tidal volume delivery (Gerhardt, et al., 2008).

Some ventilators use a set flow, either based on manufacturer’s guidelines (Bhutani, 2002) or set by the clinician. Despite recommendations about flows, there are hardly any experimental data available to support different flows. One study in 9 ventilated preterm babies with RDS (gestational age between 27 and 36 weeks, birth weight between 1,300 and 4,010 g) reported that the optimum bias gas flow was 7.7 L/min in combination with an I:E ratio of 1:4.5 (rate 60-70 breaths/min) in order to achieve the best arterial oxygenation (Owen-Thomas, et al., 1968). This study was performed before PEEP was used in neonatal care, which will affect the compliance and time constant. The inspiratory time in this study will have been less than 0.2 s at an I:E ratio of 1:4.5. It is, therefore,
questionable if in the current era a bias gas flow of 7.7 L/min would be seen as optimum bias gas flow.

The lack of empirical evidence, however, has not stopped some authors from recommending that in preterm infants a bias gas flow of 6-10 L/min is generally sufficient, in term infants 12 L/min, whereas older infants need higher flow rates (Gerhardt, et al., 2008). Experiments for anaesthetic techniques have shown that gas flow in a ventilator circuit should be ~ 2½ to 3 times the minute volume to prevent rebreathing or air dilution (Harrison, 1964). Some paediatricians recommend the bias gas flow be at least twice the minute volume (Spitzer, et al., 2003), whereas others recommend it to be approximately 8 fold the desired minute volume (Bhutani, 2002). As an approximate minute ventilation for a preterm infant is 300 mL/Kg.min (tidal volume 5 mL x ventilator rate of 60 breaths per minute), it could be postulated that a flow of even 6 L/min is excessive for an infant weighing less than 2 Kg.

High bias flows are believed to increase the risk of air leak syndrome, because maldistribution of ventilation results in a rapid pressure increase in non-obstructed or non-atelectatic airways or alveoli (Spitzer, et al., 2003). High flows may also result in decreased tidal volume delivery secondary to increased turbulence in high-resistance, small diameter endotracheal tubes (Spitzer, et al., 2003). Again, empirical evidence to support these statements is lacking.

1.6.5. Inspiratory and expiratory flow

Bias gas flow in the ventilator circuit needs to be distinguished from inspiratory and expiratory flow that traverses the endotracheal tube and which can be measured using a pneumotachograph just before entering the endotracheal tube or at the nose with a face mask or nasal probes. In healthy term babies who were breathing spontaneously, the average value for peak inspiratory flow (PIF) was 2.6-2.8 L/min and for peak expiratory flow (PEF) 2.2-2.4 L/min (Swyer, et al., 1960; Harrison, et al., 1968). Infants with severe RDS, who were breathing spontaneously but with grunting, rib recession and tachypnoea, had an average PIF of 3.1 L/min and PEF of 2.9 L/min (Harrison, et al., 1968). In these spontaneously breathing infants, the air flow travels through the nose and
pharynx, which are bypassed once an infant is intubated. Therefore, these flows are not comparable to the PIF and PEF measured during ventilation or even spontaneous breathing through the endotracheal tube.

The velocities of inspiratory flow during mechanical ventilation are dependent on the mode of ventilation. During TCPLV and FCPLV there is a steep increase in inspiratory flow, until the PIF is reached (either when set pressure or tidal volume is reached) followed by a decelerating inspiratory flow (Sinha, et al., 2003). During volume-controlled ventilation there is a set inspiratory flow, (Sinha, et al., 2003). Peak inspiratory pressure and peak volume delivery are achieved at the end of inspiration (Sinha, et al., 2008). Thus, during TCPLV and FCPLV the inspiratory flow is dependent on the lung mechanics and the bias gas flow, whereas during volume-controlled ventilation the inspiratory flow is preset by the clinician and the ventilator adjusts the pressure to achieve a constant inspiratory flow and deliver the preset tidal volume (Sinha, et al., 2008, 2003; Gerhardt, et al., 2008). Patient-ventilator asynchrony is a disadvantage of volume-controlled ventilation; if the set inspiratory flow is not enough to cover the patient demand, this can lead to ‘flow starvation’ and increased work of breathing by the patient (Sinha, et al., 2003). Volume-assured pressure support ventilation (VAPS) starts a breath in FCPLV and, if the set tidal volume has not been reached, completes a breath with volume-targeted ventilation (see volume-targeted ventilation) (Sinha, et al., 2003).

1.6.6. Wave forms

During mechanical ventilation wave forms can be recorded for pressure, volume and flow. Wave forms are different in pressure-targeted and volume-controlled ventilation (Sinha, et al., 2003). As volume-controlled ventilation is outside the scope of this thesis, I will focus on wave forms specific for pressure targeted ventilation.

In time-cycled ventilation, the length of the inspiratory time can determine the presence and/or length of an inspiratory pressure plateau. The rate of rise of inspiratory pressure is dependent on lung mechanics and the ventilator bias gas flow (Gerhardt, et al., 2008). A high bias gas flow combined with a long
inspiratory time will result in a steep rise in pressure and an inspiratory pressure plateau can occur, i.e. in IMV, SIMV or time-cycled SIPPV-mode. There is minimal inspiratory flow during this inspiratory pressure plateau (Figure 1.9); however, the inspiratory pressure is maintained for the duration of the set inspiratory time (Gerhardt, et al., 2008). The inspiratory pressure plateau, or the square pressure wave form, results in higher mean airway pressures and in better oxygenation, although overdistension and air leak syndrome might be a complication of inspiratory pressure plateaus (Kamlin, et al., 2003; Spitzer, et al., 2003).

Shortening the inspiratory time will result in similar rate of pressure rise, but the inspiratory pressure plateau will eventually disappear. Lowering the bias gas flow will result in slower rate of rise of pressure and can consequently reduce the length of a pressure plateau, or even result in a sawtooth waveform (Gerhardt, et al., 2008). An inspiratory time which is too short, or a bias gas flow which is too low will lead to inadequate ventilation where pressure and volume targets are not reached. Therefore, a proper combination of inspiratory time and bias gas flow is essential to reach the intended pressure, volume and, if desired, the intended pressure plateaus.

Flow-cycled ventilation, for example in the PSV-mode of the Babylog 8000plus or flow-cycled SIPPV, results in a sawtooth pressure waveform as inspiration is terminated once the inspiratory flow is reduced to 10, 15 or 20% of the peak inspiratory flow (Gerhardt, et al., 2008).

1.6.7. Humidification

The upper airway plays an important role in warming and humidification of air. Even at wide ranges of temperature and humidity of inhaled air, the gas arriving in the nasopharynx during quiet nasal breathing has a temperature of 32°C with 100% relative humidity (RH) (Rouadi, et al., 1999). By the time the gas reaches the trachea or main bronchi it is at core temperature at 100% RH (Williams, et al., 1996).
Humidity is defined as water vapour in a gas; water vapour consists of individual water molecules, moving independently and randomly through the gas. The amount of water vapour in a gas is expressed as absolute humidity (AH). The relative humidity is the amount of water vapour present in the gas relative to what it can hold at the given temperature. When gas is heated its capacity to hold water is increased. The maximum capacity for air is 44 mg per litre at 37°C (AH 44 mg/L), resulting in a relative humidity of 100% (100% RH) (Williams, et al., 1996).

Endotracheal intubation bypasses the normal physiological system of warming and moisturising air, potentially causing cold and / or dry air to arrive at alveolar level. This can lead to damage to the tracheobronchial epithelium, malfunction of the mucociliary transport system, thickening of secretions and heat loss of the patient (Chalon, et al., 1979). The effect of air temperature has been tested experimentally, exposing ovine trachea mounted flat in an organ bath to air at 37°C, 34°C or 30°C at 100% RH. At the lower temperatures ciliary beat frequency and mucus transport velocity were severely reduced, or failed completely within 4 hours (Kilgour, et al., 2004). At 37°C the trachea maintained its normal histology, whereas tissues exposed to 30°C had reduced numbers of epithelial cells, reduced height and shedding of columnar epithelium leaving the basement membrane exposed (Kilgour, et al., 2004). Thus, even a slight reduction in air temperature can lead to epithelial damage, indicating the necessity to heat inspiratory air when a patient is intubated.

It is now accepted practice to use humidifiers during mechanical ventilation to warm and humidify the gas before it is delivered into the patient's lungs. The optimum temperature and humidity required to prevent these deleterious changes are still controversial (Poopalalingam, et al., 2002; Williams, et al., 1996); an AH level of inspired gas of >33 mg/L is recommended by the American National Standards and International Organization of Standardization (Miyoshi, et al., 2005). In an experiment in which 4 humidifiers were tested at 32 and 37°C, only one humidifier reached this recommendation at 32°C, whereas all of them had an AH > 33 mg/L at 37°C, with the RH varying between 84 and 100% (Nishida, et al., 2001). Besides the temperature of the inhaled air, ventilator
settings also determined humidity: if the tidal volume was larger than the volume in the inspiratory circuit, the humidity decreased (Nishida, et al., 2001). In neonatal units this problem is negligible because of the small size of the babies ventilated. Current practice for neonates is to use humidifiers set at 37°C, aiming for a 100% RH. However, at these settings condensation will occur in the ventilator circuit, since the room temperature is lower than the temperature in the circuit. To prevent this, heated wires integrated into the ventilator circuits prevent condensation, enabling a better temperature and humidity control.

1.6.8. Non-invasive respiratory support

Nasal continuous positive airway pressure (NCPAP) and nasal intermittent positive pressure ventilation (NIPPV) are forms of non-invasive respiratory support, since the infants do not need to be intubated to receive this treatment. As complications of ventilation, such as subglottic stenosis and respiratory infections are avoided or less likely to occur with the use of NCPAP or NIPPV, theoretically this treatment should have an advantage over mechanical ventilation. However, both methods still deliver pressures that are supra-physiologic (Gupta, et al., 2009), which might affect development of VILI in the vulnerable immature lung.

Both NCPAP and NIPPV apply a continuous positive pressure to the airways, which supports the breathing in preterm infants via a number of mechanisms. The functional residual capacity (FRC) increases, leading to decreased ventilation-perfusion mismatch and improved oxygenation. Airway resistance reduces via dilatation of the airways, the incidence of obstructive apnoea reduces and the chest wall is better stabilised with reduction of chest distortion; the latter is especially important in preterm infants, who have very compliant chest walls (Bancalari, et al., 2006; Krouskop, et al., 1975; Richardson, et al., 1978; Davis, et al., 2008).

**CPAP**

Gregory et al. were the first to describe improved oxygenation and decreased mortality with the use of CPAP in spontaneously breathing newborn infants with
RDS (Gregory, *et al.*, 1971). Various delivery forms of NCPAP have been developed since this first report; however, short binasal prongs have been demonstrated to be superior to single nasal prongs in the neonate in reducing the rate of reintubation (De Paoli, *et al.*, 2008). Evidence for the optimal level of pressure is poor. Infants needing NCPAP to prevent apnoeic periods might need lower pressures than infants needing NCPAP as treatment for RDS, since the latter will have less compliant lungs (Davis, *et al.*, 2008). If inappropriately high NCPAP pressures are used in infants with compliant lungs, cardiopulmonary status might be compromised with reduced blood flow through the lungs, and overdistension of the airspaces. This may result in hypercapnia, secondary to increased dead space ventilation and reduced tidal volume and an increased risk of pneumothorax (Bancalari, *et al.*, 2006; Davis, *et al.*, 2008; Polin, *et al.*, 2002).

Although the application of CPAP has beneficial effects on the respiratory function, it has been demonstrated to up-regulate both a local and systemic inflammatory response in preterm lambs (133 days’ gestation; normal gestation 150 days) to a similar level as that seen following mechanical ventilation with normal tidal volume and PEEP, i.e. ‘gentle ventilation’ (Polglase, *et al.*, 2009). The inflammatory response was documented after only 3 hours of CPAP or mechanical ventilation, and a logical next question is the effect CPAP has on lung injury and / or lung development in an immature lung since, ultimately, this is the more important outcome. A study in preterm baboons, delivered at 125 days (normal gestation 185 days) and mostly supported with NCPAP for 28 days after delivery, did not demonstrate an arrest in alveolar development when compared with lung tissue from age-matched controls at 156 days’ gestation. Similar alveolar counts and alveolar surface area were documented in the two groups. Furthermore, myofibroblasts could be identified in most of the secondary septal crests and elastin was deposited at the tip of the secondary crests following NCPAP, therefore, supporting the hypothesis that NCPAP enables a normal alveolarisation in the immature lung of a baboon (Thomson, *et al.*, 2004).
Basic principles of CPAP

Positive pressure in the CPAP circuit can be generated through a neonatal ventilator with a continuous flow of at least 2.5 times the infant’s minute volume to prevent rebreathing of carbon dioxide (Polin, *et al.*, 2002). A threshold resistor exhalation valve provides resistance to exhalation, thus creating a positive pressure. An alternative method is underwater bubble CPAP, which uses a column of water to create the positive airway pressure. Besides the fact that this system does not need a ventilator, and thus is a more cost-effective option, it also generates oscillations at ~15-30 Hertz to the infant’s chest (Polin, *et al.*, 2002). This has been demonstrated to reduce the respiratory rate and minute volume compared with conventional endotracheal CPAP (Lee, *et al.*, 1998). A third mode to create the positive pressure is via a variable flow CPAP device (Infant Flow™, Yorba Linda, USA) (Polin, *et al.*, 2002; Bancalari, *et al.*, 2006; Davis, *et al.*, 2008).

During variable flow CPAP, a gas enters the system via the relatively wide gas inlet and flows into the narrow jet injector nozzle, which will increase the rate of flow and decrease the pressure in the nozzle. Subsequently, gas flows into the wider nasal prong, where flow velocity will be lower and pressure will be higher than in the nozzle (Figure 1.11) (Wiswell, *et al.*, 2003). During expiration there is a so called “fluidic flip” explained by the Coanda effect, which is the tendency of a gas or fluid to follow the curved surface of a wall. Once an infant starts to exhale, the jet of gas flow changes direction from towards the nasal prongs to the expiratory channel, the “fluidic flip”. The residual gas pressure enables stable levels of CPAP to be delivered to the infant (Wiswell, *et al.*, 2003). Therefore, the infant does not exhale against the gas flow of continuous CPAP. This has been shown to reduce the work of breathing (Pandit, *et al.*, 2001), but not the need for mechanical ventilation or the duration of CPAP treatment (Mazzella, *et al.*, 2001) compared with continuous flow CPAP.
Figure 1.11: Schematic representation of the variable flow CPAP device

During inspiration (left) gas flows from the gas inlet through the jet injector into the nasal prong. Pressure is low, but flow is fast in the jet injector nozzle. Conversely, once gas enters the nasal prong the pressure is higher, but flow slower. During expiration (right), the expiratory gas flow follows the curve of the tube. The Coanda effect causes the inspiratory flow to flip (the fluidic flip) and to leave the generator chamber via the expiratory limb together with the expiratory gas. Drawing adapted from Wiswell (Wiswell, et al., 2003).

Clinical studies on the use of CPAP and surfactant

The COIN trial (CPAP or Intubation at Birth), an international, multicentre, randomised controlled clinical trial, compared early nasal CPAP with intubation and ventilation, but not necessarily surfactant administration, in 610 infants born between 25 weeks’ and 28+6 weeks’ gestation, evaluating death or BPD at 36 weeks as the primary outcome (Morley, et al., 2008). The outcome death or oxygen treatment was significantly better at 28 days (OR 0.63, 95% CI 0.46 to 0.87, p=0.006); however, this difference disappeared at 36 weeks (OR 0.80, 95% CI 0.58 to 1.12, p=0.21). There was an increased risk of pneumothorax in the
CPAP group (9% vs. 3%, p<0.0001). Although there was a trend to fewer days of oxygen, ventilation, CPAP and hospitalisation, these were not significant (Morley, et al., 2008). This study showed that very preterm infants can be treated with CPAP from birth, with less than half of this group needing intubation or surfactant in the first days of life.

A Cochrane review has addressed whether CPAP with early intubation for surfactant administration and brief ventilation would be better than later selective surfactant administration and continued mechanical ventilation in infants of less than 37 weeks’ gestation (Stevens, et al., 2007). Four randomised controlled trials were included in this review. Early surfactant and CPAP resulted in a lower incidence of mechanical ventilation (RR 0.70, 95% CI 0.59 to 0.84), but no significant differences were found in air leak syndromes (RR 0.51, 95% CI 0.23 to 1.10) or BPD, defined as oxygen treatment at 28 days (RR 0.94, 95% CI 0.20 to 4.35) (Stevens, et al., 2007). No data on oxygen treatment at 36 weeks’ gestation were available.

The recently published multicentre Surfactant, Positive pressure, and Pulse Oximetry Randomised Trial (SUPPORT) compared CPAP treatment with surfactant administration within an hour followed by continued mechanical ventilation in 1,316 infants of a younger gestation (24 to 27+6 weeks). The primary outcome of death or BPD, according to the physiological definition of BPD, at 36 weeks’ gestation was not significantly different. However, when BPD was defined as any oxygen at 36 weeks’ gestation, the primary outcome was nearly significant (RR 0.91, 95% CI 0.83 to 1.01, p=0.07), in favour of CPAP. Furthermore, CPAP resulted in significantly lower number of ventilated days (p=0.03), mechanical ventilation at day 7 (p=0.01) and need for postnatal steroids (p<0.001). There was no difference in the occurrence in air leak syndrome between the groups (6.8% vs. 7.4% in the ventilated group) (SUPPORT Study Group of the Eunice Kennedy Shriver NICHD Neonatal Research Network, 2010a). The outcomes of both the SUPPORT trial and the COIN trial show that, although no significant reduction in BPD was seen, starting CPAP at birth has important benefits in the very preterm baby, even if it fails in some, and has no serious side effects (Morley, 2010).
The CURPAP study, an international RCT on 208 infants of 25 to 28 weeks’ gestation compared CPAP with surfactant administration, mechanical ventilation and extubation to CPAP within an hour. This study reported no difference in their primary outcome of the need for mechanical ventilation in the first 5 days of life, which was needed by 33 vs. 31.4% of the infants (p=0.80), nor in any of the secondary outcomes, such as death, pneumothorax, or postnatal steroids use. Their overall rate of a pneumothorax was 3.8%, which possibly is a reflection of a lower threshold for surfactant treatment compared with the COIN trial (Sandri, et al., 2010; Morley, et al., 2008).

The next question that was investigated in a single centre study was if intubation for prophylactic surfactant administration immediately followed by extubation and CPAP compared with only CPAP would reduce the incidence of the need for mechanical ventilation in the first 5 days in 279 babies of 27 to 31+6 weeks’ gestation (Rojas, et al., 2009). There was a significant reduction in their primary outcome with 26% babies in the surfactant group vs. 39% in the CPAP only group needing mechanical ventilation in the first 5 days (RR 0.69, 95% CI 0.49 to 0.97, p<0.05). There was a trend towards a reduction in BPD at 36 weeks’ gestation (49% vs. 59% in the CPAP only group, RR 0.84, 95% CI 0.66 to 1.05). Air leak syndrome occurred less often after surfactant treatment (2% vs. 9%, RR 0.25, 95% CI 0.07 to 0.85). Other secondary outcomes, such as mortality, number of ventilated days, hospitalisation, severe IVH and PVL were not different (Rojas, et al., 2009).

These studies demonstrate that CPAP is safe to use, possibly reduces the need for and the duration of mechanical ventilation, and has beneficial effects on the outcome of very preterm babies. Combining CPAP with surfactant treatment followed by immediate extubation seems to have a possible positive effect on both BPD and the incidence of air leak. However, this technique still requires intubation, with the potential risks such as subglottic stenosis. To avoid trauma from intubation, Kribs et al reported on a technique to install surfactant using a feeding catheter followed by NCPAP (Kribs, et al., 2007). In infants of less than 32 weeks’ gestation a lower mortality rate and incidence of BPD was seen when compared with historical controls in their centre (Kribs, et al., 2008).
Chapter 1

et al described a similar technique, using a 16G vascular catheter, in 11 preterm infants. A significant decrease in both pressure and supplemental oxygen were demonstrated 4 hours after surfactant administration. No pneumothoraces were seen and only 3 babies needed subsequent mechanical ventilation, which was necessary because of apnoea of prematurity in 2 babies (Dargaville, et al., 2010). It yet has to be determined if this technique to administer surfactant immediately followed by NCPAP is as effective in other centres and the effect on long term outcomes should be investigated in a multicentre randomised controlled trial.

**NIPPV**

NIPPV is an alternative mode of non-invasive respiratory support that combines nasal CPAP with superimposed ventilator breaths. These breaths can be synchronised (preferred) or non-synchronised (Bancalari, et al., 2006). NIPPV is applied to enhance weaning or to treat apnoea in newborn infants (Davis, et al., 2008; Lemyre, et al., 2002). There is little guidance available for the correct settings during NIPPV; the trials described in the Cochrane review on NIPPV used a rate of 10 to 25 breaths per minute and the PIP was slightly higher than the PIP used pre-extubation (Davis, et al., 2001).

**Clinical studies on NIPPV**

A systematic review on 3 studies, including 159 preterm infants, compared NCPAP with NIPPV following extubation and indicated that NIPPV might be superior to NCPAP. NIPPV reduced the need for endotracheal reintubation (RR 0.39, 95% CI 0.16 to 0.97); however, only one of the three studies was responsible for this effect. There was a trend towards a reduction in BPD (RR 0.73, 95% CI 0.49 to 1.07) (Davis, et al., 2001), which is promising; however, larger trials are needed to reliably predict the impact of NIPPV on BPD development. No significant difference was seen in abdominal distension, which necessitated discontinuation of enteral feeding (Davis, et al., 2001).

Another systematic review on 2 studies, including only 54 preterm babies, compared NIPPV with CPAP on the incidence of apnoea of prematurity. One
study was a randomised controlled trial, the other trial was a randomised 2-period crossover study, and both only examined the short-term effects (4-6 hours). There was no difference in the need for intubation and no reduction in apnoea in the pooled analysis (Lemyre, et al., 2002).

In summary, there is some evidence that NIPPV following extubation reduces the risk of extubation failure and a possibility that NIPPV reduces the risk of BPD development. Further studies are required to determine the best settings and best device to use, as well as the use of NIPPV as primary respiratory support in the preterm infant.

1.7. Biomechanical forces

Breathing is a mechanical process, entailing cyclic application of distending pressures by the chest wall and respiratory muscles on the pleural surface with transmission of these distending stresses throughout the lung. The working range of this distending pressure is 0-30 cmH\(_2\)O, with 30 cmH\(_2\)O being the pressure at which the total lung capacity (TLC) is achieved. Remarkably, this working range of the distending pressure is independent of lung size, or body mass (Fredberg, et al., 2006).

Distending pressures are necessary to prevent the natural tendency of the lung to collapse, which applies to all levels of lung architecture (airways, alveoli and intrapulmonary blood vessels) (Fredberg, et al., 2006). Collapse at the level of the alveoli is prevented by the surface tension lowering qualities of surfactant. Surface tension in alveoli follows the Laplace relationship and is, therefore, inversely proportional to the radius of the alveolus (Wood, 2003). Preterm babies are at an increased risk of atelectasis, as they are often surfactant deficient and have alveoli of reduced size (Wood, 2003).

1.7.1. Elasticity in the lung

Elastic mechanisms also prevent collapse of the lung parenchyma. In experimental models lung parenchyma is frequently modelled as an homogenous elastic continuum, undergoing only small uniform distortions. However, this approach oversimplifies the mechanics seen in lung tissue (Denny,
The lung parenchyma is a connective tissue network with blood vessels and airways embedded in this network. Elastin and collagen fibres are the most important connective tissue fibres of the cytoskeleton of the lung and their architecture aids in the stability of the lung during respiratory movements (Vlahakis, et al., 2005; Young, et al., 1980). The blood vessels and airways resist deformation during breathing to a greater extent than the surrounding parenchyma (Vlahakis, et al., 2005); therefore, part of the lung parenchyma can be distorted, for example around a constricted airway, leading to non-uniform distortion (Denny, et al., 2006).

The difference in mechanical properties potentially can result in the development of a pressure gradient between the alveolus and the adjacent blood vessel or airway (dos Santos, et al., 2006) and explains why air generally tracks along bronchovascular bundles and why oedema accumulates in perivascular cuffs during lung injury secondary to ventilation (Vlahakis, et al., 2005).

1.7.2. Mechanics of the lung

In addition to air leaks, a progressive atelectasis is seen in injured lungs. This is due to surfactant deficiency in RDS, or to oedema formation, neutrophil influx and subsequent surfactant malfunction as a result of VILI (dos Santos, et al., 2006). This progressive atelectasis leads to deteriorations in compliance and gas exchange. Normally aerated, poorly aerated and nonaerated alveoli co-exist in the injured lung, reducing the overall lung volume available for gas exchange (referred to by Gattinoni as ‘baby lung”) (Vlahakis, et al., 2005; Gattinoni, et al., 1987). The non-injured areas in the lung receive a large proportion of the tidal volume during mechanical ventilation, and are thus at a risk of overdistension, while the injured areas remain collapsed. Since all alveoli are connected, a collapsed alveolus does not only exert mechanical forces on its own walls, but also on those of the adjacent alveoli. Injured lungs are, therefore, exposed to large mechanical forces, not normally encountered in a healthy lung (Mead, et al., 1970).

The mechanical forces in the lung, as in the vascular system, can be described as shear stress (a stress component parallel to a given surface) and cyclic strain or
stretch (fractional length change across an axis) (dos Santos, et al., 2006; Resnick, et al., 2003). Shear stress in the lung describes the stress on epithelial cells as alveoli collapse and re-open, although this is more often referred to as atelectotrauma (dos Santos, et al., 2006). Shear stress is more commonly used for the frictional forces of air on the wall of the airway or blood flow on the wall of the blood vessel. Air behaves like a liquid when flowing through a tube (Tarran, et al., 2005) and the shear stress generated is proportional to air or blood flow and inversely proportional to the diameter of the tube the air or blood is flowing through (Kamiya, et al., 1980; Tarran, et al., 2005).

In computer models of the respiratory tree, heterogeneity, or non-uniform inflation, of the alveoli and airways, as is seen in injured lungs, not only leads to a change in compliance but also to an uneven distribution of the air flow in the airways down to smallest airways (Gillis, et al., 1999; Nucci, et al., 2002). During heterogeneous constriction of the airways, airflow (Nucci, et al., 2002; Gillis, et al., 1999) and shear stress (Nucci, et al., 2003) were increased markedly in the smaller airways of the model compared with the alveolar level or the large airways (comparable to the trachea, bronchi and bronchioli).

Cyclic stretch in the respiratory system can occur during inspiration when lung volume increases. During normal tidal breathing the amplitude of alveolar volume change is not clear (Vlahakis, et al., 2005). However, it is assumed that the alveolar epithelium and underlying basement membrane are not stretched but simply fold and unfold (Tschumperlin, et al., 2006), thus, probably not leading to injurious cyclic stretch. To examine the effect of stretch, frequency and duration on alveolar epithelium, type II alveolar epithelial cells (AECs) were cultured for 5 days on a silicone membrane, after which the cells have attained more of the stretch resistant characteristics of type I AECs (Tschumperlin, et al., 2000). This is important, because the alveolar epithelium in the lung consists primarily of type I AECs (Tschumperlin, et al., 2000; Ross, et al., 1989). After 5 days, the silicone membranes were subjected to an increase in membrane surface area of 25, 37 or 50%, which equates roughly to an 80, 100 and greater than 100% change in alveolar epithelial basement membrane area, or 80, 100 or greater than 100% of TLC (Tschumperlin, et al., 2000; Tschumperlin, et al.,
The cells on the silicone membrane were either exposed to one static stretch for 1 hour or 900 stretches at a frequency of 15 or 60 cycles/minute. Cyclic stretch resulted in a higher percentage of dead AECs on the silicone membrane than one static stretch, and this occurred rapidly, within the first 5 minutes of the onset of cyclic stretch. A faster rate of cyclic stretch was more injurious than a slower rate, and the number of dead AECs increased dramatically with the highest membrane stretch compared with 25 and 37% stretch. A few membranes were exposed to one brief stretch of 1 or 4 seconds, which resulted in a similar number of dead AECs as one static stretch for 1 hour. Some silicone membranes were first stretched to a new baseline, increasing the surface area to 25%, upon which a further 12% or 25% cyclic stretch was applied. Thus, similar peak increases in silicone membrane surface area to 37% or 50% were achieved; however, this occurred at a lower amplitude of cyclic stretch. Interestingly, the percentage of dead AECs significantly reduced on these silicone membranes compared with membranes exposed to cyclic stretch from 0 to 37% or 0 to 50% respectively (p<0.05) (Tschumperlin, et al., 2000).

Thus, increasing the baseline and limiting the amplitude of cyclic stretch preserved viability of AECs (Tschumperlin, et al., 2000). This is highly relevant for the respiratory care of preterm babies, where both application of CPAP, increasing the baseline, and smaller tidal volumes, reducing the amplitude, have been demonstrated to reduce lung injury or improve respiratory outcomes (Davis, et al., 2008; McCallion, et al., 2005; Singh, et al., 2006; Dreyfuss, et al., 1992). The surfactant deficient lung of a preterm baby has a higher tendency to collapse (Wood, 2003); therefore, during each inspiration many alveoli need to open from a lower inflation point, i.e. the stretch in these alveoli is larger compared with normal lungs. Treatment with CPAP prevents collapse of the lung (Davis, et al., 2008); the applied pressure increases the baseline of stretch on the alveolar epithelium. A study in baboons looked at the effect of CPAP on cell viability. Preterm baboons, mainly managed on CPAP for 28 days after delivery, had similar numbers of endothelial cells and total amount of lung parenchyma when compared to age-matched controls (Thomson, et al., 2004). Thus, the application of CPAP did not reduce cell viability, supporting the hypothesis that
CPAP may reduce lung injury in the immature lung by increasing the baseline of stretch on the alveolar epithelium.

1.7.3. Mechanical force and lung injury

In addition to cell death, inter- and intracellular gap formation and denudation of the basement membrane are seen in response to exposure of mechanical forces on the alveolar epithelium (dos Santos, et al., 2006). The hypothesis is that the mechanical forces lead to rearrangement of the cytoskeleton and generation of tensile forces within the cell. These changes result in cellular contraction, interruption of intercellular adhesion complexes and gap formation. Ultimately, this can result in a disruption of the integrity of the pulmonary endothelial barrier (dos Santos, et al., 2006), which is a plausible explanation of the increased vascular permeability observed in VILI (Webb, et al., 1974; Dreyfuss, et al., 1998).

Several experiments and articles support this hypothesis by demonstration of alveolar haemorrhage in animal models (Dreyfuss, et al., 1995; Webb, et al., 1974), damage to the alveolar-capillary membrane using electron microscopy (Dreyfuss, et al., 1998) or presence of surfactant proteins A and D in the circulation (Eisner, et al., 2003) following injurious mechanical ventilation or RDS. The permeability has been demonstrated to return to normal shortly after the removal of the mechanical forces (Dreyfuss, et al., 1998; dos Santos, et al., 2006).

Mechanical ventilation also leads to abnormal deposition of collagen and elastin fibres in lung tissue of animal paradigms of VILI and preterm infants who died as a result of BPD (Albertine, et al., 1999; Coalson, et al., 1999; Pierce, et al., 1997; Thibeault, et al., 2003), which may be a result of abnormal rearrangement of the cytoskeleton following mechanical forces the immature lung is normally not exposed to.

1.7.4. Mechanosensing

During mechanical ventilation the lung parenchyma is thus subjected to several mechanical forces, which can result in lung injury and a systemic inflammatory
response. For this to happen, the cells in the lung parenchyma have to translate the mechanical signals into cellular signals, a process referred to as mechanosensing (dos Santos, et al., 2006) or mechanotransduction (Vlahakis, et al., 2005). The discussion around cellular mechanosensing is far from unravelled and falls outside the scope of this thesis; however, a brief explanation based on both vascular and pulmonary mechanosensing will follow.

Mechanosensors have been located in the plasma membrane, close to the site of applied force and flow. In addition, mechanosensors have been recognised in various compartments of endothelial cells with mechanical forces transmitted via the cytoskeleton to other locations (Ali, et al., 2002). Membrane channels for Na⁺, K⁺ and Ca²⁺ in the plasma membrane have been demonstrated to be responsive to mechanical forces, though the specific mechanisms by which they are activated are not fully clear (Gimbrone, et al., 2000; Ali, et al., 2002). Activity of a Na⁺-pump was found to increase in proportion to the magnitude of cyclic stretching of a silicone membrane to which rat type II AECs were seeded (Fisher, et al., 2002). Some of the cells were cultured for 5 days before the cyclic stretch was applied, which probably led to a transformation of those cells into the more stretch resistant type I AECs (Fisher, et al., 2002; Vlahakis, et al., 1999). These cells only responded when cell stretch changed the surface area of the silicone membrane by 37%, corresponding to severe overdistension in lung parenchyma or approximately 100% TLC (Fredberg, et al., 2006), compared with cells not cultured for 5 days which responded to stretch of the silicone membrane of 12 and 25% (Fisher, et al., 2002). Vascular permeability increased in response to overdistension (Dreyfuss, et al., 1998) and the increased activity of the Na⁺-pump within 60 minutes after the onset of stretch may be a beneficial process in the clearance of pulmonary oedema (Fisher, et al., 2002). Mechanosensing via a K⁺-channel has been demonstrated to occur within seconds. Application of laminar flow to cultured bovine aortic endothelial cells (BAECs) resulted in hyperpolarisation of the cell membrane, mediated via the K⁺-channel and reversed by depolarisation within 35 to 160 seconds via an outward Cl⁻ ion current (Barakat, et al., 1999).
Furthermore, protein kinases, such as mitase-activated protein kinases (MAPKs), and caveolae located in the plasma membrane seem to play a role in mechanosensing (Ali, et al., 2002). Two specific MAPKs, extracellular signal-related kinase (ERK) 1 and 2, have been demonstrated to undergo rapid tyrosine phosphorylation (Ali, et al., 2002; Tseng, et al., 1995; Rizzo, et al., 1998b) and to regulate gene expression (Takahashi, et al., 1996). This response is rapid and transient, with a peak at 5 minutes and a return to basal levels by 30 minutes after exposure to laminar shear stress (Park, et al., 2000). Caveolae are membranal domains containing various signalling molecules, such as serine and tyrosine kinases, as well as NO (Kurzchalia, et al., 1999). They are found in most cell types, including fibroblasts, endothelial cells and smooth muscle cells (Park, et al., 2000). Proteins in caveolae respond to laminar shear stress within 1-2 min with a rapid phosphorylation (Chen, et al., 1999). In addition to this, acute changes in shear stress induce rapid endothelial NO release, resulting in vasodilatation (Rizzo, et al., 1998a). It is not yet known how activation of endothelial nitric oxide synthase (eNOS) occurs, but fluid shear stress leads to movement of eNOS from the caveolae to the cytoplasm (Rizzo, et al., 1998a). BAECs treated with antibodies against caveolin-1, one of the principle components of caveolae, inhibited shear stress mediated activation of ERK1/2 (Park, et al., 2000), thus supporting the role of caveolae as mechanosensors.

In addition to mechanosensors in a central location such as the plasma membrane, less centrally located mechanosensors have also been proposed. Integrins attach the cytoskeleton of the cell to the extracellular matrix (ECM) and are likely candidates in decentralised mechanosensing (Ali, et al., 2002). During stretch, integrins can transfer mechanical forces almost instantaneously throughout the whole cell via the cytoskeleton, leading to a stiffening of the cellular cytoskeleton, thereby resisting deformation by stretch. A direct relationship has been observed between mechanical forces applied by a magnetic twisting device to cell surface receptors, such as integrins, and cytoskeletal stiffness in cultured endothelial cells (Wang, et al., 1993).

One of the proteins important in cell stiffness is actin and its cross-linker filamin-A. Molecular dynamics simulation, a novel technology (Fredberg, et al., 2006),
has demonstrated that these proteins tend to bind with nearly right angular alignment, especially in fibroblasts and macrophages, thereby forming a hinge. Experiments on two actin filaments revealed that these filaments can slide, or unfold, some distance relative to each other when exposed to mechanical forces (range 50-220 piconewton), after which they spring back to their original position once the stress is removed. This reversible process is described as ‘unfolding/refolding’ and likely contributes to the cytoskeletal elasticity (Yamazaki, et al., 2002). The actin filaments are also likely to play a role in the adaptation to fluid shear stress, as was demonstrated by in vitro experiments with a collagen scaffold, vascular smooth muscle cells and an endothelial monolayer on top of the cell culture (Fisher, et al., 2001). This resembles more closely the anatomy of a blood vessel compared with cell cultures solely using a monolayer of endothelial cells. Without flow, the collagen organisation determined the orientation of the endothelial cells and actin filaments, whereas after flow exposure all layers of the cell culture were aligned with the direction of flow (Seliktar, et al., 2000).

Based on the knowledge of the behaviour of individual proteins, 3D computer models are being developed to better understand the mechanical properties of tissue in vivo, for example lung tissue. One of these studies confirmed the reorientation and shortening or lengthening of line elements in response to mechanical forces in order to maintain stability. These line elements represented the elastin and collagen fibres in the extracellular matrix of the lung parenchyma (Denny, et al., 2006). Thus, the interaction between cells seems to be an important mechanism in stability of cell shape and lung parenchyma, and, therefore, ultimately in the prevention of atelectasis.

1.7.5. Inflammatory response and gene expression

Apart from cytoskeletal remodelling, mechanical force can also lead to a modulation in gene expression that affects activation or inhibition of cell proliferation, adhesion and inflammation (Resnick, et al., 2003). These mechanisms enable the endothelial cells to exist in a dynamic state (Fisher, et al.,
Chapter 1

2001) and to adapt to an alteration in shear stress by, for example, reshaping the diameter of a blood vessel (Kamiya, et al., 1980; Kubis, et al., 2001).

It is likely that the effects of mechanical forces are important during fetal development with fetal breathing movements and accumulation of fetal lung liquid being regulatory cues for deposition of extracellular matrix in the lung. To address this hypothesis, several experiments have been performed on cultures of purified and mixed fetal lung cells (Tschumperlin, et al., 2006). Fetal cells grown in mixed epithelial and fibroblast cultures for example, responded to intermittent stretch, simulating fetal breathing movements, with an increase in mRNA levels for collagen IV, an increase in the amount of collagen I and IV (Xu, et al., 1999), and an increase in the synthesis and secretion of the extracellular matrix protein fibronectin (Mourgeon, et al., 1999) when compared with static cultures.

Cyclic stretch and shear stress, as seen during breathing and artificial ventilation, also affect non-fetal lung cells. Shikata et al demonstrated that cyclic stretch and shear stress each induce different patterns of gene expression in human pulmonary artery endothelial cells, ultimately resulting in distinct patterns of cytoskeletal remodelling using fluorescent immunostaining (Shikata, et al., 2005). Isolated lung cells, such as macrophages, endothelial cells, bronchial cells and fibroblasts, were exposed to cyclic stretch with or without intratracheal lipopolysaccharide (LPS). The macrophage was the main source of the pro-inflammatory cytokines, IL-6, CXCL8 and TNF-α and increased levels were seen within 8 hours with a synergistic effect from LPS. The other cells failed to produce CXCL8 (Pugin, et al., 1998; Dunn, et al., 1999). Human alveolar epithelial cells (A549 cells), with characteristics of type I AECs, had a fourfold increase in CXCL8 mRNA expression following 4 hours of 30% cyclic stretch compared with static controls. Furthermore, these cells released 49 ± 34% more CXCL8-protein following 30% cyclic stretch for 48 hours compared with static control cells, whereas smaller deformations of 20% stretch produced no consistent increase in CXCL8-protein (Vlahakis, et al., 1999). The inflammatory response occurred without a difference in cell viability or proliferation rate; therefore, the
amplitude of stretch, and not cell injury, was responsible for the gene expression and protein release (Vlahakis, et al., 1999).

One has to bear in mind that cultured lung cells might behave differently from the actual cells in lung parenchyma. Some studies looked at gene profiling in lung tissue of rats following mechanical ventilation at high volumes, thereby leading to stretch on lung cells. Several genes, such as EGR1, IL-1β, HSP70, were up-regulated after only 30 minutes of in vivo ventilation with high tidal volume (25 mL/Kg); surprisingly enough, all of them were localised in the bronchiolar epithelium using in situ hybridization and only HSP-70 was also localised in the alveolar epithelium (Copland, et al., 2003). Another study ventilated rat lungs ex vivo with different ventilatory strategies (high or physiological tidal volume, PEEP or no PEEP). Ventilation with high volumes and/or no PEEP enhanced expression of TNF-α and IL-6, with the mRNA localised to the bronchial, bronchiolar and alveolar epithelium within 30 min of the onset of ventilation (Tremblay, et al., 2002). Thus, a greater degree of stretch on lung tissue seems to quickly enhance pro-inflammatory gene expression.

1.7.6. Flow physics

In addition to the cyclic stretch of tidal breathing, the lung is also exposed to the frictional forces of air flowing through the airways. Flow effects can vary depending on the magnitude, nature (steady, oscillatory or pulsatile) and distribution of the flow (Balcells, et al., 2005); however, cell responses to shear stress are tissue specific, as was demonstrated by exposing BAECs, rat lung epithelial cells, rat small intestine epithelial cells and human umbilical vein endothelial cells (HUVECs) to flows at different frequency and measuring the resulting cell proliferation and metabolic response, such as nitric oxide synthase activity (Balcells, et al., 2005).

Shear stress will be greater if the flow pattern is turbulent rather than laminar (Kamiya, et al., 1980). Vascular endothelial cells in vivo are normally exposed to shear stress in the narrow range of 15-20 dyn/cm² (1 Pa = 10 dyn/cm² = 1.033 x 10⁻² cmH₂O) (Fredberg, et al., 2006; Kamiya, et al., 1980). To maintain the level of shear stress within this narrow range, the vascular system is able to adapt to
acute changes in blood flow by rapid modulating of the vascular tone through endothelial compounds acting on the underlying smooth muscle cells, leading to vasodilatation or vasoconstriction (Resnick, et al., 2003). The release of NO from the endothelium mentioned above is an example of a vasodilator (Rizzo, et al., 1998a). The vascular system also seems to be able to adapt to chronic changes in blood flow by altering the diameter of the vessel, as illustrated by the following two studies. A chronic increase or decrease in blood flow in the common carotid artery, achieved by constructing an arteriovenous shunt to the jugular vein in dogs 6-8 months prior to the measurements, led to either an increase or a decrease in the internal diameter of the carotid artery, thus maintaining a stable level of shear stress (Kamiya, et al., 1980). Similar observations were made in humans with an occlusion of the internal carotid artery, mostly based on atherosclerosis (Kubis, et al., 2001). The common carotid artery feeds the internal carotid artery, and on the occluded side blood flow in the common carotid artery was reduced compared with the non-occluded side or compared with the blood flow in healthy controls. Wall cross-sectional area, an indicator for the internal diameter, was positively correlated to blood flow. Values for shear stress in the common carotid artery were identical in all arteries (Kubis, et al., 2001), therefore supporting the hypothesis that the artery can adapt to chronic changes in mechanical forces by adjusting its diameter in order to maintain shear stress within a narrow range.

1.7.7. Haemodynamics

A certain amount of shear stress is important to maintain the vascular endothelium in a quiescent state, thereby preventing alteration of gene expression, initiation of an inflammatory response or cellular proliferation and apoptosis. The levels of shear stress, however, can vary enormously in the vascular system; the branching geometry of the vascular system and the fact that many vessels have one or several curves all result in areas of complex flow (Ali, et al., 2002). The mechanosensors register the changes in blood flow, activate the endothelium and initiate an inflammatory response, which can ultimately lead to atherosclerotic lesions (Chappell, et al., 1998; Araim, et al., 2001) and a thrombotic tendency (Kar, et al., 2005; Dong, 2005).
Computer models can be used to study the effect of bifurcations or curvatures on the level of shear stress. One model simulated a 90 degree bifurcation, with 2 daughter vessels of similar size, one straight, the other curved (non-planar). Velocity profiles and flow patterns were different between the planar and non-planar vessel (Lu, et al., 2002). Beyond the bifurcation, flow was skewed towards the inner wall in both daughter vessels and shear stress was increased in this area compared with levels of shear stress in the outer wall. As flow continued along the two daughter vessels, the curvature in the non-planar vessel created a swirling motion of the fluid away from the inner wall, resulting in lower shear stress in the inner wall compared with the outer wall or the same section in the straight daughter vessel (Lu, et al., 2002). Using viscous fluid compared with non-viscous fluid accentuated these findings (Gijsen, et al., 1999; Chen, et al., 2004) and pulsatile flow resulted in more shear stress during systole compared with diastole (Chen, et al., 2006).

*In vitro* studies have been used to study the effect of one particular flow pattern per experiment, such as pulsatile, turbulent or oscillatory flow patterns (Resnick, et al., 2003), since the complex flow patterns as seen *in vivo* are difficult to simulate. Exposing HUVECs to oscillatory shear stress (5 dynes/cm², 1 Hz) significantly increased oxygen free radical formation when compared with steady shear stress (De Keulenaer, et al., 1998) and induced a 7.5-11 fold up-regulation in the expression of several intercellular adhesion molecules (vascular cell adhesion molecule-1, ICAM-1 and E-selectin) when compared to statically cultured cells (Chappell, et al., 1998), indicating an activation of the endothelium in response to complex flow. Anti-oxidant treatment of cultured cells led to inhibition of these results, indicating the important role oxygen free radicals play in cellular activation and intracellular signal transduction (Chappell, et al., 1998; De Keulenaer, et al., 1998).

Another variable of shear stress is the temporal nature of the stress. For example, the increase in shear stress can be rapid or slow and gradual (White, et al., 2001; Resnick, et al., 2003). In HUVECs, rapid temporal shear stress was created using an instant increase in shear stress from 0 to 16 dynes/cm², after which the shear stress was maintained at 16 dynes/cm² for 10 min, or a pulsatile
shear stress (0 to 16 dynes/cm$^2$) was applied every 3 seconds for 10 min. Both stimulated the ERK1/2 activity (300% vs. 800% increase, $p<0.05$) when compared with static controls (Bao, et al., 2000). Conversely, a slow and gradual increase of shear stress to 16 dynes/cm$^2$ led to a 50% reduction in ERK1/2 activity compared with controls (Bao, et al., 2000). Furthermore, pulsatile shear stress resulted in a significant increase in the cell proliferation index compared with static controls, an effect that was abolished in the presence of an ERK-specific antagonist (PD98059), suggesting an important role for ERK1/2 activation in cellular proliferation (Bao, et al., 2001).

Another experiment with HUVECs confirmed the positive effect of pulsatile shear stress (10 dynes/cm$^2$, 1 Hz) on the cellular proliferation index compared with slow and gradual increase of shear stress (White, et al., 2001). Care has to be taken in the interpretation of these studies, since HUVECs in a normal physiological condition are only exposed to steady shear stress (Balcells, et al., 2005). Even so, the results confirm that complex blood flow in the blood vessels leads to an altered cellular response compared with steady flow and is a step towards understanding the pathophysiological mechanisms associated with atherosclerosis and thrombosis in response to complex flow.

1.7.8. Air flow in the lung

The respiratory tree is characterised by a three dimensional, asymmetrical branching pattern (Cebral, et al., 2004), with a number of generations of which the trachea corresponds to generation 0 (Tarran, et al., 2005). The branching system rapidly increases the cross-sectional area of the parallel airways and gives rise to a large alveolar surface area (Cebral, et al., 2004). Airflow through the airways is dependent on the geometry of the airways and on the respiratory phase, inspiration or expiration (Cebral, et al., 2004). The air flow through the airways behaves according to the continuity and Navier-Stokes equations for an incompressible Newtonian fluid (Cebral, et al., 2004), and as for blood flowing through the blood vessels, imparts shear stress to the walls of the airways (Tarran, et al., 2005). During tidal breathing, there is an inspiratory flow and an expiratory flow travelling through the airways, each resulting in shear stress.
Therefore, 2 cycles of shear stress occur per breath (Tarran, et al., 2005). As a result of the three dimensional and phasic character of the flow, calculation of the level of shear stress on the pulmonary surfaces is extremely difficult (Tarran, et al., 2005).

According to calculations based on the Horsfield model (Horsfield, et al., 1968), shear stress in all generations of the respiratory tree is roughly equal, since the diameter of the tube decreases following branching, while the flow has to be divided over both daughter branches and, therefore, also reduces (Fredberg, et al., 2006). Shear stress is proportional to flow, but inversely proportional to the diameter of the tube, therefore resulting in similar values for shear stress throughout the entire respiratory tree (Tarran, et al., 2005). However, the calculation that shear stress varies little in the respiratory tree, is based on a laminar flow pattern.

From the observations in the cardiovascular system it is clear that bifurcations and curvatures lead to areas with complex flow: flow velocity can increase and decrease and flow can become turbulent. Since gas behaves like a fluid (Cebral, et al., 2004), we can assume that gas flow in the airways will not always be laminar. Calculations in patients with known narrowing of the airways demonstrated clearly that flow characteristics altered along the length of the airway, with highly skewed velocity profiles and, therefore, increased shear stress at the level of a stenosis or at a curvature, pressure drops at stenoses and reversal of pressure gradients between inspiration and expiration leading to dynamic changes in flow pattern (Cebral, et al., 2004).

1.8. Scope of the thesis

As discussed, flow dynamics in the cardiovascular system have a big impact on the mechanical forces the vessel wall is exposed to. An increase or decrease in shear stress leads to signalling events by the mechanosensors (Fisher, et al., 2001), triggering functional responses (Ali, et al., 2002) that enable the vascular endothelial cells to adapt to the alteration in shear stress. On the other hand, shear stress may be related to important pathophysiological processes such as

The role of shear stress in the airways has been less well studied, but it is becoming clear that gas flow through the respiratory tubes also leads to shear stress (Tarran, et al., 2005), possibly down to the smallest airways (Nucci, et al., 2003). Cells in the alveolar epithelium and the underlying basement membrane have been demonstrated to have mechanosensing qualities (Fredberg, et al., 2006; Fisher, et al., 2002; Mourgeon, et al., 1999; Xu, et al., 1999; Pugin, et al., 1998; Dunn, et al., 1999; Vlahakis, et al., 1999) with the magnitude of the response being dependent on the stretch, frequency and duration (Tschumperlin, et al., 2000).

Mechanical ventilation involves cyclical and forcible injection of gas into the lungs during inspiration. To achieve this, most ventilators apply a continuous or variable bias gas flow within the ventilator circuit. During inspiration, the expiratory valve closes and pressure in the ventilator circuit will rise (Rees, 1950; Goldsmith, et al., 2003). Once this pressure exceeds the pulmonary pressure, gas will flow into the patient's lung (Wood, 2003). A faster continuous bias gas flow in the ventilator circuit will lead to faster rise in inspiratory pressure and a faster inspiratory flow (Gerhardt, et al., 2008).

In the cardiovascular system, a higher blood flow velocity, a turbulent flow pattern, or a rapid change in flow generates increased shear stress, which activates the endothelium and can induce inflammatory and pathological responses (Ali, et al., 2002; Bao, et al., 2000).

Despite the fact that certain settings for ventilator bias gas flow are recommended (Gerhardt, et al., 2008), no scientific evidence exist for the correct bias gas flow during ventilation of the preterm baby, nor are there any data on whether higher bias gas flow results in increased shear stress and lung injury in the immature lung. Normally, surfactant proteins appear to modulate the effects of shear stress injury in the lung, altering elasticity and viscosity, particularly at the changes during phase of respiration, that is, from inspiration to expiration (Wustneck, et al., 2001). In preterm infants, surfactant production is severely
deficient (Jobe, 2006), thereby potentially exposing the preterm lung to increased shear stress injury.

This thesis investigates the effect of different ventilator bias gas flows on ventilator dynamics and ventilator-induced lung injury. The hypothesis is that a high bias gas flow will lead to a faster inflation of the lung, altered ventilator parameters, and more evidence of ventilator-induced lung injury as assessed by expression of early response genes and histological changes in lung structure compared with ventilation at a lower ventilator bias gas flow.
Chapter 2. Materials and methods

2.1. General methodology

2.1.1. Animal supply

All animal experiments were approved by the University of Auckland Animal Ethics Committee. Romney ewes were time-mated with Dorset rams to guarantee a known and consistent gestational age of the lamb. Dorset rams were used because of their proven performance and reliability. Ewes were scanned to confirm pregnancy and fetal number twice after mating (day 42 and 56). Twin-pregnancies were used in all studies, which allowed for collection of age-matched control tissue in the preterm studies.

The ewes were kept at Ngapouri Sheep Research Station. Ewes (and term lambs for the study described in chapter 3) were brought indoors two days before the experiment. The term lambs were studied in the first 10 days after spontaneous delivery. They were separated from the ewe before the experiment and returned to the ewe at the end of the experiment. Ewes received corticosteroids before surgical delivery of the preterm lambs. Due to an institutional policy change, betamethasone (Celestone Chronodose, Schering-Plough, Wellington, New Zealand, 11.4 mg i.m.) was given to the animals described in chapter 4 at 48 and 24 hours before delivery and dexamethasone (Dexa 0.2, Southern Veterinary Supplies, Palmerston North, New Zealand, 0.25 mg/Kg i.m.) was given to the animals described in chapter 5 at 24 and 12 hours before surgical delivery. The ewes and term lambs were fasted from 12 hours before the experiment.

2.1.2. Hysterotomy

For the studies involving preterm lambs, lambs were delivered by hysterotomy at 131-133 days’ gestation (Chapter 4) or 110-112 days’ gestation (Chapter 5; term 147 days). Before surgery, anaesthesia was induced with alfaxalone (Alfaxan-CD RTU, JUROX Pty. Ltd, Australia, 2-4 mg/Kg; chapter 4) or, due to an institutional policy change, thiopentone (AFT Pharmaceuticals Ltd, Auckland,
New Zealand, 10-15 mg/Kg; chapter 5) given intravenously via the jugular vein. After induction the ewe was intubated with a cuffed endotracheal tube size 8.0 (Mallinckrodt, Athlone, Ireland) and anaesthesia was continued by using inhaled halothane (Halothane Vet, AFT Pharmaceuticals Ltd, Auckland, New Zealand, 1-5%) in the earlier studies (chapter 4) or isoflurane (Isoflurane, Lunan Better Pharmaceutical Co, ShanDong, China, 3 L/min) in the later studies (chapter 5) due to an institutional policy change. Inhaled anaesthesia was administered in a mixture of air and oxygen to maintain normal transcutaneous oxygen saturations (SaO₂ > 90%; chapter 4) in the ewe during delivery of the lamb or to maintain normal arterial oxygenation (PaO₂ 90-140 mmHg; chapter 5) in the ewe during the two hours of fetal ventilation. Arterial oxygenation was chosen in the study described in chapter 5 to prevent oxytrauma in the fetal lung; supplemental oxygen was only given to the ewe in order to maintain the arterial oxygenation within physiological levels for the duration of the experiment.

The abdomen of the ewe was shorn, disinfected with iodine and hibitane and covered with sterile drapes. A midline incision was used to access the uterus. After locating the head of the fetus, the uterus was opened and the lamb (chapter 4) or the fetal head of the experimental lamb (chapter 5) was delivered. During this latter preterm study the head of one lamb was exteriorised and ventilated whilst still attached to the placental circulation (chapter 5). The twin lamb, used for age-matched, non-ventilated control tissue, was delivered shortly after the first lamb was delivered and stabilised (chapter 4) or at the end of the experiment (chapter 5). The ewe was euthanised with a lethal dose of pentobarbitone (Pentobarb 300, Provet NZ, New Zealand, 90 mg/Kg i.v.), followed by immediate delivery of the lamb to be used for control tissue.

In studies in which the lamb was ventilated whilst still attached to the placental circulation (chapter 5), a jugular venous line and carotid arterial line (Single Lumen Dural Tubing 040, Tyco Electronics, Pennsylvania, USA) were inserted in the ewe for fluid or drug administration in case of future contingencies and for measurement of arterial blood gases in the ewe.
2.1.3. Anaesthesia of the lamb

The *ex-utero* ventilated lambs (chapter 3 and 4) received anaesthetic drugs, whereas the fetuses, ventilated when still attached to the placenta (chapter 5) received their anaesthetic drugs via the maternal circulation.

Anaesthesia was induced intravenously with alfaxalone (JUROX Pty. Ltd, Rutherford, Australia, 4 mg/Kg), maintained with midazolam (Roche, Auckland, New Zealand, 0.4 mg/Kg) and ketamine (Parnell Laboratories, Auckland, New Zealand, 4 mg/Kg), and muscle relaxation was maintained with pancuronium (AstraZeneca, North Ryde, Australia, 0.1 mg/Kg). Lower dosages were used in the term lambs, since they had to regain spontaneous breathing shortly after the experiment was completed. These lambs received a loading dose of midazolam (0.2 mg/Kg) followed by a continuous infusion of midazolam (30 µg/Kg.h) and muscle relaxation was maintained using boluses of 25-50 µg/Kg pancuronium as necessary.

2.1.4. Intubation and catheterisation of the lamb

Following induction of the lamb, or after delivery of the head, the lambs were intubated with a cuffed endotracheal tube (Mallinckrodt, Athlone, Ireland) size 4.5 in the term lambs, size 4.0 in the preterm lambs. A cuffed endotracheal tube was used to avoid significant leakage around the tube, which would impact on the tidal volume delivered. The tube was inserted orally in the lambs ventilated *ex-utero* using a laryngoscope and Magill forceps and via tracheostomy for the fetuses ventilated whilst attached to the placenta. The tracheostomy was performed by making a 2 cm vertical incision, starting just under the glottis. The trachea was dissected free from surrounding tissue and two silk threads size 1/0 (Resorba, Nürnberg, Germany) were placed underneath it; care was taken to avoid damaging the laryngeal nerves. An incision was made between two cartilage rings of the trachea, approximately 2-3 cm caudal to the glottis. The endotracheal tube was inserted 6 cm and secured with the silk threads. If applicable, lung fluid was drained spontaneously and by gentle suction via the tube.
All animals received venous and arterial lines to enable fluid and medication administration using the venous line and blood sampling and blood pressure measurement using the arterial line. In *ex-utero* ventilated lambs umbilical arterial catheters (Single Lumen Dural Tubing, inner diameter 0.40 mm, Tyco Electronics, Pennsylvania, USA) were preferred. If placement was unsuccessful, a peripheral catheter was placed (Surflo IV catheter, 20G, Terumo, Laguna, Philippines). Using a sterile technique, the umbilical cord was cut 5 cm from the skin at delivery, the catheter was inserted 20-25 cm and secured to the umbilical cord with silk suture material size 4/0 (Resorba). In fetuses ventilated whilst still attached to the placental circulation, a jugular venous catheter (inner diameter 0.40 mm) and a carotid arterial catheter (inner diameter 0.30 mm) were inserted using the tracheostomy incision and secured with 4/0 silk ties.

The *ex-utero* ventilated lambs received 10% dextrose (term lambs 25 mL/h, preterm lambs 10 mL/h i.v.) for the duration of the experiment and all lambs received NaCl 0.9% plus heparin 10 U/mL at 2-5 mL/h via the arterial line.

2.1.5. Ventilation of the lamb

Ventilation was provided with a VIP Bird ventilator (Viasys Healthcare Inc., Palm Springs, CA, USA) for the term animals and a Babylog8000plus (Dräger Medical, Lübeck, Germany) for the preterm animals. Ventilation commenced as soon as anaesthesia was started or after catheterisation and tracheostomy were completed.

Medical air and oxygen were connected to the ventilator and a mixture of gas, depending on the experiment, was heated and humidified using a humidifier with the temperature set at 37°C (Fisher & Paykel Healthcare Ltd, Auckland, New Zealand).

Further details about mode of ventilation and the settings on the machine are given in the relevant chapters.

The development of a pneumothorax is one of the complications of positive pressure ventilation. If a pneumothorax was suspected, based on asymmetrical or symmetrical enlargement of the chest, abnormal breathing sounds,
tachycardia or sudden change in blood pressure, oxygenation or saturation, drainage was performed using a butterfly needle (Hospira, Donegal Town, Ireland; 19-gauge) with the tubing submersed in a container with water. If a pneumothorax was confirmed, the drain was left in situ until the end of the experiment.

2.1.6. Measurement of physiological data

Blood pressure, heart rate and transcutaneous oxygen saturations were measured continuously using a Hewlett Packard Merlin Omnicare 2.4 (HP-company, Palo Alto, CA, USA) in the term lambs. Data for blood pressure, heart rate, saturation (SaO₂), supplemental oxygen (FiO₂), positive inspiratory pressure (PIP), inspiratory time (Ti) and tidal volume (TV) were recorded every 3 min. Rectal temperature was monitored every 15 min.

In the preterm animals a Powerlab® data acquisition system (ADinstruments, Dunedin, New Zealand) connected to a laptop computer (Latitude D620, Dell Ltd, Palmerston North, New Zealand) was used to record physiological data continuously. The arterial catheter was connected to a pressure transducer (MLT844), which was connected to a Bridge Amplifier to amplify the signals. Pressure values were derived electronically, and heart rate was calculated from these values. Temperature was measured using a thermistor pod (ML309) connected to a nasal temperature probe (MLT415/A), which was inserted 5-8 cm into one of the nostrils of the lamb. Inspiratory and expiratory flows were measured using a respiratory flow head (MLT300L) and a spirometer (MLT141), which together function as a pneumotachometer. Saturation was measured using the Hewlett Packer monitor as the signal could be obtained more reliably than on Powerlab®.

PowerLab® data were recorded at 1,000 Hertz. Ventilator data (PIP, positive end expiratory pressure (PEEP), TV, minute volume, rate, Ti, compliance, resistance and FiO₂) were recorded continuously using Ventview (Version 2.1, Dräger Medical, Lübeck, Germany). Ventilatory parameters were recorded every 10 seconds using Ventview. In the experiment described in chapter 5, analogue
leads connected the ventilator to PowerLab® to record ventilator pressures and tidal volumes.

2.1.7. Blood samples

Arterial blood gases (0.3 mL) were taken every 15 min. In the study where fetuses were ventilated whilst attached to the placenta, maternal arterial blood gases were also taken every 15 min. Further blood samples were taken in the preterm animals; blood samples for full blood counts (1-2 mL collected in EDTA tube) at time 0, 30, 60 and 120 min in the ex-utero ventilated lambs and blood samples for molecular analysis (5 mL collected in EDTA tube) at time 15 and 60 min in the fetuses ventilated whilst attached to the placenta.

The latter were immediately processed using the LeukoLOCK™ Total RNA Isolation System Kit (Ambion Inc., Austin, USA) according to the manufacturer's instructions. The kit contained all filters and chemicals required. Briefly, this consisted of isolation and stabilisation of the leukocytes by passing the blood through the LeukoLOCK filter into a collection tube not containing any anti-coagulant. The filter was then flushed with 3 mL of phosphate-buffered saline and 3 mL of RNAlater®, the ports of the filter were sealed with stops and the filter was frozen at -80°C. The filters were shipped to the Monash Institute in Melbourne, where collaborators are currently establishing a technique to measure the early response genes (CTGF, CYR61 and EGR1) in small blood samples. All samples were taken from the arterial line.

2.2. Post mortem protocol

At the end of the experiment in the preterm lambs a lethal dose of pentobarbitone (Pentobarb 300, Provet NZ, New Zealand, 90 mg/Kg i.v.) was given to kill the animals humanely. Catheters were occluded and the endotracheal tube was disconnected from the ventilatory circuit. Partly exteriorised lambs were delivered via hysterotomy, the umbilical cord was clamped, and sex and weight were recorded. Jugular veins and carotid arteries were cut to exsanguinate the lamb. A midline Y-incision was made in the chest to expose the sternum and lower costal border. The abdominal cavity was opened
to check for pneumothoraces. This was recorded as being positive left and/or right sided when the diaphragm was bulging towards the abdominal cavity. The thorax was opened by cleaving the sternum, the mediastinum checked for pneumomediastinum, and the lungs were exposed. The trachea was clamped and cut just below the cricoid cartilage. The oesophagus, inferior vena cava and abdominal aorta were cut and the lungs were removed from the chest. Lung weight was recorded after the heart, oesophagus and great vessels were removed.

The left main bronchus was ligated and the left lung was removed distal to the ligature. The left lower lobe was cut into small pieces (~0.5 x 0.5 cm) and these were snap-frozen in a dry ice and isopentane (Across Organics, New Jersey, USA) slurry. Samples were stored in -80°C freezer until further processing.

The right lung was pressure fixed by instillation of 4% paraformaldehyde at 20 cmH₂O into the trachea. To prepare 1 L of 4% paraformaldehyde 40 g paraformaldehyde (Scharlau, Sentmenat, Spain) were dissolved in 400 mL of milliQ water and heated to maximum 60°C. A few drops of 6-8 pellets of NaOH (Scharlau, Sentmenat, Spain) dissolved in 10 mL of milliQ water were added until solution cleared. A 0.2 M phosphate buffer was added (3.174 g of NaH₂PO₄.H₂O dissolved in ~100 mL milliQ water (Scharlau, Sentmenat, Spain) and 10.929 g of Na₂HPO₄ dissolved in ~350 mL milliQ water (AppliChem, Darmstadt, Germany)). The solution was buffered to a pH of 7.4, topped up to 1 L and filtered, before storage at 4°C.

2.3. Analysis of early response genes in tissue

2.3.1. Total RNA extraction

Total ribonucleic acid (RNA) was extracted from lung tissue using a Qiagen RNeasy maxi-kit (Qiagen, Clifton Hill, Victoria, Australia). Tissue (~600 mg) was placed in a 50 mL collection tube and homogenised (Ultra-Turrax T25; Janke & Kunkel, IKA labor technik, Germany) in 15 mL Buffer RLT (RNeasy Lysis Buffer) supplied in the maxi-kit, which contained β-mercaptoethanol (150 μL of 14.3 M β-mercaptoethanol). Samples were centrifuged (Allegra X-15R centrifuge,
Beckman Coulter) for 10 min at 5,000 G at 23°C. The same settings for the centrifuge were used throughout this protocol. The supernatant containing the RNA was carefully transferred to a clean 50 mL collection tube; 15 mL of 70% ethanol was added followed by vigorous shaking to prevent precipitation. The fluid was then transferred to maxi-columns, supplied in the maxi-kit, and centrifuged for 5 min. Binding of RNA to the silica-gel-based membrane, inside the maxi-column, occurred during this step and flow-through was discarded.

Digestion of DNA during RNA purification was performed using a Qiagen RNase-Free DNase set (Qiagen, Clifton Hill, Victoria, Australia). All buffers and solid DNase I were provided with the kit.

To wash the maxi-columns 7.5 mL of a buffer containing guanide isothiocyanate and ethanol (Buffer RW1), were added on top of the membrane of the maxi-column, and columns were centrifuged for 5 min. Flow through was discarded. DNase I stock solution was prepared by dissolving solid DNase I (1,500 Kunitz units) in 550 μL of RNase-free water. DNase I (30 μL) was mixed with buffer RDD (210 μL) and added on top of the membrane of the maxi-column and left to incubate at room temperature for 15 min. DNase was then removed by adding 7.5 mL of buffer RW1 to the column for 5 min followed by centrifugation for 5 min. Flow through was discarded and the maxi-column was reinserted into the same collection tube. Buffer RPE is supplied as a concentrate in the maxi-kit and was diluted with 4 volumes of 100% ethanol. To wash the maxi-columns, 10 mL of this solution was added to the column followed by centrifugation for 2 min. Flow through was discarded. This step was repeated a second time, with a longer centrifugation time of 10 min to dry the membrane.

Maxi-columns were transferred to a new tube. To elute the RNA, 1.0 mL of RNase-free water was added on top of the silica-membrane, left for 1 min and centrifuged for 3 min. This elution step was repeated to optimize RNA yield. To obtain a higher total RNA concentration, the flow through (1st eluate) was replaced onto the membrane to re-elute the RNA. This was left for 1 min, before centrifugation for 3 min.
The concentration and purity of RNA in the second eluate was determined by measuring the absorbance at 230, 260, 280 and 320 nm (A230, A260, A280 and A320) in a spectrophotometer (Eppendorf, Hamburg, Germany). RNA was diluted with RNase free water (1 μL RNA in 50 μL water). The measured concentration of RNA was on average ~600 – 1,000 ng/μL. The sample was considered of acceptable purity if the A260/280 was between 1.6 and 2.0, and contamination was excluded if A320 was low.

Due to a laboratory policy change, an agarose gel assay was performed for the study described in chapter 5 to determine the purity of RNA, before cDNA synthesis was started. A 1% gel was made by adding 0.4 g of agarose (Scientifix, Cheltenham, Australia) to 40 mL of TAE buffer. TAE buffer stock solution was made by adding 242 g of TRIS base (Merck, Darmstadt, Germany), 57.1 mL glacial acetic acid and 100 mL EDTA 0.5M. Solution was topped up to 1 L by adding milliQ water and autoclaved. TAE buffer solution was diluted with milliQ water (10 mL of stock solution to 490 mL of milliQ water) to obtain TAE buffer. The 1% gel was heated briefly in the microwave (~5 s), 0.4 μL of ethidium bromide (Sigma, Chemical Co., St Louis, MO, USA) was added, mixed and poured onto a gel support tray and left to set for 20 - 60 min. RNA was diluted with RNase free water (1 μL RNA in 3 μL water), heated for 5 min at 65°C, after which 2 μL of Northern loading buffer was added to each sample. Samples were added to the gel, the gel tray was connected to the electrophoresis power pack and run for 30 min at a voltage of 80 V. The gel was then transferred to a gel documentation unit (Syngene darkroom; Synoptics Ltd., Cambridge, England), where a picture of the gel was taken using Gene Snap 6.00.26 (Synoptics Ltd.), which was printed using a Mitsubishi P91D (Mitsubishi, Japan) (Figure 2.1). Gel electrophoresis allowed detection of 28S, 18S and 5S. Additional bands suggested contamination.
Figure 2.1: Agarose gel assay

To determine the purity of RNA, an agarose gel assay was performed, allowing detection of 28S, 18S and 5S. Samples with additional bands (arrows) suggested contamination and were discarded and RNA extraction was repeated.

2.3.2. cDNA synthesis

Synthesis of cDNA was performed using a M-MLV Reverse Transcriptase kit (Promega, Madison, USA). DNase treated RNA (800 ng, diluted with nuclease-free water to 10 μl) was incubated with random hexamers (250 ng [500 μg/ml]; Promega, Madison, USA) at 70°C for 5 min and then cooled on ice for 5 min. Mastermix was made using M-MLV reverse transcriptase 5 x reaction buffer (5 μl), dNTPs (dATP, dCTP, dGTP, dTTP; each 10 mM or 0.125 μl; Promega, Madison, USA) and nuclease-free water (8.5 μl). Mastermix (14 μl) followed by M-MLV reverse transcriptase enzyme (0.5 μl) were added to annealed primer-template, and left to incubate at room temperature for 10 min, then at 42°C for 1 ½ h. To inactivate the reaction, incubation at 70°C for 15 min followed. The
concentration of single stranded DNA (ssDNA) was tested using a spectrophotometer (Eppendorf, Hamburg, Germany). ssDNA was diluted with milliQ water (1μL ssDNA in 50 μL water). The measured concentration of ssDNA was on average ~1,200 – 2,000 ng/μL. The sample was considered to be of acceptable purity if the A260/280 was between 1.6 and 2.0 and contamination was excluded if A320 was low.

The cDNA attained was used as template for PCR amplification of selected genes (*CTGF*, *CYR61* and *EGR1*) plus for 18S as the housekeeping gene.

### 2.3.3. Quantitative real-time polymerase chain reaction

Levels of mRNA were determined using quantitative real-time polymerase chain reaction (qRT-PCR) for *CYR61*, *EGR1* and *CTGF*.

The optimal primer and cDNA concentration at the start of amplification and the primer annealing temperature for each gene were already established by the Department of Physiology, Monash University, Melbourne and these conditions were used throughout (Table 2.1). The primer sequences are shown in Table 2.2.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer concentration</th>
<th>cDNA concentration</th>
<th>Melting temperature</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>CYR61</em></td>
<td>10 μM</td>
<td>200 ng/μL</td>
<td>60°C</td>
<td>35</td>
</tr>
<tr>
<td><em>CTGF</em></td>
<td>4 μM</td>
<td>1000 ng/μL</td>
<td>60°C</td>
<td>35</td>
</tr>
<tr>
<td><em>EGR1</em></td>
<td>4 μM</td>
<td>500 ng/μL</td>
<td>59°C</td>
<td>35</td>
</tr>
<tr>
<td>18S</td>
<td>10 μM</td>
<td>200 ng/μL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.1:** Optimal conditions for qRT-PCR

Optimal conditions for the three early response genes *CYR61*, *CTGF* and *EGR1* and the housekeeping gene 18S to perform qRT-PCR.
Table 2.2: Primer sequences used in qRT-PCR

Primer sequences for CYR61, CTGF and EGR1, the early response genes analysed using qRT-PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Upstream primer 5’-3’</th>
<th>Downstream primer 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYR61</td>
<td>AGGGTCACTGTGGAAGGTC</td>
<td>GCAGCTGAAGTCAAAGGAA</td>
</tr>
<tr>
<td>CTGF</td>
<td>TATAGTCCAGCGACAGCTC</td>
<td>AGGAACTTGACTCAGCCTCA</td>
</tr>
<tr>
<td>EGR1</td>
<td>ATCGTCCAAACAACCTCGTG</td>
<td>GGTAACGGGTGGAGATAC</td>
</tr>
</tbody>
</table>

qRT-PCR analysis was performed by adding the templates in triplicate to a 96 well plate (Eppendorf, Hamburg, Germany), which was heat sealed and run using a Mastercycler ep realplex real-time PCR system (Eppendorf, Hamburg, Germany).

To ensure there was no DNA contamination in any of the solutions used, a control reaction for the gene of interest and 18S was performed in each plate. This well contained all the components of the reaction except the cDNA template.

The PCR programme started with a denaturation phase at 95°C for 2 min, followed by 35 cycles of a denaturation phase at 95°C for 3 s, an annealing phase at 60°C for 20 s and an elongation phase at 72°C for 20 s. This was followed by a melt-curve analysis, to confirm that only the specific product of interest was amplified and all other PCR products were denatured. After the specified number of cycles was completed, the temperature increased to 95°C for 15 s, followed by a decrease to 75°C for 45 s, after which the temperature was increased 0.5°C between each acquisition of fluorescence up to 95°C.

PCR products were detected using SYBR Green (Platinum SYBR Green qPCR Supermix-UDG, Invitrogen, California, USA), a fluorescent dye which binds to double stranded DNA. The detection occurs at 494 nm during the elongation phase of every cycle, and since amplification of PCR products is an exponential process, the fluorescence increases over time. The level of fluorescence is plotted against the number of cycles, producing an amplification plot for each sample.
An efficient amplification of the gene of interest occurs if a steep curve and a high plateau are seen, plus if the number of cycles needed for amplification occurs in the log-linear phase of the curve and the number of cycles needed is below 35 cycles.

**Figure 2.2: Amplification plot**

Amplification plot of CYR61 (left) and 18S (right) as housekeeper gene during qRT-PCR procedure on a Mastercycler ep realplex real-time PCR system. The red line represents the threshold line.

### 2.3.4. Quantitative real time PCR analysis

qRT-PCR was performed on each sample in triplicate. The threshold line is the level of detection, or the point at which a reaction reaches a fluorescent intensity above background fluorescence, and falls on the beginning of the linear part of the curve of an amplification plot (Figure 2.2). The point at which each sample crosses this line is called the ‘cycle threshold’ or ‘C\(_T\) value’. At this point fluorescence is detectable, and this is used to calculate the initial amount of starting template for each sample. When a low C\(_T\) value is found, high levels of the gene of interest were in this sample at the start of the process.
18S was used as the housekeeping gene. Subtraction of the \( C_T \) value for 18S from the \( C_T \) value of the gene of interest for each sample gives a \( \Delta C_T \) value, which adjusted for minor differences in the amount of cDNA template per reaction:

\[
\Delta C_T \text{ value of the gene of interest} = C_T \text{ value of the gene of interest} - C_T \text{ value for 18S}
\]

Since samples from all animals did not fit in a 96 well plate, a maximum of 4 plates per gene were used in total. To compare the values of each run, levels of the gene of interest and 18S were measured in a calibration fluid that was run in each assay (in quintuplicate). The calibration fluid was a mixture of cDNA from several animals and the same calibration fluid was used in each plate. An average value for the \( \Delta C_T \) in the calibration fluid (averaged over the 5 wells containing calibration fluid) was calculated.

Calculation of the \( \Delta\Delta C_T \) value was done by subtracting the average \( \Delta C_T \) value for the calibration fluid from the \( \Delta C_T \) value of each sample:

\[
\Delta\Delta C_T \text{ value} = \Delta C_T \text{ value of each sample} - \text{average } \Delta C_T \text{ value for the calibration fluid}
\]

This value was normalised using the formula \( 2^{\Delta\Delta C_T} \) and expressed relative to the mean value for the mRNA levels of the gene of interest in controls:

\[
\text{mRNA value of gene of interest in sample} = 2^{\Delta\Delta C_T} \text{ value for sample} / \text{mean value of } 2^{\Delta\Delta C_T} \text{ of controls}
\]

The result obtained is the mRNA level of the gene of interest for a specific animal (fold change of control) (Wallace, et al., 2009) and enabled comparison of values from treatment groups with those from control tissue.

**2.4. Histology and immunohistochemistry**

After 20 min of pressure-fixation of the right lung with paraformaldehyde at post-mortem, the trachea was ligated and the lung immersed in a container filled with fresh paraformaldehyde for 24 h at 4°C. The next day the right lung was
transversely cut into 0.5 cm thick slices for further analysis. The trachea and main bronchus were discarded.

2.4.1. Estimation of lung volume

All slices were divided into upper, middle and lower lobes and placed on a cutting board, with the cut surface of the tissue facing upwards to estimate lung volume. A transparency with a printed 1 cm square grid was placed on top of the tissue slices and volume calculated as shown in Figure 2.3.

![Volume measurements of lung tissue](image)

**Figure 2.3: Volume measurements of lung tissue**

Lung tissue slices (0.5 cm thick) were placed on a cutting board, with the cut surface facing upwards. If the right upper corner of the grid (1 cm squares) was overlaying tissue, this was counted as a tissue point (i.e. 1st slice has 8 tissue points, 2nd slice 5 and 3rd slice 6). The volume for these 3 slices is calculated as $(8 + 5 + 6) \times 1 \times 0.5 = 9.5$ mL.
The volume was calculated as follows:

\[
\text{Volume (mL)} = \text{total number of tissue points counted} \times \text{area per square (1 cm}^2\text{ for used grid)} \times \text{thickness of sections (cm)}
\]

With the area per square being 1 cm\(^2\) and the thickness of a slice being 0.5 cm, the following calculation was used:

\[
\text{Volume (mL)} = \text{total number of tissue points counted} \times 1 \times 0.5
\]

2.4.2. Preparation of fixed tissue

Following estimation of lung volume, three sections of tissue per lobe (approximately 1 cm\(^3\) each) were randomly selected. A total of 9 sections per animal were taken. Tissue was placed in labelled tissue cassettes and immersed in Zamboni’s fixative overnight. Zamboni’s fixative was made by adding 200 mL of formaldehyde (37%) (Scharlau, Sentmenat, Spain) to 1 L of 0.2 M phosphate buffer, followed by 300 mL of saturated picric acid (Chem supply, Adelaide, Australia). This solution was topped-up to 2 L with milliQ water, filtered and stored in a brown glass bottle at room temperature.

The next morning Zamboni’s fixative was discarded. Tissue cassettes were rinsed twice with 70% ethanol and then immersed in 70% ethanol. The tissue sections were embedded in paraffin-wax blocks in an automatic tissue processing machine (ATPTM, Triangle Biomedical Sciences, Durham, NC, USA).

Briefly, this entailed dehydration of the tissues in increasing concentrations (70%, 80% and 95% twice) of ethanol for 30 min each, followed by \(2 \times 25\) min washes in 100% ethanol, removal of ethanol with Xylene (30 min) (Labchem, Pittsburgh, Pennsylvania, USA), and then embedding in paraffin wax (4 changes of paraffin wax; 30 min each) (Paraplast Tissue Embedding medium, McCormickTM Scientific, St. Louis, Missouri, USA).

Tissue sections were cut at 5 µm thickness on a microtome (Jung Biocut 2035, Geprüfte Sicherheit, Germany) and mounted on microscopic slides (Superfrost plus, Menzel Gläser, Braunschweig, Germany).
All slides were baked in a 70°C oven for 2 h. Before commencing staining or immunohistochemistry, slides were dewaxed in xylene (Xylenes Sulfur Free LR, Chem Supply, Adelaide, Australia), dehydrated in 100% ethanol and rinsed in tap water to prepare them for the specific staining protocol. After completion of staining or immunohistochemistry, slides were dehydrated in 100% ethanol, dipped in xylene and coverslipped.

2.4.3. Hart’s resorcin-fuschin stain

Hart’s resorcin-fuschin stain was used to stain elastin fibres black.

The stock for this stain was made by boiling 2 g basic fuschin, 4 g resorcin and 200 mL of distilled water for 1 min in a large size beaker, followed by adding 25 mL 30% aqueous ferric chloride and a further boiling of the solution for 5 min. After allowing the solution to cool it was filtered, the filtrate was discarded and the filter with precipitate was returned to the large beaker. The beaker, containing the filter and 20 mL of 95% ethanol was gently heated until the precipitate dissolved. The filter paper was discarded, 4 mL of concentrated HCl was added and the volume of the solution restored to 200 mL with 95% ethanol. The working solution was made by combining 10 mL of the resorcin-fuschin stock solution with 100 mL of 70% ethanol and 2 mL of 30% HCl.

Dehydrated tissue slides were immersed in 0.25% potassium permanganate (0.75 g in 300 mL) for 5 min. Sections were rinsed twice in tap water and briefly (~3 sec) bleached in 5% oxalic acid (15 g in 300 mL), before being rinsed in tap water (3 times). Slides were immersed in Hart’s resorcin-fuschin working solution for 1-6 hours to stain the elastin tissue black. Quality of elastin staining in septal crests was checked under the light microscope at 40x magnification; if adequate, slides were rinsed in tap water (3 times ~30 sec), followed by a rinse in distilled water. Tartrazine 0.25% in saturated picric acid (0.75 g in 300 mL) was used as counterstain for 3 min, staining the rest of the tissue yellow.
Figure 2.4: Hart’s resorcin stain

Hart’s resorcin stain was used to stain elastin tissue black and a counterstain (Tartrazine in picric acid) was used to stain the rest of the tissue yellow. Air space is white. A. Magnification 40x. Bar represents 25 μm. B. Magnification 100x, bar represents 10 μm. Two secondary septal crests are indicated using an arrow and two alveoli are marked.

2.4.4. Gordon and Sweet stain

Paraffin-embedded tissue sections were stained with Gordon and Sweet stain to visualise collagen I and III fibres as black fibres (Shoemaker, et al., 1984).

To perform this stain acidified potassium permanganate and Wilder’s Silver bath were prepared. Acidified potassium permanganate was made by combining 95 mL of 0.5% aqueous potassium permanganate with 5 mL of 3% sulphur acid aqueous followed by filtering. Wilder’s Silver bath was made by adding drops of ammonium hydroxide to 5 mL of 10% silver nitrate until the precipitate was nearly dissolved. Subsequently, 5 mL of 3% sodium hydroxide was added followed by continuous drop-wise titration of ammonium hydroxide, until the solution was almost clear. MilliQ water was added to make up to 50 mL, after which the solution was filtered and stored in a dark bottle.
Chapter 2

After dehydration the slides were immersed in acidified potassium permanganate (5 min), rinsed in tap water and bleached by immersing them in 1% oxalic acid in 10% hydrobromic acid (10-60 s, until the brown colour faded). The slides were washed twice in tap water (20 s each) and immersed in 2% ferric ammonium sulphate (5 min), before washing the slides three times in tap water (20 s each) and dipping them in Wilder’s silver bath (5 s). Tissue sections were washed three times in tap water (20 s each) and dipped in 10% neutral formalin (~ 10-30 s). This step colours the collagen in the tissue black / brown. Eosin was used as a counterstain to colour the background tissue pink.

Figure 2.5: Gordon and Sweet stain

Gordon and Sweet stain was used to stain collagen I and III fibres black. An eosin counterstain stained the remaining tissue pink. Picture was taken with a magnification of 100x, bar represents 10 μm.

2.4.5. Staining for alpha smooth muscle actin

Myofibroblasts, containing alpha smooth muscle actin (αSMA), were identified using immunohistochemical staining for αSMA (Leslie, et al., 1992; Leslie, et al., 1990). Lung sections were de-paraffinised in two washes of xylene (5 min) and re-hydrated in 3 min washes, first with ethanol (100% twice, followed by 95%
and 75%), then with double-distilled H₂O. Antigen retrieval was performed by heating sections in a microwave for 20 min immersed in a solution of 10 mmol/L tris and 1 mmol/L ethylenediaminetetra acetate (EDTA) at pH 9.0. Slides were washed 3 times with phosphate buffered saline (PBS) (5 min each). Endogenous peroxidase activity was blocked using 3% hydrogen peroxide (5 min). The tissues were rinsed with double-distilled H₂O and washed twice with PBS (5 min each). Non-specific binding was blocked by incubation with 20% normal goat serum / 0.1 % Triton X-100 in 0.05 M Tri-hydrochloric acid (Tris-HCl) pH 7.2 (30 min; 200 μL per slide). The primary antibody (mouse monoclonal anti-αSMA; 1:200 dilution; DakoCytomation, Denmark; Cat No:M0851, clone 1A4) was then added to the sections (100 μL per slide) and incubated (60 min) at room temperature prior to washing in PBS containing 0.1% Tween 20 (3 x 5 min). The sections were incubated at room temperature (60 min) with the secondary antibody (goat polyclonal anti-mouse biotinylated immunoglobin; 1:500 dilution; DakoCytomation, Denmark), after which they were again washed in PBS containing 0.1% Tween 20 (3 x 5 min). The sections were further incubated (30 min; 200 μL per slide) with avidin-biotin complex (1:150 dilution in PBS, Vector Laboratories, Inc., Burlingame, CA, USA), washed in PBS (5 min), followed by DAB (5-7 min; 200 μL per slide, Sigma Fast™, St. Louis, MO, USA) to visualise the stained myofibroblasts under the microscope. Slides were washed with PBS (5 min), counterstained with haematoxylin, washed in tap water, dipped in ammonia treated water and dehydrated in series of ethanol (70%, 95%, 100% twice, each for 3 min). Ethanol was removed by immersing slides in xylene (5 min twice), before slides were mounted in distyrene plasticiser xylene (DPX; British Drug House Chemicals, United Kingdom).

This technique stained αSMA brown and enabled identification of myofibroblasts, whereas the rest of the lung tissue was stained blue using haematoxylin (Figure 2.6). One slide was used as negative control; all the steps detailed above were followed, except that primary antibody was not added to this slide. Specificity of immunostaining for αSMA was confirmed by omission of the primary antibody on this slide, i.e. no cells with brown staining were seen on this slide.
Figure 2.6: Identification of myofibroblasts

Immunohistochemical staining of αSMA identified myofibroblasts (stained brown) in lung tissue (stained blue using a haematoxylin counterstain). Magnification 100x, bar represents 10 μm.

2.4.6. Staining for proliferating cells using Ki67 antigen

Immunostaining for proliferating cells was performed using Ki67 antigen (Gerdes, et al., 1984; Gerdes, et al., 1983). Lung sections were de-paraffinised in two washes of xylene (5 min each), after which they were re-hydrated in ethanol washes (100% x 2, 95% and 75%; 3 min each), double-distilled H₂O (3 min) and PBS (5 min). Antigen retrieval was then performed by heating the sections, immersed in 10 mmol/L sodium citrate (pH 6), in a microwave on high (20 min). After washing the slides twice in PBS (5 min each), endogenous peroxidase activity was blocked by immersing the slides in 3% hydrogen peroxide (5 min). Tissues were rinsed with distilled water and washed twice with PBS (5 min each), before non-specific binding was blocked by incubation with 25% normal goat serum / 0.1% Triton X-100 / 3% skim milk powder in 0.05 M Tris-HCl pH 7.2 (30 min; 200 μL per slide). The primary antibody (mouse monoclonal anti-Ki67; 1:100 dilution; DakoCytomation, Denmark; Cat No:M7240, clone MIB-1) was then added to sections (100 μL per slide) and incubated (90 min) at room temperature. The sections were washed in PBS containing 0.1% Tween 20 (3 x 5
min), and then incubated (60 min) with secondary antibody (goat polyclonal anti-mouse biotinylated immunoglobulin; 1:500 dilution; DakoCytomation, Denmark; 100 μL per slide), after which the sections were again washed in PBS containing 0.1% Tween 20 (3 x 5 min). The sections were then incubated (30 min; 100 μL per slide) with avidin-biotin complex (1:150 dilution in PBS, Vector Laboratories Inc., Burlingame, CA, USA), washed in PBS (3 x 5 min), followed by DAB (5-7 min; 200 μL per slide, Sigma Fast™, St. Louis, MO, USA) to visualise the proliferating cells under the microscope. Slides were washed with PBS (5 min), counterstained with haematoxylin, washed in tap water, dipped in ammonia treated water and dehydrated in series of ethanol (70%, 95%, 100% twice, each for 3 min). Ethanol was removed by immersing the slides in xylene (5 min twice), before slides were mounted in distyrene plasticiser xylene (DPX; British Drug House Chemicals, United Kingdom). Proliferating cells stained brown and the rest of the lung tissue stained blue (Figure 2.7). Specificity of immunostaining for Ki67 was confirmed by omission of the primary antibody, i.e. no brown cells on the negative control slide.

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**Figure 2.7: Identification of proliferating cells**

Proliferating cells were identified using immunohistochemical staining with Ki67 antigen. Proliferating cells stained brown, the rest of the lung tissue was stained blue using a haematoxylin counterstain. Magnification 100x, bar represents 10 μm.
2.4.7. Quantification of staining

For each animal, 1 tissue block per lobe was chosen at random (3 blocks per animal in total) and cut into sections. Sections were viewed under a light microscope (Nikon Eclipse 80i, Tokyo, Japan) and 5 fields of view per section were randomly selected to be captured using a digital camera (RT slider, SpotTM Advanced Diagnostic Instruments Inc, Sterling Heights, MI, USA), except for Ki67 for which 10 fields of view were captured. Magnification with oil (100x) was used for analyses of elastin, collagen, Ki67 and αSMA. For the analysis of the septal crest density sections were viewed at a magnification of 40x.

All analyses were performed on the distal regions of the airways, with care taken to avoid the major airways and blood vessels. All analyses were done by one observer (KPB) with the identification obscured; treatment group was disclosed after completion of the analysis.

Quantification of elastin and collagen density

The Hart’s elastin stained slides were used to measure elastin density and the Gordon and Sweet stained slides to measure collagen I and III density. Images were analysed using Image-Pro Plus (Media Cybernetics, Bethesda, MD, USA). For each field of view the tissue stained with the stain of interest was marked and the background tissue was marked separately. Subsequently, a density calculation was performed by expressing the marked area with the primary stain as a percentage of total tissue in that field of view with the following calculation:

\[
\% \text{ primary stain} = \left( \frac{\text{area of primary stain}}{\text{total area of tissue}} \right) \times 100
\]

Secondary crest density

The Hart’s elastin stain was also used to calculate secondary crest density. Five images per section were randomly selected and each was displayed on the computer screen. The image on the screen was covered with a transparency with a printed 1 cm square grid at a similar size as the image. The grid contained 19 x 26 dots; thus, the total number of dots was 494.
When a dot overlaid a secondary crest, identified by the black elastin bundles at the tip, this was counted as secondary crest. When a dot overlaid normal tissue, not being a secondary crest, this was counted as tissue. If a dot overlaid airspace, this was not counted as a tissue point.

**Figure 2.8: Technique for calculating septal crest density**

A grid, the same size as the image on the computer screen, was placed on the screen. Dots overlaying tissue were counted as tissue (green √), dots overlaying septal crests were counted as septal crest (aqua √). Dots overlaying air and not tissue were not counted (red χ).

The total amount of tissue was calculated by adding secondary crest dot count to the tissue dot count.

The secondary crest density was calculated as follows:

Secondary crest density = (# dots on secondary crest / total amount of tissue (in dots)) x 100

**Quantification of immunohistochemistry**

Sections stained for αSMA were used to visualise distal lung myofibroblasts. For each field of view the tissue stained positive for αSMA was marked and the background tissue was marked separately. Subsequently, a density calculation was performed by expressing the marked area with the αSMA stain as a percentage of total tissue in that field of view with the following calculation:
% primary stain = \( \frac{\text{area of primary stain}}{\text{total area of tissue}} \times 100 \)

Sections, stained using Ki67 antigen, were used to calculate the percentage of proliferating cells in distal lung tissue. The number of proliferating cells were counted and expressed as a proportion of the total number of cells to determine a labelling index.

### 2.5. Data analysis

Physiological data were entered by hand to be analysed with StatView (SAS Institute Inc., Cary, version 5.0.2) for the term lambs. For the preterm lambs, physiological data were downloaded from Powerlab® and Ventview. PowerLab® data were analysed using a computer software package (Chart v.7, ADinstruments, Dunedin, New Zealand). Statistical analysis for the preterm lamb studies was performed using JMP7 (SAS Institute Inc., Cary, USA), except for the histology and immunohistochemistry data that were analysed using SPSS (Version 3.3, SPSS Incorporated, USA). All data are represented as mean ± standard error of the mean (SEM). Statistical significance was taken for all analyses when \( p < 0.05 \).

Data for the term lambs were analysed using repeated measures ANOVA. The post-hoc Bonferroni / Dunn test was performed when appropriate.

Physiological data for the preterm lambs were analysed in 10 min epochs, starting 5 min before each blood gas, except for data from the beginning and the end of the experiment (two 5 min epochs; the first starting at the beginning of ventilation until 5 min into the experiment and the last starting after 115 min and finishing with the completion of the experiment). Data were analysed with ANOVA, including analysis of effect of time. The Tukey post-hoc test was performed when appropriate. mRNA levels of the early response genes were analysed with ANOVA, with Tukey post-hoc tests when appropriate. Histology and immunohistochemistry data were grouped into individual animal, treatment, lobe, region and field of view. Region was defined as a subdivision of the treatment group into tissue originating from the upper, middle or lower lobe. Thus, 4 treatment options (for example flow 8, 18 and 28 and non-ventilated
control as described in chapter 4) resulted in 12 regions. Field of view was treated as a random effect. These data were analysed using nested ANOVA, to account for differences within or between lobes, and a post hoc (least significant difference (LSD) test.
Chapter 3. Ventilator gas flow affects inspiratory time and ventilator efficiency index in term lambs

3.1. Introduction

Respiratory distress syndrome and bronchopulmonary dysplasia (BPD) continue to be major contributors to morbidity and mortality in the preterm infant (Ambalavanan, *et al.*, 2006). Although survival of the preterm baby has improved, the incidence of BPD has not decreased (Donn, *et al.*, 2003). Mechanical ventilation, used to support the preterm baby, involves repetitive and forcible injection of gas into the lungs, which can lead to ventilator-induced lung injury (VILI) (Donn, *et al.*, 2006; van Kaam, *et al.*, 2007). Barotrauma, volutrauma, atelectotrauma and biotrauma have all been shown to play a role in the development of VILI (Webb, *et al.*, 1974; Slutsky, 1999; Dreyfuss, *et al.*, 1992; Hernandez, *et al.*, 1989; Speer, 2006a). An additional possible mechanism is rheotrauma, which refers to damage imparted by the flow of gas along the respiratory tree (Donn, *et al.*, 2006); however, no evidence on the relationship between rheotrauma and VILI in the immature lung has been published as yet. Variations in bias gas flow (Simbruner, *et al.*, 1981) and different methods of providing volume-targeted ventilation (Sharma, *et al.*, 2007) have been shown to affect airway pressure profiles; in turn, airway pressure profiles with a long inspiratory plateau have been associated with a possible increased risk of pneumothorax (Greenough, 1988).

The rate of the ventilator bias gas flow in neonatal units is often set at 6-10 L/min, without any evidence supporting this as the correct setting. High bias flow often results in a rapid pressure upstroke and, in modes with a preset inspiratory time, a prolonged plateau phase. We hypothesised that a high circulating bias flow may also result in very short inspiratory times in ventilatory modes that allow the end of inspiration to be determined by the lung mechanics of the infant. One example of this is the pressure support ventilation + volume guarantee mode (PSV+VG) on the Babylog8000plus ventilator (Dräger Medical,
Lübeck, Germany). PSV allows inspiration to cease when the lungs are nearly fully inflated (when inspiratory flow falls to 15% of peak inspiratory flow), and is a form of triggered expiration. VG allows a targeted tidal volume (TV) to be delivered at the lowest possible peak inspiratory pressure (PIP). During PSV+VG, high circulating flows could potentially result in short inspiratory times, reflecting rapid entry of gas into the lungs. If this is the case, then high circulating bias flow may also contribute to rheotrauma.

The study we describe in this chapter was designed firstly, to confirm the hypothesis that the rate of the ventilator gas flow affects Ti in an artificial lung, and, secondly, to test this hypothesis in a ventilated newborn animal. As this preliminary study aimed to provide evidence of proof-of-principle of the association between bias flow and ventilatory parameters, healthy term lambs that could return to their mothers at the end of the experiment were used.

3.2. Material and methods

3.2.1. Artificial lung experiments

An artificial lung (Bellows K complete, Dräger Medical) was ventilated with a Babylog8000 plus ventilator using PSV+VG. Initial ventilator settings were maximum inflation time (Ti) 1.2 s, maximum PIP 25 cmH₂O, rate 30/min, TV 5 mL. The ventilator flow was commenced at 2 L/min and increased by steps of 1 L/min, up to 8 L/min, followed by flows of 10, 12, 15 and 20 L/min. Each flow was maintained for 60 seconds. The procedure was repeated with 1 mL incremental increases in TV to a maximum of 10 mL. A TV of 5 to 10 mL is representative of those used in preterm infants. Recordings of inflation and deflation time, PIP and TV were obtained via in-built monitoring devices in the ventilator and stored at 10 s intervals via BabyView software (Dräger Medical). Six consecutive data points from each combination of ventilator settings were used for analysis.

3.2.2. Animals

Following induction of anaesthesia, intubation and catherisation, as described in chapter 2, lambs were ventilated using a VIP Bird ventilator (Viasys Healthcare
Inc., Palm Springs, CA, USA). Initial ventilator settings were: assist control mode with termination sensitivity set at 15% (thereby replicating the triggered expiration of PSV mode, as used in the Babylog ventilator); bias gas flow 10 L/min; PEEP 6 cmH₂O; PIP set to attain TV 10 mL/Kg (maximum PIP 25 cmH₂O), and rate 40 breaths/min. Rectal temperature was maintained between 38.5 and 39.5°C using blankets.

**Experimental protocol:** Once the lamb was stabilised with a normal arterial blood gas after at least 10 min on a bias flow of 10 L/min, gas flows were altered every 15 min and an arterial blood gas sample taken at the end of each flow period. Alternate lambs had gas flows either increased in a stepwise fashion to 12, 15, 20 and 25 L/min, followed by a return to 10 L/min for 15 min and then a stepwise decrease to 8, 5 and 3 L/min, or gas flows were decreased first, returned to 10 L/min for 15 min and then increased using identical values for flow. The alternate approaches were used to minimise any potential confounding of the data caused by either alveolar recruitment or atelectasis as flows were increased or decreased. Oxygen delivery (FiO₂) was altered to maintain transcutaneous O₂ saturation recordings (SpO₂) above 95%; other ventilator settings were not altered. Values for heart rate, blood pressure, SpO₂, FiO₂, Ti, PIP and TV were recorded every 3 min and were averaged over the 15 min period at each flow. Ventilatory efficiency index (VEI), used to relate ventilation to respiratory support in the absence of spontaneous breathing, was calculated as follows: 

\[
\text{VEI} = \frac{3800}{(\text{PIP} - \text{PEEP}) \times f \times \text{PaCO}_2}
\]

where 3800 is a constant for carbon dioxide production (mL.Torr/Kg.min), PIP and PEEP are the measured peak inspiratory and positive end expiratory pressures in Torr (1 Torr = 1.332 cmH₂O), \(f\) is the ventilator rate and \(\text{PaCO}_2\) is the partial pressure of CO₂ in arterial blood in Torr (1 Torr = 1 mmHg) (Ikegami, *et al.*, 1997; Notter, *et al.*, 1985).

At the end of the experiments all medication was stopped, the lambs were ventilated on initial settings until fully awake, and then returned to the ewe.
3.2.3. Statistics

Artificial lung data were analysed by ANOVA using StatView (SAS Institute Inc., Cary, version 5.0.2). Based on the variability in physiological data obtained from preliminary experiments, and the fact each lamb was studied at each value for flow, we calculated that 8 animals would be required to detect a 20% difference in inspiratory time with different flows. Animal data were analysed using repeated measures ANOVA, with the order of change of flow (increase or decrease first) as an independent variable. As the increase in flow from 10 L/min to 25 L/min was temporally separated from the decrease in flow from 10 L/min to 3 L/min two repeated measures ANOVAs were performed: one analysing data from the increasing flows and a second analysing data from the decreasing flows.

Significance for the ANOVA was taken as p<0.05; the Bonferroni / Dunn post-hoc test was then used to determine significant comparisons with a significance level of p<0.005 to account for multiple comparisons. Data are presented as mean ± SEM.

3.3. Results

3.3.1. Artificial lung experiments

Inflation time was inversely related to bias gas flow (p<0.0001) up to 15 L/min, with no further decrease in Ti when bias flow increased from 15 to 20 L/min (Figure 3.1). For any given bias flow, Ti increased as set TV increased (p<0.0001; Figure 3.1). Compliance of the artificial lung increased from 0.53 mL/cmH2O at a flow of 2 L/min and a TV of 10 mL to 0.60 mL/cmH2O at a flow of 20 L/min and a TV of 5 mL, with a mean coefficient of variation (CV) of 0.8%. Resistance of the artificial lung increased from 31.1 cmH2O /L.s at a flow of 2 L/min and a TV of 5 mL to 69.6 cmH2O /L.s at a flow of 20 L/min and a TV of 10 mL, with a mean CV of 1.8%.
Figure 3.1: Inflation time of an artificial lung as a function of ventilator bias gas flow

Each line represents a set tidal volume (TV). Data are mean ± SEM.

3.3.2. Animal experiments

Eight term neonatal lambs (6.1 ± 2.1 Kg) were studied. Data were not significantly different between animals in which bias flow was increased first compared with those in which bias flow was initially decreased. Results are therefore presented for all animals together. Ti decreased as bias flow increased between 3 and 12 L/min; at higher gas flow there was no further significant decrease in Ti (Figure 3.2A). TV per kilogram bodyweight at 3 L/min was significantly reduced (p<0.0001; Figure 3.2B), and the set PIP was not reached at this ventilator bias gas flow (mean 19.8 ± 1.9 versus 21.3 ± 2.3 cmH₂O at 10 L/min) (p<0.005 compared with PIP at 10 L/min).
Figure 3.2: Inspiratory time and tidal volume as a function of ventilator bias gas flow

(A) Inspiratory time (Ti) and (B) tidal volume (TV) as a function of ventilator bias gas flow. Bias gas flow 10^d and 10^i refer to flows of 10 L/min before flows were decreased (d) or increased (i) respectively. Points with different superscript letters are different at the p < 0.005 level. Data are presented as mean ± SEM, n = 8.

PaCO₂ and FiO₂ were significantly greater, and VEI significantly lower, at a bias flow of 3 L/min compared with all other flows (p<0.0001; Figure 3.3). PaCO₂ was greater (p=0.005; Figure 3.3A) and VEI (p=0.005; Figure 3.3C) lower at a bias flow of 25 L/min than at 10 L/min. Cardiorespiratory measurements were stable, except for a lower heart rate at 3 L/min (mean 191 ± 45 bpm) when compared to the heart rate at 10 L/min (mean 238 ± 34 bpm; p=0.003; Figure 3.4A). Blood pressures were not significantly different at different bias gas flows (Figure 3.4B)
Figure 3.3: PaCO$_2$ supplemental oxygen and ventilator efficiency index as a function of ventilator bias gas flow

(A) PaCO$_2$, (B) oxygen delivery (FiO$_2$) and (C) ventilation efficiency index (VEI) as a function of ventilator bias gas flow. Bias gas flow 10$^d$ and 10$^i$ refer to flows of 10 L/min before flows were decreased (d) or increased (i) respectively. Points with different superscript letters are different at the p < 0.005 level. Data are presented as mean ± SEM, n = 8.
Figure 3.4: Heart rate and blood pressure as a function of ventilator bias gas flow

(A) Heart rate (HR) and (B) mean arterial blood pressure (BP) as a function of ventilator bias gas flow. Flow $10^d$ and $10^i$ refer to flows of 10 L/min before flows were decreased (d) or increased (i) respectively. Points with different superscript letters are different at the $p < 0.005$ level. Data are presented as mean ± SEM, $n = 8$.

3.4. Discussion

In this chapter we have shown that there is an inverse relationship between ventilator bias gas flow and Ti in both an artificial lung model and term lambs. We believe that this is the first demonstration that ventilator gas flow affects respiratory parameters in the newborn lung.

This study is, therefore, the first step in testing the hypothesis that continuous ventilator bias gas flow may play a part in ventilator dynamics in ventilated newborns. As lambs in this study all received each bias gas flow, this study was not designed to assess the effect of different rates of gas flow on lung injury, but
simply to confirm the hypothesis that different rates of gas flow affect ventilatory dynamics.

We have assessed the effect of changing the continuous ventilator bias gas flow. Inspiratory flow at the level of the endotracheal tube will also depend on other factors such as lung compliance and airway resistance, and thus the time constant. Although in this study we did not measure the rate of inspiratory flow into the lungs, which should not be confused with the rate of the bias gas flow in the ventilator circuit, the fact that inspiratory times decreased with increasing bias flow strongly suggest that inspiratory flow increased with bias flow. A minimum circulating flow is necessary to provide positive end expiratory pressure. Circulating bias flow in addition to this minimum flow generates a rise in pressure. Lower circulating bias flow produces a gradual rise in inspiratory pressure, whereas a high bias flow produces a rapid rise in pressure in the ventilator circuit. Thus, as the bias flow increases, the airway pressure profile during inspiration changes from a waveform with a gradual upslope to one with a “square wave” pattern. Our results are, therefore, of relevance to constant flow ventilators in which continuous bias flow is determined by the clinician. Other types of ventilators use a variable flow; nevertheless, the principle that bias flow affects ventilatory dynamics will be universal.

We used flows between 3 and 25 L/min in this study. At flows above 12 L/min we did not see a further decrease in Ti and the shortest Ti in our study was 0.5 s. This is longer than the Ti observed in human preterm neonates with respiratory distress syndrome (RDS) (South, et al., 1992). These findings may be explained by the fact that we studied healthy term lambs with compliant lungs, whereas neonates with RDS have less compliant lungs. A low compliance is directly correlated to a shorter time constant as time constant = compliance x resistance (Wood, 2003). As compliance should not have significantly changed in these term lambs over time, the lack of effect on Ti with increasing flows above 12 L/min may be due to increased airway resistance. Increasing the flow rate through an endotracheal tube can result in a turbulent flow and exponential increase in resistance, when a critical flow is exceeded (Cave, et al., 1968). It is
also possible that the flow sensor may not have been sensitive enough to detect further shortening of the Ti at the low values seen with high gas flows.

The longest Ti in this study was 1.25 seconds; this was the maximum value we preset and was determined by the respiratory rate of 40/min, which was kept constant. This maximum time was reached at the lowest flow of 3 L/min, when the ventilator was not able to reach its default PIP (and thus TV) within the set time limit for inspiration. In the artificial lung, TV was maintained, even at the lowest flows. The difference is probably explained by characteristics of the in vitro and in vivo systems, demonstrated by the different inflation times in each at given flows. These differences will depend, in part, on characteristics of compliance and resistance. In the lambs we limited Ti to 1.25 seconds; if we had decreased the rate, enabling a longer Ti, the ventilator would most likely have reached its default PIP and TV. At this lowest flow, all cardiorespiratory parameters except blood pressure deteriorated. This could be due to inadequate ventilation, since the target TV of 10 mL/Kg was not reached, or because this low flow approximated or equalled the minute ventilation in the lamb, therefore not providing sufficient flow to remove exhaled CO₂.

Flows as low as these are unlikely to be used in neonatal units, and at all higher rates gas exchange and cardiorespiratory parameters were unchanged, suggesting that the rate of gas flow may be set within a wide range without adverse effects on gas exchange.

Lambs in this study had healthy lungs. Immature lungs have reduced compliance and therefore a shorter time constant. Inflation times will, therefore, be less for any given bias flow. Thus, it is unlikely that the long inflation times seen with low flows in both the artificial lung and in the term lambs would be seen in preterm lambs or, indeed, humans. This is clearly important, as few preterm infants are muscle relaxed during ventilation and several modes of neonatal ventilation allow the baby to determine the rate of assisted breaths. We are not aware of any studies on the effect of reducing continuous bias flow on spontaneous respiratory rates in preterm infants.
At both the lowest (3 L/min) and highest (25 L/min) bias flows the ventilator efficiency index (VEI) was significantly lower compared with the VEI at flows between 5 and 20 L/min, reflecting decreased ventilator efficiency at the extremes of rates used for bias flow in this study. As we were unable to utilise flows greater than 25 L/min due to the limitations of the ventilator, we are unable to determine whether even greater flows have any further impact on ventilator efficiency. Clearly, optimal flows in the preterm lung may be different from those in term lungs due to different lung compliance and airway resistance.

There are currently no data on the optimum level of continuous bias flow when using ventilators with a constant bias flow. Our interest in this parameter arises from the fact that flowing gas behaves like a liquid and, therefore, imparts shear stress on the walls of the tubular structures through which it flows (Cebral, et al., 2004; Tarran, et al., 2005). Although there are no data on the role of shear stress injury in VILI in the immature, ventilated lung, increasing inspiratory flow may result in increasing shearing forces, or so called rheotrauma. This possibility, and the possibility that bias gas flow may, therefore, be related to lung injury, merits further study. Such effects are likely to be magnified in immature lungs with reduced compliance, shorter inflation times and increased respiratory rates and, therefore, experimental studies are necessary to determine the effect ventilator bias gas flow has on ventilator dynamics and lung injury in the immature lung.

With the studies described in this chapter, we have demonstrated, in vitro and in vivo, that pulmonary ventilatory parameters are influenced by continuous bias gas flow of the ventilator. In the ventilated lambs, ventilation efficiency appeared to be maintained at bias gas flows lower than those commonly used in neonatal intensive care settings. We propose the possibility that bias gas flow, and therefore inspiratory flow, may affect rheotrauma within the lung. A greater understanding of the role bias gas flow plays in ventilatory dynamics, and whether this contributes to lung injury, may lead to the development of ventilatory strategies that are of greater benefit to preterm infants. We, therefore, performed two studies in preterm lambs, described in chapters 4 and 5, with the aim of quantifying lung injury in the immature lung as a function of the ventilator bias gas flow.
Chapter 4. High bias gas flows increase lung injury in the ventilated preterm lamb

4.1. Introduction

As described previously, mechanical ventilation can cause VILI with barotrauma, volutrauma, atelectotrauma and biotrauma all playing a role (Webb, et al., 1974; Slutsky, 1999; Dreyfuss, et al., 1992; Hernandez, et al., 1989; Speer, 2006a). Although this knowledge has led to major advances in perinatal care (Roberts, et al., 2006; Crowther, et al., 2006; Sinn, et al., 2002), respiratory disease remains the most common complication of preterm birth and the incidence of BPD has not decreased (Bancalari, et al., 2003). Clearly, additional mechanisms must be involved, which may include shear stress injury caused by high rates of bias gas flow.

Histologically, BPD is characterized by poor alveolarisation (Albertine, et al., 1999; Coalson, et al., 1999), abnormal elastin deposition (Coalson, et al., 1999; Albertine, et al., 1999; Pierce, et al., 1997), fibrosis (Thibeault, et al., 2003; Coalson, et al., 1999), an increase in relative tissue space in the lung, likely resulting from cellular proliferation (Coalson, et al., 1999; Allison, et al., 2008), and abnormal capillary growth (Coalson, et al., 1999). Recent studies in preterm lambs have demonstrated that even a brief period of mechanical ventilation results in up-regulation of the early response genes early growth response factor 1 (EGR1), cysteine rich-61 (CYR61) and connective tissue growth factor (CTGF) (Wallace, et al., 2009), which are known to play a role in adult human lung injury (Zhang, et al., 2000; Ning, et al., 2004; Pan, et al., 2001).

Mechanical ventilation applies a continuous or variable bias gas flow through the ventilator circuit, which is used to inflate the lungs during inspiration and maintain positive end-expiratory pressure during expiration. Neonatal ventilators applying a continuous bias gas flow are commonly set to flows of 6-12 L/min, but there is little evidence to support the use of these flows (Gerhardt, et al., 2008).
Chapter 3 reports data demonstrating that bias gas flow is inversely related to inspiratory time and that optimal ventilator efficiency was obtained at bias gas flows much lower than currently used in many neonatal intensive care units (Bach, et al., 2009b). It is not known, however, whether the velocity of the bias gas flow also affects lung injury or ventilatory dynamics in the poorly compliant immature lung.

Therefore, the hypothesis tested in this chapter was that high ventilator bias gas flow both decreases ventilator efficiency and increases acute lung injury in the preterm lamb by randomly assigning preterm lambs to ventilation with high, medium or low bias gas flows. Physiological and ventilatory parameters, as well as histological and molecular markers of lung injury were assessed.

4.2. Methods

4.2.1. Experimental protocol

As described in chapter 2, ewes received antenatal corticosteroids to mimic the clinical setting, where treatment with antenatal corticosteroids during threatened preterm labour is proven to reduce the occurrence of RDS and death in the neonate (Roberts, et al., 2006).

Hysterotomy was performed at 131-133 days’ gestation (term 147 days). The lamb was orally intubated, the umbilical cord was cut and the lung fluid was allowed to drain by briefly inverting the lamb. If lung fluid was still visible in the endotracheal tube following drainage it was removed by gentle suction. The lamb was weighed before it was transferred to a heat table to maintain a body temperature of 38-39°C. Umbilical catheters were placed after ventilation was commenced with the randomly assigned bias gas flows of 8 (n=13), 18 (n=12) or 28 L/min (n=14). The lambs were ventilated for 2 h on a Babylog8000plus, (Dräger Medical, Lübeck, Germany) in pressure support ventilation + volume guarantee (PSV+VG) mode. Settings were: TV 7 mL/Kg; PEEP 6 cmH₂O; maximum PIP 50 cmH₂O; maximum inspiratory time (Ti) 0.8 s, and frequency 40 breaths/min (increased to 50 and 60 breaths/min if PaCO₂ > 60 mmHg or pH <
7.25). Supplemental oxygen was commenced if SaO₂ was < 90%. Pneumothoraces were promptly drained percutaneously.

At the end of the experiment lambs were killed humanely and lung tissue was collected and processed as described in chapter 2. Eight additional non-ventilated lambs were euthanised prior to delivery to provide control tissue.

4.2.2. Analyses

Analyses were performed as described in chapter 2. Briefly, physiological data were continuously recorded using Powerlab and Ventview systems. Arterial blood gases were taken every 15 min and blood samples for a full blood count were taken at 0, 30, 60 and 120 min after commencing ventilation. The left lower lobe of the lung was used to measure mRNA levels of EGR1, CYR61 and CTGF with quantitative real-time PCR and expressed relative to mean levels of the gene of interest in control tissue. The right lung was prepared for histological (elastin, collagen, septal crest density) and immunohistochemical (αSMA, Ki67) analyses. Proportion of tissue stained positive for elastin, collagen and αSMA and the percentage of lung occupied by tissue and secondary septal crest density were calculated by a single observer (KPB) blinded to the experimental group. Proliferating cells, staining positive for Ki67, were counted and expressed as a proportion of the total number of cells to determine a labelling index (Allison, et al., 2008).

4.2.3. Data analysis

Data were normalised when necessary and are represented as mean ± SEM. Physiological data were analysed in 10 min epochs, commencing 5 min before each blood gas measurement, and were analysed by ANOVA, including analysis of effect of time. Histological data were analysed by nested ANOVA and mRNA levels by one-way ANOVA. The Tukey post-hoc test, or the LSD post-hoc test for histological data, were used when appropriate. Data were analysed using JMP7 (SAS Institute Inc., Cary, NC, USA) or SPSS 14.0 (SPSS Inc., Chicago, IL, USA) and significance was taken at p<0.05.
4.3. Results

4.3.1. Effect of bias gas flow on cardiorespiratory parameters

Three of 39 animals died prior to completion of the 2-hour ventilation period; two were ventilated at 8 L/min, one at 18 L/min. All three died from pneumothoraces.

There was an inverse relationship between inspiratory time and bias gas flow (Table 4.1). Peak inspiratory pressure was lowest and ventilator efficiency index highest in lambs ventilated with the lowest bias gas flow (8 L/min) (Table 4.1). Inspiratory flow through the endotracheal tube increased with increasing bias gas flow (Table 4.1). The rate of rise of inspiratory flow was most rapid, expiratory time was shortest and expiratory flow was greatest in lambs ventilated at 18 L/min (Table 4.1). Examples of the resulting wave forms for pressure, flow and volume, downloaded from Ventview, are shown in Figure 4.1. The gentler slope of the pressure wave at 8 L/min is explained by the significantly lower rate of rise for flow at 8 L/min compared with 18 and 28 L/min (Table 4.1) (Figure 4.1). Effective pulmonary compliance was similar for all animals (1.8-1.9 mL/cmH$_2$O) but pulmonary resistance was significantly higher, and tidal volume significantly lower, in lambs ventilated at 28 L/min compared with lambs ventilated at 8 L/min (Table 4.1). An increase in tidal volume and a decrease in expiratory flow and resistance were seen in the first 30 minutes of the experiment; as these differences were of small magnitude and a similar degree was observed for all groups, data in Table 4.1 are averaged for all the time points.

All animals had a gradual increase in ventilator rate (40 breaths/min at t0 vs. 50 breaths/min at t120 min) and a gradual decrease in expiratory time (1.12 s at t0 vs. 0.8 s at t120 min) during the experiment, independent of bias gas flow ($p<0.05$ for effect of time). PaCO$_2$ was highest in lambs ventilated at 18 L/min (Table 4.2), despite these lambs also having significantly higher ventilator rates in response to the PaCO$_2$ values (Table 4.1). Heart rate, but not blood pressure, was higher in the 18 L/min group compared with the other two groups (Table 4.2). PaO$_2$, but not mean airway pressure or oxygenation index, was also highest.
in lambs ventilated at 18 L/min (Table 4.2). FiO₂ tended to be higher in these lambs, but this was not statistically significant (Table 4.2). Pneumothorax rates tended to be higher in lambs ventilated at 18 L/min (66%) compared with lambs ventilated at 28 L/min (43%) and 8 L/min (31%) (Table 4.2).

Blood white cell count and neutrophil count were not different amongst groups; however, blood white cell count at 60 and 120 minutes were decreased in all three groups compared with baseline values (3.6 at t0 vs. 2.5 at t60 vs. 2.7 x 10⁹/L at t120; p<0.05).

Figure 4.1: Wave forms for pressure, flow and tidal volume at 3 different ventilator bias gas flows

Wave forms were downloaded using Ventview and illustrate the wave forms for pressure, flow and tidal volume over a time period of 3 seconds at a bias gas flow of 8, 18 and 28 L/min. The tidal volume is different in the 3 examples, since tidal volume was set at 7 mL/Kg and the body weight was highest in the lamb ventilated at 8 L/min and lowest in the lamb ventilated at 28 L/min.
### Table 4.1: Effect of bias gas flow on ventilatory parameters

Values are mean ± SEM for inflation time (Ti), expiratory time (Te), peak inspiratory pressure (PIP), mean airway pressure (MAP), ventilator rate, tidal volume (TV), inspiratory flow, expiratory flow, rate of rise for inspiratory flow (ROR inspiratory flow), ventilator efficiency index (VEI) and resistance during ventilation at ventilator bias gas flow rates of 8, 18 or 28 L/min. Data are averaged for all time points. *p<0.05; **p<0.01 vs. flow 18 L/min, †p<0.05 and ‡p<0.01 vs. flow 28 L/min.

<table>
<thead>
<tr>
<th>Bias gas flow (L/min)</th>
<th>8</th>
<th>18</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ti (s)</td>
<td>0.52 ± 0.01**†</td>
<td>0.41 ± 0.01‡</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>Te (s)</td>
<td>0.95 ± 0.03</td>
<td>0.90 ± 0.02‡</td>
<td>1.04 ± 0.03</td>
</tr>
<tr>
<td>PIP (cmH₂O)</td>
<td>24.5 ± 0.7**†</td>
<td>29.2 ± 0.7</td>
<td>29.5 ± 0.6</td>
</tr>
<tr>
<td>MAP (cmH₂O)</td>
<td>11.5 ± 0.2</td>
<td>12.7 ± 0.2</td>
<td>12.1 ± 0.2</td>
</tr>
<tr>
<td>Rate (breaths/min)</td>
<td>42 ± 1**</td>
<td>47 ± 1†</td>
<td>44 ± 0</td>
</tr>
<tr>
<td>TV (mL/Kg)</td>
<td>6.91 ± 0.07†</td>
<td>6.70 ± 0.07</td>
<td>6.65 ± 0.06</td>
</tr>
<tr>
<td>Inspiratory flow (L/min)</td>
<td>14.9 ± 1.7†</td>
<td>20.8 ± 1.7‡</td>
<td>28.2 ± 1.4</td>
</tr>
<tr>
<td>Expiratory flow (L/min)</td>
<td>-23.9 ± 1.3**</td>
<td>-30.6 ± 1.2†</td>
<td>-26.6 ± 1.1</td>
</tr>
<tr>
<td>ROR inspiratory flow (L/s²)</td>
<td>1.88 ± 0.2**†</td>
<td>3.07 ± 0.18‡</td>
<td>2.46 ± 0.17</td>
</tr>
<tr>
<td>VEI (ml.Torr/kg.min)</td>
<td>0.13 ± 0.01**†</td>
<td>0.08 ± 0.01</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Resistance (cmH₂O/L/s)</td>
<td>72.5 ± 3.1‡</td>
<td>77.7 ± 2.8</td>
<td>86.0 ± 2.5</td>
</tr>
</tbody>
</table>

### Table 4.2: Effect of bias gas flow on cardiorespiratory parameters

Values are mean ± SEM for heart rate (HR), mean arterial blood pressure (BP), PaO₂, PaCO₂, FiO₂ and pneumothorax (PNX) during ventilation at ventilator bias gas flow of 8, 18 or 28 L/min. Data are averaged for all time points. *p<0.05 vs. flow 18 L/min, †p<0.05 vs. flow 28 L/min.

<table>
<thead>
<tr>
<th>Bias gas flow (L/min)</th>
<th>8</th>
<th>18</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>151.8 ± 4.6*</td>
<td>184.2 ± 4.1†</td>
<td>158.8 ± 3.8</td>
</tr>
<tr>
<td>Mean BP (mmHg)</td>
<td>73.7 ± 3.3</td>
<td>66.2 ± 2.9</td>
<td>65.5 ± 2.6</td>
</tr>
<tr>
<td>PaO₂ (mmHg)</td>
<td>54.4 ± 6.6*</td>
<td>82.3 ± 5.5</td>
<td>72.5 ± 4.7</td>
</tr>
<tr>
<td>PaCO₂ (mmHg)</td>
<td>59.3 ± 2.0†</td>
<td>69.9 ± 1.5</td>
<td>65.1 ± 1.3</td>
</tr>
<tr>
<td>FiO₂ (%)</td>
<td>57.8 ± 3.2</td>
<td>66.1 ± 3.0</td>
<td>58.3 ± 2.7</td>
</tr>
<tr>
<td>PNX (n/total)</td>
<td>4/13</td>
<td>8/12</td>
<td>6/14</td>
</tr>
</tbody>
</table>
4.3.2. Effect of bias gas flow on mRNA levels of acute response genes and on pulmonary histology

Ventilation resulted in significant up-regulation of *CYR61*, *CTGF* and *EGR1* mRNA levels. Consistent with the physiological data, mRNA levels of all 3 genes were highest in lambs ventilated at 18 L/min; *CTGF* and *EGR1* mRNA levels were ~200% of those seen in lambs ventilated at 8 L/min (*p* < 0.05) (Figure 4.2).

Quantitative histological analysis of lung sections demonstrated that ventilation at 8 and 28 L/min increased collagen density and decreased percentage of space occupied by tissue compared with unventilated control lambs (Table 4.3). Ventilation at 28 L/min also increased alpha smooth muscle actin (αSMA) staining density compared with control lambs (Table 4.3). Ventilation at 18 L/min had the most marked effects on quantitative histological analysis, with decreased elastin density compared with control lambs and lambs ventilated at 28 L/min, decreased αSMA staining density compared with all other groups and an increased proportion of each field of view occupied by tissue compared with lambs ventilated at 8 and 28 L/min. In lambs ventilated at 18 L/min, collagen density (Figure 4.3) and the proportion of Ki67 positive cells (Figure 4.4) were not significantly different from unventilated control tissue (Table 4.3). There was no significant difference amongst groups in number of secondary septal crests (Table 4.3) (Figure 4.5).

Qualitative analysis demonstrated that in control tissue elastin and αSMA staining was predominantly located at the tips of the secondary septal crests. Following ventilation, septal crests appeared thicker and shorter and increased elastin and αSMA staining was seen in the alveolar wall with differentiated myofibroblasts more randomly distributed throughout the interstitium (Figure 4.3). These changes were most apparent in lambs ventilated at flows of 18 and 28 L/min (Figure 4.3). Collagen fibres, seen as thick parallel fibres in the airway wall in control tissue, appear more tortuous following ventilation with a fine meshwork of fibres in the thickened interstitium. Again, these changes were most apparent in lambs ventilated at 18 and 28 L/min (Figure 4.3).
### Table 4.3: Quantitative analysis of the effect of bias gas flow on pulmonary histology

The proportion of lung tissue stained positive for elastin, αSMA and collagen, the proportion of Ki67-positive cells (representing mitotic cells), the proportion of each field of view occupied by tissue rather than air space and secondary septal crest density (both using a point counting technique) (Allison, *et al.*, 2008) for age matched non-ventilated controls and animals ventilated at 8, 18 and 28 L/min. § *p*<0.05 vs. control tissue, *p*<0.05 vs. flow 18 L/min, † *p*<0.05 vs. flow 28 L/min.

<table>
<thead>
<tr>
<th>Bias gas flow (%) of lung tissue</th>
<th>Control</th>
<th>8 L/min</th>
<th>18 L/min</th>
<th>28 L/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elastin</td>
<td>11.4 ± 0.5</td>
<td>10.8 ± 0.5</td>
<td>10.0 ± 0.6§†</td>
<td>11.6 ± 0.6</td>
</tr>
<tr>
<td>αSMA (%) of lung tissue</td>
<td>17.8 ± 0.8</td>
<td>18.7 ± 1.2*</td>
<td>14.9 ± 1.0§†</td>
<td>21.3 ± 1.1§</td>
</tr>
<tr>
<td>Collagen (%) of lung tissue</td>
<td>0.27 ± 0.01</td>
<td>0.32 ± 0.01§*</td>
<td>0.27 ± 0.01†</td>
<td>0.32 ± 0.01§</td>
</tr>
<tr>
<td>Ki67 (%) of total cells</td>
<td>6.5 ± 0.3</td>
<td>5.8 ± 0.4†</td>
<td>6.7 ± 0.6</td>
<td>7.5 ± 0.4</td>
</tr>
<tr>
<td>Tissue ratio (%) of total area</td>
<td>40.0 ± 3.0</td>
<td>34.2 ± 2.9§*</td>
<td>41.9 ± 2.8†</td>
<td>33.7 ± 3.1§</td>
</tr>
<tr>
<td>Septal crests (%) of total area</td>
<td>9.8 ± 0.4</td>
<td>9.6 ± 0.5</td>
<td>10.7 ± 0.5</td>
<td>9.6 ± 0.6</td>
</tr>
</tbody>
</table>
Figure 4.2: Effect of bias gas flow on mRNA levels of *CTGF*, *EGR1* and *CYR61*

mRNA levels, expressed as fold change relative to mRNA levels in age-matched non-ventilated control tissue, of *CTGF* (A), *EGR1* (B) and *CYR61* (C) in lung tissue of lambs ventilated with a ventilator bias gas flow of 8, 18 or 28 L/min. *p*<0.05 vs. flow 18 L/min, § *p*<0.05 vs. control tissue and §§ *p*<0.01 vs. control tissue.
Figure 4.3: Representative photomicrographs of lung tissue from age matched non-ventilated controls and ventilated animals

Columns demonstrate elastin (stained black with Hart’s resorcin stain; A-D), differentiated myofibroblasts (stained brown using immunohistochemistry; E-H), and collagen type I and III fibres (stained black with Gordon-Sweet’s stain; I-L). Unventilated controls, A, E, I; lambs ventilated at 8 L/min, B, F, J; at 18 L/min, C, G, K, and at 28 L/min, D, H, L. Bar 10 μm. Arrows (↑) demonstrate secondary septal crests with elastin (A) or myofibroblasts (E) visible at the tip. Solid arrowheads (▲) demonstrate abnormal deposition of elastin and open arrowheads (△) demonstrate thickened interstitium (C, D, H) containing a finer meshwork of collagen fibres in tissue ventilated at 18 and 28 L/min (K, L).
Figure 4.4: Representative photomicrographs of lung tissue stained using Ki67

Photomicrographs of: A. age matched non-ventilated controls; B-D. Lambs ventilated with a bias gas flow of 8 L/min (B); 18 L/min (C), and 28 L/min (D). Proliferating cells stained brown, the rest of the lung tissue was stained blue using a haematoxylin counterstain. Bar represents 10 μm.
Figure 4.5: Representative photomicrographs of lung tissue used for septal crest analysis

Photomicrographs of: A. age matched non-ventilated controls; B-D. Lambs ventilated with a bias gas flow of 8 L/min (B); 18 L/min (C), and 28 L/min (D). Hart’s resorcin stain was used to stain elastin tissue black and a counterstain (tartrazine in picric acid) was used to stain the rest of the tissue yellow. Bar represents 25 μm.

4.4. Discussion

This study reports the novel finding that the rate of ventilator bias gas flow affects lung injury in the preterm lung. Higher bias gas flows have adverse effects on ventilatory parameters, increase mRNA levels of acute early response genes and result in histological evidence of lung injury. The findings occurred after only
2 hours of ventilation; it remains to be determined whether adverse effects would increase with increasing duration of ventilation.

Many factors are known to contribute to the development of VILI in the preterm lung, including barotrauma (Webb, et al., 1974; Slutsky, 1999), volutrauma (Dreyfuss, et al., 1992; Hernandez, et al., 1989), atelectotrauma (Slutsky, 1999) and oxytrauma (Bancalari, et al., 2003; Jobe, et al., 2001a). Volutrauma is considered to be more injurious than barotrauma (Dreyfuss, et al., 1992; Hernandez, et al., 1989); therefore, volume guarantee ventilation was used to ensure delivery of a consistent tidal volume independent of changes in compliance or resistance. Effective compliance was not different amongst groups, although resistance was lowest in lambs ventilated at 8 L/min and highest in lambs ventilated at 28 L/min. Lambs ventilated at 18 and 28 L/min had lower tidal volumes (by 0.3 mL/Kg; less than 5% of the set tidal volume) than lambs ventilated at 8 L/min but they required higher PIP values. Apart from flow-related parameters (including the inspiratory time which is closely correlated to flow) (Bach, et al., 2009b), the only ventilatory parameter that was different between lambs ventilated at 18 and 28 L/min was the ventilator rate. This was increased in lambs ventilated at 18 L/min in response to the increased PaCO₂ in this group. As there is a clear pattern of increased lung injury in animals ventilated at 18 L/min compared with lambs ventilated with 28 L/min, despite having similar TV and PIPs, it appears that the injury is unrelated to either barotrauma or volutrauma.

The contribution of hyperoxia induced trauma (Jobe, et al., 2001a; Bancalari, et al., 2003) cannot be excluded as PaO₂ was highest at 18 L/min. This may be in response to alterations in ventilator rate and FiO₂ in response to clinical condition, as lambs ventilated at 18 L/min tended to have higher PaCO₂ values and a greater incidence of pneumothorax. However, the likelihood that oxytrauma is solely responsible for the observed injury is not high, since the mean PaO₂ in the lambs ventilated at 18 L/min was still within physiological limits (Bland, et al., 2000).
A change in the shape of the pressure wave may contribute to lung injury by altering the rate of lung inflation (Spitzer, *et al.*, 2003). A slower pressure rise can be achieved by altering various ventilatory parameters (Greenough, 1988; Simbruner, *et al.*, 1981; Boros, *et al.*, 1984), including reducing the bias gas flow. A study in adult sheep demonstrated that, in the presence of high pressure ventilation (PIP=50 cmH₂O), a decreased bias gas flow, with its attendant altered pressure wave pattern, protected against lung injury (Rich, *et al.*, 2000). However, there are no studies in neonates reporting advantages of one pressure wave pattern over another.

The inspiratory time was inversely related to bias gas flow in the preterm lambs in this study, similar to findings in term lambs with compliant lungs (Bach, *et al.*, 2009b). A long inspiratory time has been associated with a 50% increase in the risk of pneumothorax in preterm babies with RDS (Greenough, 1988; Kamlin, *et al.*, 2003). However, babies in those studies were ventilated with time-cycled pressure-limited ventilation in which there is a significant risk of active expiration against an inspiratory pressure triggered by the ventilator. PSV+VG, the mode we used in this study, is a flow-cycled mode of ventilation in which the expiratory phase commences when inspiratory flow falls to less than 15% of peak inspiratory flow. There was no significant difference in the rate of pneumothorax with different bias gas flows in this study, although there was a trend for more pneumothoraces in animals ventilated at 18 L/min.

Although inspiratory time was inversely related to bias gas flow, the rate of rise of inspiratory flow and the expiratory flow were both highest in lambs ventilated with 18 L/min bias gas flow, rather than in those ventilated with 28 L/min. The finding of most marked lung injury and highest mRNA levels of the acute response genes in lambs ventilated with 18 L/min therefore supports the hypothesis that lung injury may be related to rate of rise of flow and / or expiratory flow.

The lower rate of rise of inspiratory flow in lambs ventilated at 28 L/min compared with those ventilated at 18 L/min is probably explained by a turbulent gas flow pattern in the endotracheal tube at a flow of 28 L/min. Flow of gas at
37°C and at a barometric pressure of 760 mmHg becomes turbulent through a size 4.0 endotracheal tube once the flow exceeds 18 L/min (Jarreau, et al., 1999). Thus, at the highest flow of 28 L/min the flow pattern will have been turbulent, paradoxically leading to a lower flow in the respiratory airways. Furthermore, a turbulent flow pattern leads to a larger pressure drop from the T-piece to the endotracheal tube. The pressure drop between two cross sections of a tube is caused by changes in kinetic energy and friction pressure drop between the gas and the wall of the tube. Both a mathematical model and actual measurements of flow and pressure demonstrated a larger pressure drop with turbulent flow compared with laminar flow (Jarreau, et al., 1999). Thus, even though PIP values measured by the sensor at the T-piece were similar in lambs ventilated with flows of 18 and 28 L/min, the pressure in the endotracheal tube is likely to have been higher at 18 L/min when there is laminar flow than at 28 L/min when there is turbulent flow. Lambs ventilated at 18 L/min also had the greatest expiratory flows. The reason for this is not clear, but may result from higher expiratory airflow in the endotracheal tube creating a Venturi-like effect at the intersection between the comparatively narrow tube and wider ventilator circuit. When gas or fluid is flowing from a narrow tube into a wider tube, the velocity of the flow will decrease while pressure will increase compared with measurements in the narrow tube. It could also be due to reduced pulmonary compliance as a result of more serious lung injury in these lambs.

However, effective compliance was not different for lambs ventilated at 18 or 28 L/min. Effective compliance was calculated by linear regression, recalculated on a new ventilation stroke every few seconds. For this stroke, the Babylog measured airway pressure and volume at 120 Hz. The resulting compliance was displayed on the Babylog (Draeger Medical). The latest value was downloaded to Ventview every 10 seconds, averaged per time block and analysed. The dynamic compliance was not measured in this study. The dynamic compliance is obtained by measuring the change in volume and in transpulmonary pressure, the latter approximated by measuring the pressure at the mouth and in the oesophagus (Wood, 2003).
Heart rate was also significantly higher in animals ventilated with a bias gas flow of 18 L/min. As tidal volume or PIP in animals ventilated at 18 L/min were not different from animals ventilated at 28 L/min, this is unlikely to be due to a response to altered diastolic filling of the heart. Pulmonary disease, such as emphysema or asthma, or positive pressure ventilation leads to an increase in intrathoracic pressure. This can result in a pulsus paradoxus, a more than 10 mmHg difference in systolic arterial pressure during the respiratory cycle, caused by a decreased filling of the heart during inspiration, thus leading to a decrease in cardiac output and a subsequent tachycardia (Bernstein, 1996). The higher heart rate may reflect the higher PaCO$_2$ values in this group which, despite increasing the ventilator rate, remained above 60 mmHg. Since the animals were muscle relaxed, they were not able to increase their breathing rate spontaneously to compensate for higher PaCO$_2$, but may have increased their heart rate via the chemoreceptor reflex in an attempt to eliminate surplus arterial CO$_2$ (Seeley, et al., 1992).

The early response genes CTGF, CYR61 and EGR1 have recently been identified as potentially useful biomarkers of VILI in the preterm newborn lamb (Wallace, et al., 2009). They are also up-regulated in lungs of adults with chronic obstructive pulmonary disease and pulmonary fibrosis (Pan, et al., 2001; Ning, et al., 2004; Zhang, et al., 2000), in response to stimuli promoting fetal lung growth in sheep (Sozo, et al., 2006) and in response to hyperoxia in mice (Perkowski, et al., 2003). CTGF and CYR61 both belong to the CCN family of cysteine rich proteins (Brigstock, 2003). These proteins form interactions between extracellular matrix and cell adhesion molecules and they promote cell proliferation and differentiation, including lung fibroblast proliferation and myofibroblast differentiation, tissue regeneration and synthesis of extra-cellular components, including collagen, are involved in embryonic angiogenesis and up-regulate pro-inflammatory cytokines, such as IL-1 (Kubota, et al., 2007; Chen, et al., 2001). CTGF is immunolocalised to activated fibroblasts and type II alveolar epithelial cells (Pan, et al., 2001) whereas CYR61 is an extracellular matrix associated protein (Zhou, et al., 2005; Sakamoto, et al., 2004) and is up-regulated when subjected to cyclic mechanical stretch (Zhou, et al., 2005). CYR61 mediates
expression of αSMA in smooth muscle cells through cytoskeletally based mechano-transduction (Zhou, et al., 2005).

Expression of the transcription factor EGR1 is rapid and of short duration after acute tissue injury (Zhang, et al., 2000), hypoxia (Yan, et al., 2000) and pneumonectomy (Landesberg, et al., 2001), although a prolonged expression has been found in smooth muscle cells of bronchial and vascular walls and in alveolar macrophages in lung tissue from patients suffering from advanced stage emphysema (Zhang, et al., 2000). Thus, EGR1 is involved in both immediate early responses and more chronic lung destruction.

These 3 early response genes may all contribute to the pathogenesis of BPD, which includes an inflammatory response, hypercellularity, fibrosis and dysmorphic capillary growth, by inducing pro-inflammatory cytokines, by directly stimulating fibroblasts and epithelial cells, by stimulating collagen formation and by upsetting the normal balance of angiogenic factors (Wallace, et al., 2009), especially since mRNA levels of these three genes in the lung were up-regulated after 2 hours of ventilation. This is consistent with the study of Wallace et al, in which peak levels were reported 15 minutes after the onset of injurious ventilation in preterm lambs with levels remaining elevated after 2 hours of ventilation (Wallace, et al., 2009). In the study reported in this chapter, highest levels (~10 fold of control values) were measured in animals ventilated at 18 L/min, consistent with the physiological data. In both the study of Wallace et al and this study, up-regulation of these genes was seen shortly after the onset of ventilation in preterm lambs, with higher expression levels after ventilation with larger volumes or at higher bias gas flows. Therefore, altered levels of these early response genes may reflect the level of injury in the preterm lung and might potentially predict the level of injury and risk of BPD development in the preterm baby.

The histological changes that are described here after only two hours of ventilation are consistent with changes found in babies (Thibeault, et al., 2003; Margraf, et al., 1991; Thibeault, et al., 2000) and animals (Allison, et al., 2008; Albertine, et al., 1999; Coalson, et al., 1999) with BPD and are consistent with the
physiological and gene expression data. Septal crests appear as part of normal lung development, giving rise to alveoli. Poor alveolarisation is seen during BPD (Albertine, et al., 1999; Coalson, et al., 1999), with abnormal deposition of elastin (Albertine, et al., 1999; Coalson, et al., 1999; Pierce, et al., 1997) and collagen fibres (Thibeault, et al., 2003; Coalson, et al., 1999), decreased number of secondary septal crests (Allison, et al., 2008) and increased cellular proliferation (Allison, et al., 2008; Coalson, et al., 1999).

Collagen and elastin fibres provide the stabilising framework for the lung (Young, et al., 1980) and both are important for septal crest formation and airway branching (Wright, et al., 1999; Heine, et al., 1990). Elastin is essential for lung recoil (McGowan, 1992). Deposition of elastin fibres occurs primarily at the tip of secondary crests; however, abnormal deposition (increased deposition and altered spatial pattern) has been described in VILI (Pierce, et al., 1997; Coalson, et al., 1992; Albertine, et al., 1999). Allison et al suggested that VILI destroys immature secondary septal crests, causing them to flatten with the consequence that elastin fibres located at the tip of the secondary crest are re-absorbed into the primary alveolar wall giving the appearance of abnormal deposition (Allison, et al., 2008).

We described altered deposition of both elastin and collagen following ventilation, which was most marked at the higher flows. Quantitative analysis demonstrated that elastin and collagen densities were lowest after ventilation at 18 L/min. This may reflect disruption of fibres and reabsorption into the primary alveolar wall. Alternatively, it is possible that the altered deposition, with less discrete areas of staining, resulted in an underestimation of the degree of staining by the image analysis software.

Differentiated myofibroblasts, which play a role in connective tissue fibre deposition and secondary septal crest formation, were identified by immunohistochemical staining of alpha smooth muscle actin (Leslie, et al., 1992; Leslie, et al., 1990). The pattern of these cells followed that of elastin, with most cells located at the tip of the secondary septal crest in control animals but a more random distribution throughout the interstitium following ventilation,
consistent with the hypothesis of re-absorption of septal crests into the primary wall (Allison, et al., 2008). However, we did not find decreased secondary septal crest density in this study, possibly because of the short duration of the study and the immediate collection of lung tissue afterwards. The appearance of shorter and thicker septa after ventilation, especially at the higher flows, might reflect early lung injury and inflammation, as described in baboons with BPD (Coalson, et al., 1999). An actual decrease in density in our study might have been found after a longer duration of ventilation.

Increased cellular proliferation is another characteristic feature of BPD, and has been described in baboons with BPD (Coalson, et al., 1999) and following in-utero ventilation of 110 day lamb fetuses (Allison, et al., 2008). Recruitment of inflammatory cells to the lungs, with a consequent fall in circulating neutrophil count (Carlton, et al., 1997), has also been reported with ventilation resulting in an increased DNA concentration in the lung (Allison, et al., 2008). Our findings of increased numbers of proliferating cells, increased space occupied by tissue in the lung and reduced circulating white blood cells are consistent with these reports.

The results in this chapter indicate that lower flows, even for a short duration of ventilation, have the potential to be beneficial in reducing ventilator-induced lung injury in the preterm lung. In chapter 3 it was demonstrated that flows as low as 5 L/min were safe and efficient in healthy term lambs. Furthermore, optimal ventilator efficiency occurred at flows between 1.5-3 L/Kg.min (bodyweight ~ 6 Kg). Lambs of our breed are ~ 4.5-5 Kg at 131 days' gestation; therefore, we chose 8 L/min to be the lowest flow in the study described in this chapter. The Dräger Babylog 8000plus is a continuous flow ventilator with a range from 2-30 L/min. Theoretically 30 L/min is the maximum flow on the Babylog; however, at these flow rates the flow sensor is inconsistent in its measurements and we therefore used a flow of 28 L/min as the highest flow. The effect of a further reduction in bias gas flow on lung injury in the immature lamb lung is investigated in chapter 5. Randomised studies in preterm babies will be necessary to determine whether bias gas flow may play a role in lung injury in humans.
In summary, to investigate the role of ventilator bias gas flow on VILI preterm lambs were ventilated with three different bias gas flows. High ventilator gas flows lead to more severe lung injury, altered ventilator parameters and up-regulation of biomarkers of VILI in the preterm lamb compared to lower gas flows, although our data suggest that turbulent gas flow at the highest flow velocity might ameliorate the adverse effect of fast laminar flow. We have thus confirmed that the velocity of ventilator gas flow is important in the development of VILI. The next chapter examines the effect rheotrauma has on the more immature lung combined with the effect of ventilator bias gas flow at different modes of ventilation.
Chapter 5. The effect of bias gas flow during different modes of ventilation on lung injury in the preterm sheep fetus

5.1. Introduction

One of the major complications after preterm delivery is the development of bronchopulmonary dysplasia. As discussed previously, many factors associated with mechanical ventilation are known to cause VILI and thus to play a role in the aetiology of BPD. Concern about VILI has led to the development of newer modalities of ventilation in an attempt to protect the immature lung from the harmful effects of ventilation. For example, volume targeted ventilation is now possible with current flow sensors being sensitive enough to measure the small tidal volumes used during ventilation of the preterm baby (McCallion, et al., 2008). This guarantees the tidal volume delivered to the baby, even during conditions when the baby's lung compliance may be changing rapidly due to clinical condition or the treatment given, and thus potentially can limit volutrauma. Volume targeted ventilation has been shown to result in a reduction in the number of days on the ventilator, the rate of pneumothorax and severe intracranial haemorrhage and it appears to have a favourable effect on development of BPD when compared to pressure-limited ventilation (McCallion, et al., 2005).

Furthermore, synchronised ventilation, which uses a patient derived trigger to initiate and/or end a mechanical breath, has been the subject of ongoing development with newer techniques of synchronisation reducing the lag time between the onset of the baby's breath and initiation of the ventilator inspiratory phase. Although evidence for long-term benefits of synchronised versus non-synchronised ventilation in preterm babies is currently lacking, synchronisation is the preferred option for most clinicians, since babies supported by synchronised ventilation have a decreased risk of air leaks (Gupta, et al., 2007), shorter duration of ventilation (Greenough, et al., 2008), a more uniform tidal
volume delivery (Gupta, et al., 2007; Jarreau, et al., 1996) and better oxygenation (Cleary, et al., 1995).

Two types of patient-triggered ventilation commonly used in neonatal units are synchronised intermittent positive pressure ventilation (SIPPV), a time-cycled mode of ventilation, and pressure support ventilation (PSV), a flow-cycled mode of ventilation. During both SIPPV and PSV all spontaneously initiated breaths are supported by the ventilator. The difference between the two modes of ventilation is that during SIPPV the inspiratory pressure is maintained for the duration of the inspiratory time set by the clinician (Gupta, et al., 2007), often resulting in an inspiratory plateau phase or square pressure wave (Sinha, et al., 2008). In contrast, during PSV expiration occurs once the inspiratory flow has decreased to a certain percentage of the peak inspiratory flow (for example, 15% for the Babylog 8000plus), thus providing full synchronisation between patient and ventilator (Sinha, et al., 2008). The inflation time of the lung (equivalent to the inspiratory time recorded by the ventilator in PSV mode) is dependent on the time constant of the respiratory system, calculated by multiplying compliance x resistance (Gerhardt, et al., 2008). The time constant can lengthen rapidly, for example when surfactant is given to a baby with severe RDS. Inflation time is thus dependent on lung mechanics, whereas maximum inspiratory time is the time set on the ventilator by the health professional. Thus, the inspiratory time equals the maximum inspiratory time during SIPPV, whereas the inspiratory time during PSV is normally shorter than the maximum inspiratory time set by the clinician.

Longer inspiratory times, resulting in square wave ventilation with a plateau phase, were widely adopted in neonatal practice (Kamlin, et al., 2003) in the 1970s after reports demonstrated that the resulting higher mean airway pressures were associated with better oxygenation (Herman, et al., 1973; Reynolds, 1971). However, when the inspiratory time is long enough to result in an inspiratory pressure plateau, the baby may attempt active expiration whilst the ventilator is maintaining the inspiratory pressure. This leads to an increased risk of pneumothoraces (Kamlin, et al., 2003; Greenough, 1988) and interstitial emphysema (Gerhardt, et al., 2008) in babies with RDS.
A change in the pressure wave-form can also be achieved by altering other ventilator settings in addition to the inspiratory time (Greenough, 1988; Boros, et al., 1984; Simbruner, et al., 1981). A higher ventilator bias gas flow, for example, leads to a steeper incline of the pressure upstroke, as was discussed in chapter 4 of this thesis. Previous chapters in this thesis have demonstrated an inverse relationship between bias gas flow and inflation time of the lung in both term (chapter 3) and preterm (chapter 4) lambs. Preterm lambs ventilated in PSV mode with high bias gas flows had shorter inflation times but higher mean airway pressures and more severe lung injury than those ventilated at the lowest bias gas flow (chapter 4).

Thus, different levels of bias gas flow result in altered pressure waveforms and different degrees of lung injury. To date, there are no studies investigating the impact of varying bias gas flows in different ventilator modes in the immature lung.

The hypothesis of the study described in this chapter is that both mode of ventilation and bias gas flow affect lung injury in the immature lung. Specifically, that ventilation with a long inspiratory time and an inspiratory pressure plateau phase at high ventilator bias gas flows leads to higher expression levels of early response genes of lung injury and more severe histological lung injury compared to ventilation without an inspiratory plateau phase and at lower bias gas flows.

This hypothesis was tested by randomising preterm fetuses to different modes of ventilation at two different ventilator bias gas flows. The fetal lambs remained connected to the placental circulation to remove any confounding factors due to ventilatory difficulties, including oxytrauma, in such an immature lung. Physiological and ventilatory parameters, as well as histological and molecular markers of lung injury were assessed.

5.2. Methods

5.2.1. Experimental paradigm

Hysterotomy was performed at 110-112 d gestation (term = 147 d) as described in chapter 2; however, only the fetal head of the experimental animal was
delivered and placental circulation was maintained for the duration of the experiment.

A jugular venous catheter and a carotid arterial catheter were inserted in the fetal neck, followed by a tracheostomy using a size 4.0 endotracheal tube (see chapter 2). If lung fluid was visible in the endotracheal tube following intubation, a gentle suction was performed to clear the fluid before the onset of ventilation. The head of the fetus was covered with a NeoWrap™ (Fisher & Paykel Health Care, Auckland, New Zealand) to maintain a body temperature of 38-39°Celsius.

Animals were randomised to ventilation for 2 hours with a Babylog 8000plus (Dräger Medical, Lübeck, Germany) with one of two ventilatory modes: pressure support ventilation (PSV) mode or synchronised intermittent positive pressure ventilation (SIPPV) mode, and at one of two bias gas flows: 4 L/min or 18 L/min, thus yielding four groups of animals (PSV4, PSV18, SIPPV4 and SIPPV18). Settings were: volume guarantee with TV 10 mL (expected bodyweight of the fetus was 2 Kg, thus TV~5 mL/Kg); positive end expiratory pressure (PEEP) 6 cmH₂O; maximum PIP 50 cmH₂O; maximum inspiratory time (Ti) 1.0 s, and frequency 30 breaths/min. During ventilation of the first six fetuses, used to establish the experimental paradigm, it had become apparent that a prolonged inflation was necessary to recruit FRC. Data from these fetuses were not included in the analyses. Therefore, all fetuses included in the experiment received an initial manual inflation of 20 s at 40 cmH₂O with a flow of 8 L/min using a Neopuff (Fisher & Paykel Health Care, Auckland, New Zealand), followed directly by initiation of ventilation with the mode and bias gas flow appropriate for the arm to which they were randomised. Medical air was used to run the Babylog ventilator; no supplemental oxygen was given to the fetus. No adjustments were made to the ventilator settings throughout the experiment. The room temperature was kept at 25°C during the experiments.

At the end of the experiment a lethal dose of pentobarbitone (Pentobarb 300, Provet NZ, New Zealand, 90 mg/Kg i.v.) was administered to the fetus, followed by complete delivery and a post-mortem. To provide control tissue from age matched, non-ventilated fetuses a further six fetuses, twins of the experimental
animals, were humanely killed with pentobarbitone and tissues rapidly collected as described in chapter 2.

5.2.2. Analyses

Analyses were performed as described in chapter 2. Arterial blood gases were taken every 15 min, starting at the beginning of the experiment, in both the fetus and the ewe. Furthermore, blood samples (5 mL) taken from the fetus after 15 and 60 min were passed through a LeukoLOCK filter as described in chapter 2. Filters were then frozen at -80 °C and shipped to collaborators in the Monash Institute in Melbourne, where measurement of early response genes in small blood samples is currently being validated.

To record temperature and relative humidity in the ventilator circuit, a modified EasyLog USB-2 hygrometer (Lascar Electronics Ltd., Salisbury, UK) was inserted into the ventilator circuit, just proximal to the spirometer. Recordings were made once per min and stored using EasyLog USB version 3.10. Room temperature and room humidity were recorded every 15 min using a thermometer/hygrometer.

The post-mortem was performed as described in chapter 2, subheading 2.2.

5.2.3. Data analysis

All physiological data, including hygrometer data, were analysed in 10 min epochs as described in chapter 2. Data were analysed using repeated measures ANOVA. Interaction effects for mode x flow, time x mode, time x flow and time x mode x flow were analysed using repeated measures ANOVA. A one-way ANOVA and Tukey post-hoc test was performed reporting the effect of ventilation groups or time, if the p-value of the relevant repeated measures ANOVA was p<0.05.

The mRNA levels of the early response genes in tissue of experimental and control fetuses were analysed with a one-way ANOVA, with Tukey post-hoc tests when appropriate. Data were analysed using JMP7 (SAS Institute Inc., Cary, USA).
Histology and immunohistochemistry data were analysed by nested ANOVA with a post-hoc LSD test utilising SPSS (Version 3.3, SPSS Incorporated, USA) (for details see chapter 2).

All data are represented as mean ± SEM.

5.3. Results

5.3.1. Effect of bias gas flow and mode of ventilation on cardiorespiratory parameters

In total, 34 fetuses were ventilated, of which the first 6 were used to establish the experiment. The remaining 28 animals were randomised to ventilation at PSV4 ($n=6$), PSV18 ($n=6$), SIPPV4 ($n=7$) and SIPPV18 ($n=9$). Fetal weight was not significantly different amongst groups (1,958 ± 98; 1,944 ± 98; 1,974 ± 91 and 1,842 ± 98 g for PSV4, PSV18, SIPPV4 and SIPPV18 respectively). Three animals randomised to SIPPV18 died; 1 was dead upon delivery of the head, 1 was moribund at delivery of the head and was humanely killed after the pH of the first blood gas was 6.8 and 1 fetus died 60 min after the onset of ventilation. This fetus was found to have a pneumothorax and abnormal cardiac anatomy (truncus arteriosus) at post mortem and was excluded from further analysis. Lung tissue was collected from 6 age-matched, non-ventilated fetuses.

The two different ventilatory modes resulted in different pressure waveforms, with an inspiratory plateau phase during ventilation in SIPPV mode but not in PSV mode (Figure 5.1). PIP was highest in animals ventilated in PSV mode, with the highest levels in the PSV18 group (Figure 5.2). However, MAP values in PSV18 were half those in animals ventilated in SIPPV4 or 18 (Figure 5.2). PIP values decreased in the first 15 min for animals ventilated in SIPPV mode ($p = 0.015$) and MAP values decreased for all groups during this time ($p = 0.0017$). The mean tidal volume achieved was not significantly different amongst groups (5.2 ± 0.4; 5.2 ± 0.3; 5.1 ± 0.2 and 5.4 ± 0.2 mL/Kg for PSV4, PSV18, SIPPV4 and SIPPV18 respectively), except for time zero, when it was significantly higher in the PSV18 group. The recorded inspiratory time during PSV was shorter than the set inspiratory time for SIPPV, with animals in the PSV18 group having the
shortest inspiratory times (Figure 5.3). The ventilator rate was set at 30 breaths per minute and except for a slightly lower value in the first 5 min this was well maintained (Figure 5.3).

Figure 5.1: Representative pressure wave forms

Representative pressure wave forms during ventilation with pressure support ventilation (PSV) or synchronised intermittent positive pressure ventilation (SIPPV) at ventilator bias gas flows of 4 or 18 L/min (data captured from Babylog 8000 plus using Ventview). Respective inspiratory times (Ti), at the time data were captured, are given per pressure wave.
Figure 5.2: Ventilation pressures and tidal volume as a function of time

Groups: Δ PSV4, ▲ PSV18, □ SIPPV4 and ■ SIPPV18. Values are mean ± SEM. A. Peak inspiratory pressure (PIP); B. mean airway pressure (MAP); C. tidal volume (TV). Significant differences are represented as follows: group effect, different lower case letters (p < 0.0001); †††, mode effect (p < 0.0001); §§, flow effects (p < 0.01); *, time x mode interaction (p < 0.05), and # for time (p < 0.05). PIP in SIPPV mode, TV in PSV18 and MAP in all groups were significantly higher at time 0 compared with later time points.
Figure 5.3: Inspiratory time, ventilator frequency, compliance, resistance, time constant and FiO₂ as a function of time

Groups: △ PSV4, ▲ PSV18, □ SIPPV4 and ■ SIPPV18. Values are mean ± SEM. A. Inspiratory time (Ti); B. ventilator frequency; C. compliance; D. resistance; E: time constant and F: FiO₂. Significant differences are represented as follows: group effect, different lower case letters (p < 0.0001); § p < 0.05, §§ p < 0.01 and §§§ p < 0.0001 for flow effect; † p < 0.05, †† p < 0.01 and ††† p < 0.0001 for mode effect; *, time x mode interaction (p < 0.05), ‡, time x mode x flow interaction (p < 0.05), and # for time (p < 0.05). Ventilator rate, resistance and time constant at time 0 were significantly different from all other time points; compliance at time 0 was significantly different from time 105 and 120 min. The results for Ti were identical for SIPPV4 and SIPPV18.
Compliance and resistance fell in all fetuses between time zero and 15 minutes. Fetuses ventilated with PSV18 tended to have lower compliance and resistance than fetuses in other experimental groups (p = 0.06 and p = 0.07 respectively) (Figure 5.3). Compliance was significantly greater with SIPPV mode (p = 0.0005) and the lower flow of 4 L/min (p = 0.009). Resistance was significantly greater with SIPPV (p = 0.02) and a flow of 4 L/min (p = 0.03). The time constant, a product of multiplying compliance and resistance, was consequently significantly shorter for PSV18 (Figure 5.3).

A bias gas flow of 18 L/min resulted in significantly higher peak inspiratory and expiratory flows and a greater rate of change for pressure in both inspiration and expiration (all p<0.05) (Figure 5.4). However, although the rate of pressure rise was significantly greater with a flow of 18 L/min in both SIPPV and PSV modes, the rate of pressure fall during expiration was only greater in the PSV18 group (Figure 5.4), consistent with the shortest time constant in this group (Figure 5.3). Rate of pressure fall in the PSV4 group was significantly less than the PSV18 group, but significantly greater than in both SIPPV groups (both p<0.01). Tidal volume entered the lung at a faster rate during ventilation at a high flow or in the SIPPV mode, whereas tidal volume was expired faster at a low flow (Figure 5.4).

Heart rate and blood pressure were not significantly different amongst groups (Figure 5.5).

Arterial blood gases values were not significantly different, neither amongst groups nor over time (Figure 5.6). No fetus received supplemental oxygen (Figure 5.3F). Some ewes received supplemental oxygen in order to maintain a maternal PaO₂ between 90 and 140 mmHg (Figure 5.6F).

The humidifier was set to achieve a temperature of 37 °C; however, the temperature only stabilised to 34.2 ± 0.2 °C for PSV and 35.3 ± 0.01 °C for SIPPV 30 minutes into the experiment (p<0.05) (Figure 5.7). A bias gas flow of 4 L/min resulted in a significantly lower relative humidity and dew temperature at the Wye piece (p<0.0001) with an even further decrease over time (Figure 5.7). The median room temperature during the experiment was 25.4°C (range 23.8 –
26.0°C). The median room humidity was 40% (range 34-47%, due to different weather conditions).

**Figure 5.4: Inspiratory and expiratory flows and rates of change for pressure and volume as a function of time**

Groups: Δ PSV4, ▲ PSV18, □ SIPPV4 and ■ SIPPV18. Values are mean ± SEM. A. Peak inspiratory flow (PIF); B. peak expiratory flow (PEF); C. rate of rise for pressure; D. rate of drop for pressure; E. rate of rise of volume and F. rate of drop of volume. Significant differences are represented as different lower case letters for group effects (p < 0.01), ††† for mode effect (p < 0.0001), § p < 0.05, §§ p < 0.01 and §§§ p < 0.0001 for flow effect and * for time x mode interaction (p < 0.05).
Figure 5.5: Mean arterial blood pressure and heart rate as a function of time

Groups: △ PSV4, ▲ PSV18, □ SIPPV4 and ■ SIPPV18. Values are mean ± SEM. A. Mean arterial blood pressure (BPmean) and B. heart rate (HR). There were no significant differences amongst groups.
Figure 5.6: Blood gas values for lamb (left) and ewe (right) as a function of time

Groups: Δ PSV4, ▲ PSV18, □ SIPPV4 and ■ SIPPV18. Values are mean ± SEM. A. pH in the ventilated fetus; B. pH in the ewe; C. PaCO₂ in the ventilated fetus; D. PaCO₂ in the ewe; E. PaO₂ in the ventilated fetus and F. PaO₂ in the ewe. There were no significant differences amongst groups.
Figure 5.7: Temperature, humidity and dew temperature in the ventilator circuit as a function of time

Groups: △ PSV4, ▲ PSV18, □ SIPPV4 and ■ SIPPV18. Values are mean ± SEM. A. temperature; B. relative humidity (RH) and C. dew temperature. Significant differences are represented as † p < 0.05 for mode effect, §§§p < 0.0001 for flow effects, * p < 0.01 for time x flow interaction in B, p < 0.0001 for time x flow and p < 0.05 for time x mode x flow in C, and # p < 0.05 for time. Time 0 and 15 were significantly different from times between 30 and 120 min for temperature; time 0 was significantly different from time 15, time 15 was significantly different from times between 75 and 120 min for relative humidity, and time 0 was significantly different from time 15 and 30 for dew temperature in all groups.

5.3.2. Effect of bias gas flow and mode of ventilation on mRNA levels of early response genes in lung tissue

mRNA levels of EGR1 were significantly higher in PSV18, SIPPV4 and SIPPV18 animals compared to age-matched non-ventilated control tissue (p<0.01; Figure 5.8). The levels in animals ventilated in PSV4 were not different from the levels
in control tissue. Both CYR61 and CTGF tended to have the highest levels after ventilation with PSV18 and SIPPV4 (p 0.086 and p 0.065 respectively; Figure 5.8).

**Figure 5.8: mRNA levels of early response genes**

A. Early growth response 1 (EGR1); B. cysteine-rich 61 (CYR61); C. connective tissue growth factor (CTGF) mRNA levels in lung tissue expressed as fold change of the mean level in tissue from age matched non-ventilated control animals. Bars with different letters are significantly different (p<0.01).
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5.3.3. Effect of bias gas flow and mode of ventilation on histological and immunohistochemistry analysis in lung tissue

The proportion of differentiated myofibroblasts, detected with αSMA staining, was increased after ventilation in SIPPV mode with a bias gas flow of 4 or 18 L/min compared with control animals and animals ventilated with PSV18 (p<0.05) (Figure 5.9). Ventilation with SIPPV18, but not SIPPV4, also resulted in increased αSMA staining compared with PSV4. The location of myofibroblasts also appeared to be altered, with fewer myofibroblasts visible at the tip of septal crests in animals ventilated with SIPPV and this was most obvious at high flows (Figure 5.9).

Septal crest density was less, and percentage of lung area occupied by lung tissue was greater, following ventilation with PSV18 or SIPPV4 compared with PSV4, SIPPV18, or control tissue (Figure 5.10; p<0.05 for all comparisons, except SIPPV4 vs PSV4 for septal crest density [p>0.05]). The septal crests in PSV18 and SIPPV4 fetuses also appeared to be more blunted and not as finely demarcated as the septal crests in control animals or those ventilated with PSV4 (Figure 5.10). No difference was seen amongst ventilation groups in the number of proliferating cells expressed as a percentage of total number of cells per field of view (Figure 5.11).

In ventilated animals, areas with normal aeration were observed next to areas with thickened saccule walls, or to areas with saccules that appeared to be larger with thinner walls. Thus, there was a more non-uniform inflation in lung tissue from ventilated animals compared with tissue from non-ventilated animals. This was more obvious following ventilation with high flows and least obvious following PSV4 (Figure 5.12).
Figure 5.9: Differentiated myofibroblasts in lung tissue detected with αSMA staining

A. Deposition of αSMA positive tissue expressed as a percentage of total field of view. Bars with different letters are significantly different (p<0.05). Representative photomicrographs of B. age matched non-ventilated controls; C. fetuses ventilated at 4 L/min in PSV mode; D. at 4 L/min in SIPPV mode; E. at 18 L/min in PSV mode; and F. at 18 L/min in SIPPV mode. Cells positive for αSMA stained brown, the rest of the lung tissue was stained blue using a haematoxylin counterstain. Bar represents 10 μm.
Figure 5.10: Analyses of septal crest density and space occupied by tissue

See next page for legend.
A. Septal crest density expressed as a percentage of tissue per group and B. space occupied by tissue expressed as a % of the field of view per group. Bars with different letters are significantly different (p<0.05). Representative photomicrographs of C. age matched non-ventilated controls; D. fetuses ventilated at 4 L/min in PSV mode; E. at 4 L/min in SIPPV mode; F. at 18 L/min in PSV mode; and G. at 18 L/min in SIPPV mode. Hart’s resorcin stain was used to stain elastin tissue black and a counterstain (Tartrazine in picric acid) was used to stain the rest of the tissue yellow. Bar represents 25 μm.
Figure 5.11: Proliferating cells in lung tissue, detected by staining for Ki67

A. Number of proliferating cells expressed as a percentage of total number of cells per group. There were no significant differences amongst groups. Representative photomicrographs of B. age matched non-ventilated controls; C. fetuses ventilated at 4 L/min in PSV mode; D. at 4 L/min in SIPPV mode; E. at 18 L/min in PSV mode; and F. at 18 L/min in SIPPV mode. Proliferating cells stained brown, the rest of the lung tissue was stained blue using a haematoxylin counterstain. Bar represents 10 μm.
Figure 5.12: Non-uniformity in tissue of ventilated animals.

See next page for legend.
Representative photomicrographs of tissue stained for analysis of Ki67. Each row of photomicrographs (both left and right image) was taken from the same animal and tissue from the same tissue section was used. A, B: fetuses ventilated at 4 L/min in PSV mode; C, D: at 18 L/min in PSV mode; E, F: at 4 L/min in SIPPV mode; and G, H: at 18 L/min in SIPPV mode. Arrow illustrates areas with thinner saccule walls and larger surrounding airspaces, arrowhead illustrates areas with thick saccule walls. Bar represents 10 μm.

5.4. Discussion

This study has investigated the effect of bias gas flow, mode of ventilation and the interaction between flow and mode on ventilatory parameters and lung injury in a paradigm using preterm sheep fetuses. The hypothesis that both ventilator bias gas flows and mode of ventilation affect lung injury in the preterm lamb was confirmed. For animals ventilated with PSV mode, the higher flow led to the lowest compliance, more severe lung injury and a trend towards higher mRNA levels of the early response genes. Animals ventilated with SIPPV mode had better compliance and a longer time constant, but, surprisingly, a lower bias flow led to fewer septal crests compared with the high flow group and controls. Ventilation with PSV4 resulted in better lung mechanics than PSV18 and most lung injury markers were not significantly different from non-ventilated controls. The results were seen after only 2 hours of ventilation. It remains to be seen if these findings will change after a longer duration of ventilation, or if ventilation continues after the transition from intra-uterine to extra-uterine life has been completed and the lung fluid has been cleared.

Since volutrauma is recognised to be more harmful than barotrauma in the development of VILI (Dreyfuss, et al., 1992; Hernandez, et al., 1989), this study used volume guarantee ventilation to ensure the delivery of a consistent tidal volume, independent of changes in compliance or resistance. The tidal volume was set at 10 mL, resulting in a TV of 5.1-5.4 mL/Kg, which is within the generally accepted range for the tidal volume during ventilation of a preterm
lamb (Albertine, et al., 1999; Wallace, et al., 2009; Crossley, et al., 2007). Therefore, volutrauma is unlikely to be a major complicating factor in this paradigm, particularly as ventilation was initiated with a prolonged inflation (see below). The fetus was connected to the placental circulation throughout the experiment and the arterial oxygenation of the ewe was maintained within physiological limits. This also resulted in physiological values of arterial oxygenation in the fetus; therefore, oxytrauma was effectively avoided in this paradigm.

Peak inspiratory pressures were much higher in fetuses ventilated in the PSV mode compared with the SIPPV mode and were higher in PSV fetuses ventilated with high bias gas flow. In contrast, PIP values in fetuses ventilated with SIPPV mode were similar with high and low bias gas flows. High pressures per se do not lead to more severe lung injury as long as cells and/or lung tissue are not overstretched even for a brief period of time (Copland, et al., 2003; Korones, 2003). Comparison of ventilation with high or low airway pressures, but similar tidal volumes, achieved by restricting thoraco-abdominal expansion in rats or immature rabbits, did not lead to more severe lung injury (Hernandez, et al., 1989; Dreyfuss, et al., 1992; Dreyfuss, et al., 1988). The observed changes in early lung injury markers and lung histology in this experiment are, therefore, not likely a result of barotrauma.

PIP values were higher in the PSV mode, with the highest PIP during ventilation with PSV18. This may simply have been caused by the short inspiratory time with PSV18, during which the target TV was delivered. On the other hand, the higher PIP values in fetuses ventilated with PSV, may indicate that in this mode the lungs were less compliant. Both compliance and the time constant, the product of compliance and resistance (Wood, 2003), deteriorated over time in all experimental groups and the lowest values were seen with ventilation with PSV18. Lower compliance and a short time constant are seen in babies with RDS or lung disease, whereas values are higher for the more mature and healthy lung (Wood, 2003). The deterioration of lung mechanics over time in this study is suggestive of the occurrence of ventilator-induced lung injury and the lower values at PSV18 are possibly suggestive of more severe lung injury in this group.
The rates of pressure and volume rise were significantly greater at the high flows, supporting the hypothesis that high bias gas flows result in a more abrupt opening of the lung. Rate of pressure rise has been reported to be dependent on the bias gas flow and lung mechanics (Gerhardt, et al., 2008). In the current experiment, bias gas flow seemed to play a bigger role, since the shorter time constant in PSV18 compared with SIPPV18 was not reflected in a significantly greater rise of pressure.

Peak inspiratory flows, traversing the endotracheal tube, were also significantly greater during ventilation with high bias gas flow. Interestingly, bias gas flows of 4 L/min generated peak inspiratory flows of 11.1 ± 0.5 for PSV4 and 12.0 ± 0.5 L/min for SIPPV4, whereas bias gas flows of 18 L/min generated only slightly higher peak inspiratory flows of 21.2 ± 0.5 for PSV18 and 19.9 ± 0.5 L/min for SIPPV18. Flow dynamics may have limited the increase in peak inspiratory flow at a bias gas flow of 18 L/min, since it has been demonstrated that flow of gas through a size 4.0 endotracheal tube becomes turbulent once the bias gas flow exceeds 18 L/min (Jarreau, et al., 1999).

In the PSV mode, the expiratory phase was initiated once the inspiratory flow fell to 15% of the PIF, whereas in the SIPPV mode the PIP was maintained for the duration of the inspiratory time resulting in an inspiratory pressure plateau. This plateau phase accounts for the higher MAPs in the SIPPV mode compared with the PSV mode. Only one animal in the SIPPV mode developed a pneumothorax, which is a well-described complication of ventilation with a prolonged inspiratory pressure plateau (Greenough, 1988; Kamlin, et al., 2003). The low incidence of a pneumothorax may be explained by the use of a prolonged inflation breath in every fetus before the start of ventilation in the experiment described in this chapter. Theoretically, this leads to more even alveolar recruitment and better pulmonary compliance. The prolonged inflation breath was implemented after initial experiments, used to establish the protocol (but not reported in this chapter), showed that the set tidal volume was achieved after a few breaths in the SIPPV mode, whereas this tidal volume was not reached at all in the PSV mode. However, following an inflation breath of 20 s all fetuses achieved their set tidal volume. The fluid-filled lung will have a long time
constant (Milner, 2001), due to the high resistance of moving liquid through the airways compared with air (te Pas et al 2009) and prolonging the inflation will likely have helped to establish an FRC. Observations in the first few minutes of life in spontaneously breathing preterm infants demonstrated that nearly 80% of the breaths had a prolonged expiration, mainly characterised as an expiratory breath hold (te Pas, et al., 2008b). The first breaths were characterised by a short deep inspiration followed by a prolonged expiratory phase, initially with low or zero expiratory flow ending with an expiratory flow peak or multiple expiratory flow peaks (expiratory braking) (te Pas, et al., 2008a). Similar patterns for the first few breaths are observed in spontaneously breathing term infants. These breathing patterns are recognised as a mechanism to maintain elevated intrapulmonary pressure, possibly leading to enhanced fluid clearance from the lung, distribution of gas within the lung and splinting of the alveoli and airways (te Pas, et al., 2008b; Mortola, et al., 1982). The 20 s duration of the prolonged inflation was based on the results of a study in preterm rabbits. Inflation times of 1, 5, 10 and 20 s demonstrated that only an inflation time of 20 s led to a full recruitment of FRC and uniform inflation of the lung at 20 s, without leading to overdistension, as evaluated by phase contrast x-ray imaging (te Pas, et al., 2009). Therefore, this recruitment strategy likely avoided the occurrence of the 'baby lung' as described by Gattonini et al (Gattinoni, et al., 1987), where the tidal volume delivered is received in only a small part of the lung, thus easily leading to over-inflation and possibly to pneumothorax in this part of the lung.

The peak expiratory flows were significantly greater during ventilation at the high flow compared with the low flow. Surprisingly, peak expiratory flows for the low flow groups were greater than the peak inspiratory flows. In spontaneously breathing infants, expiratory flows are lower than inspiratory flows (Bhutani, et al., 2003). This observation is probably related to the different lung mechanics in our fetuses, caused by the fetal position during ventilation, as well as the fact that these fetuses were intubated. Normally, expiration is a largely passive process that is reliant on elastic recoil of lung tissue and surface tension forces (Wood, 2003). In this experiment, very immature fetuses with stiff lungs were used; therefore, lung recoil was expected to be high, leading to high
expiratory flows. In addition, the chest of the fetus was *in utero*, while only the head and part of the neck were *ex utero*. The uterus and abdominal wall of the ewe may have led to higher compressing forces, with a resultant faster expiration.

The rate of pressure drop was faster in PSV18 compared with PSV4. PSV18 had the shortest time constant and since it takes three time constants to expel 95% of the tidal volume (Wood, 2003; Kamlin, *et al.*, 2003), the air should leave the lung faster in this group compared with the others. Furthermore, PSV18 had the highest value for PIP, and thus the biggest pressure differential between PIP and PEEP; therefore, the rate for pressure drop in this group had to be highest. Animals ventilated in the SIPPV mode, independent of the bias gas flow, had similar values for PIP, compliance and time constant and, indeed, also had similar values for the rate of pressure drop. A possible explanation can be found in the inspiratory pressure plateau in these fetuses. The pressure of the plateau phase is more representative of the alveolar pressure than the PIP (Sinha, *et al.*, 2003). Theoretically, three time constants should be long enough to achieve 95% of an equilibrium between airway and alveolar/saccular pressures, since it has been reported that 95% of the pressure applied to the airway is delivered to the alveoli within three time constants (Wood, 2003). However, when inflation of the lung is non-uniform, for example secondary to surfactant deficiency or ventilator-induced lung injury, different areas of the lung will have different time constants (Wood, 2003). Therefore, having a pressure plateau possibly allowed the lung more time to reach this equilibrium between airway and alveolar/saccular pressures, likely leading to the better compliance and longer time constant in the SIPPV group.

The rate of volume drop during expiration was faster following ventilation at a low flow, which can possibly be explained by a lower resistance in the ventilator circuit at low bias gas flows. The lower resistance allowed a faster onset of expiration with an earlier occurrence of the peak expiratory flow, thereby possibly allowing most or all of the expiratory tidal volume to leave the lung in a shorter period of time.
The low bias gas flows led to much lower values of relative humidity, all well below the recommended humidity values as specified by the American National Standards and International Organization of Standardization (Miyoshi, et al., 2005). It is well described that ventilation with dry air leads to damage to the tracheobronchial epithelium, malfunction of the mucociliary transport system, thickening of secretions and heat loss of the patient (Chalon, et al., 1979). A study in preterm lambs (140 d; term 150 d) demonstrated a trend towards increased IL-1β mRNA expression in lung tissue following 3 hours of ventilation with cold and dry air compared with heated and humidified air (Pillow, et al., 2009), thus suggesting that ventilation with dry air promotes inflammation. We did not measure cytokine expression in this study. Only minimal, conflicting data on the temperature and relative humidity achieved in the circuit during ventilation at different flows in babies are available (Tarnow-Mordi, et al., 1986; Schumann, et al., 2007). Despite this, different bias gas flows are already applied by some neonatologists during neonatal ventilation (C. J. Morley, C. A. Kuschel, F.H. Bloomfield, W.P.F Fetter, personal communication, 2006). One study has reported that humidity is affected by bias flow (Tarnow-Mordi, et al., 1986), but another found that humidity was independent of the inspiratory flow, but dependent on the rate of breathing (Schumann, et al., 2007). However, these studies used different humidifiers. A test report on the performance of the humidifier used in the experiment described in this chapter contains a graph with data for absolute humidity at different bias gas flows. Extrapolation of these data suggests that at bias gas flows of 4 and 18 L/min, the absolute humidity should be expected to be approximately 30 and 35 mg/mL respectively (Fisher and Paykel Healthcare, 1998). However, verified data are not available. In the current experiment, the circuit temperature was not significantly different amongst groups. Therefore, it can be concluded that the relative humidity was also dependent on the bias gas flow, with a lower rate of flow leading to lower values for relative humidity.

Condensation in the ventilator circuit can explain a relative humidity of less than 100% in the high flow groups. Dew temperature is the temperature at which water vapour condenses into water. In the high flow groups, the dew
temperature in the circuit was higher than the average room temperature leading to rain-out in the ventilator circuit and resulting in a lower than 100% relative humidity. In the low flow groups, the dew temperature was lower than the average room temperature, which explains why condensation was not observed. Thus, the low relative humidity in the low flow groups was likely due to technical limitations of the humidifier at low flows and not of condensation. The results of this study warrant careful assessment of the temperature and humidity of the gas delivered during ventilation of the neonate, especially when ventilating at low bias gas flows. It will be important to determine whether current humidifiers can achieve desired levels of temperature and humidity at lower flows. For both future studies and clinical care, it is worth considering incorporating a hygrometer in the ventilator circuit close to the patient to monitor humidity and temperature closely. However, it is important to note that lower bias flows, both in chapter 4 and in this chapter, tend to result in lesser degrees of lung injury. If humidification is less effective at these flows, then the true effect size of bias gas flow on lung injury may have been underestimated in these studies.

High bias gas flows resulted in a 9-10 fold increase in mRNA levels of the early response gene EGR1 compared with control tissue, whereas the mRNA levels for PSV4 were not significantly different from controls. EGR1 has been demonstrated to up-regulate rapidly after, for example, acute tissue injury, hypoxia and pneumonectomy (Zhang, et al., 2000; Yan, et al., 2000; Landesberg, et al., 2001). Therefore, the higher levels can be assumed to be a reflection of more severe lung injury following ventilation at high flows. The two other early response genes tested, CYR61 and CTGF, showed trends towards an interaction effect between flow and mode of ventilation, with higher levels following ventilation at a high flow in the PSV mode or a low flow in the SIPPV mode. CYR61 is known to up-regulate the expression of inflammatory genes, including IL-1. Higher levels of inflammatory cytokines, such as IL-1β, IL-6 and CXCL8, were demonstrated after injurious ventilation (Wallace, et al., 2009; Chiumello, et al., 1999; von Bethmann, et al., 1998) and a trend towards higher IL-1β mRNA expression was observed after ventilation with dry non-humidified air (Pillow, et
The trend towards higher levels of *CYR61* at SIPPV4 might, therefore, be a reflection of the combination of more injurious ventilation with air at a low relative humidity. These genes are members of the CCN family and promote cell proliferation and differentiation, tissue regeneration and synthesis of extracellular components (Brigstock, 2003; Kubota, *et al.*, 2007; Chen, *et al.*, 2001). Cell proliferation was not different amongst groups in this study, as assessed by the number of proliferating cells expressed as a percentage as the total number of cells, which possibly is a reflection of the immediate collection of lung tissue following the 2 hours of ventilation. Another group documented a significant increase in proliferating cells following 1 hour of *in utero* ventilation. However, these fetuses were left *in utero* for another 11 hours before tissue was collected (Allison, *et al.*, 2008). Furthermore, antenatal corticosteroids, which are known to alter the fetal lung structure, decrease VILI and decrease inflammatory cytokine mRNA expression, were not used (Willet, *et al.*, 2001; Hillman, *et al.*, 2010). In the experiment described in this chapter, *CYR61* and *CTGF* were not significantly up-regulated compared with non-ventilated controls, which might have been different if antenatal corticosteroids had not been used. Other explanations for the low levels of these genes may be found in the inability of the immature immune system at a gestational age of 110 days to up-regulate them any further, or a down-regulation of the fetal inflammatory response during pregnancy, as suggested in animal experiments and in the human fetus with chorioamnionitis (Kramer, *et al.*, 2005; Kallapur, *et al.*, 2006; Kallapur, *et al.*, 2005). Since the fetuses were connected to the placental circulation, this may have decreased the inflammatory response in the fetus and subsequently affected levels of early response genes and histological findings in this study. Ventilation of an immature lung after delivery may potentially result in an increased up-regulation of these early response genes leading to more abnormal lung histology.

A decreased density of septal crests was seen following ventilation at a high flow in the PSV mode or a low flow in the SIPPV mode. A similar interaction between flow and mode was seen for the percentage of space occupied by tissue, which was increased in these two groups. These findings, as well as the blunter
appearance of the septal crests and the non-uniformity of lung tissue, occurring after only two hours of ventilation, are consistent with injurious changes observed in other paradigms of BPD (Allison, et al., 2008; Coalson, et al., 1999; Pierce, et al., 1997; Albertine, et al., 1999). Authors of another preterm sheep paradigm observed a decrease in septal crest density after only 1 hour of in utero ventilation (Allison, et al., 2008). They proposed the theory that this occurs as a result of reabsorption of the secondary septal crests into the primary alveolar wall, accounting for the reduction in number of secondary crests and the simplification of the distal airspace (Allison, et al., 2008), or the poor alveolarisation, as seen following VILI (Albertine, et al., 1999; Coalson, et al., 1999).

In the study reported in this chapter, the proportion of differentiated myofibroblasts, visualised using an immunohistological technique to stain αSMA (Leslie, et al., 1992; Leslie, et al., 1990), were highest after ventilation in the SIPPV mode. Qualitative analysis revealed a more random location of differentiated myofibroblasts throughout the interstitium after ventilation at the high flows, a similar finding to ventilation at 18 L/min in chapter 4. Normally, myofibroblasts are located at the tip of a septal crest (Leslie, et al., 1992; Leslie, et al., 1990). The reabsorption of septal crests, reflected in a lower density and a more blunted appearance, especially after ventilation with PSV18 or SIPPV4, may, therefore, have affected the location of the differentiated myofibroblasts in the interstitium.

The reason why an interaction effect between flow and mode was seen for septal crest density and the percentage of space occupied by tissue and why a similar trend was observed for two of the early response genes is not clear. The interaction between flow and mode is apparently not a linear, straightforward relationship, but a more complex relationship. The hypothesis that high flows, especially when combined with an inspiratory pressure plateau phase, would be more injurious on the immature lung has not been demonstrated. On the contrary, the use of a plateau phase resulted in better values for lung compliance and time constant compared with ventilation at a high flow in the PSV mode. It is possible that the initial ventilation after delivery, until an FRC has been recruited
and the lung fluid has been cleared, may be better with a longer inspiratory time or an inspiratory plateau. Overall though, ventilation with PSV4 resulted in least severe markers of lung injury, often without significant differences compared with non-ventilated controls. Therefore, with the current knowledge, this seems to be the preferred mode of ventilation in the immature sheep lung.

In conclusion, this chapter has shown that bias gas flow, mode of ventilation and the interaction between these two affect ventilatory parameters and histology of the immature sheep lung. More research needs to be done, to determine if the interaction between bias gas flow and mode still exists after the lung fluid has been cleared during the transitional phase from intra-uterine to extra-uterine life, and if this interaction possibly affects long-term injury, such as BPD. Close attention needs to be paid in these studies to the achieved humidity of the air, especially if lower flows are used.
Flow at lower rates

Does bias gas flow in ventilated preterm babies affect lung injury or the risk of bronchopulmonary dysplasia?

A feasibility study

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6.1. Abstract

**Background:** The proportion of babies born preterm is increasing throughout the developed world. Improved neonatal care has decreased mortality in preterm babies; however, the rate of bronchopulmonary dysplasia (BPD) has not decreased. BPD is multifactorial in origin, with factors such as prematurity, infection (antenatal and / or postnatal), oxygen treatment and mechanical ventilation all playing a role. The adverse effect of some of these factors is likely mediated via activation of inflammatory pathways in the preterm lung. An inflammatory response occurs rapidly following onset of mechanical ventilation, and increased concentrations of pro-inflammatory cytokines in tracheal secretions are associated with risk of later BPD. Studies in preterm lambs have demonstrated that higher bias gas flows are associated with increased lung injury and decreased ventilatory efficiency. Historically, bias gas flows have not been altered routinely; in the light of recent research more neonatal paediatricians are altering bias gas flows, although there are no clinical data to support optimum levels.

**Aims:** To determine (i) the effect of ventilator bias gas flow on inflammatory markers predictive of later BPD; (ii) whether a multicentre RCT of low vs. high bias gas flow is feasible and indicated.

**Methods:** We propose a randomised, multicentre pilot study to test the feasibility of randomising preterm babies to ventilation at two different flows. Babies born at less than 28 weeks’ gestation and / or with a birth weight less than 1,000 g and who require ventilation in the first week of life will be randomly assigned to a ventilator bias gas flow of 4 L/min (low flow) or 10 L/min (current standard flow). Primary outcomes will be CXCL8 concentrations in tracheal aspirates (TA) and number of ventilated days. Secondary clinical outcomes will be BPD, defined as ongoing respiratory support and / or supplemental oxygen at 36 weeks’ postmenstrual age, duration and nature of respiratory support and a composite outcome of neonatal death and serious neonatal morbidity. We will also measure concentrations of other biomarkers of lung injury in TA (IL-1β, IL-6, TNF-α and 3-chlorotyrosine) and blood. Blood
samples will be stored in anticipation of the validation of an assay for mRNA levels of acute early response genes. Furthermore, we will collect data from the medical records of the follow-up clinics, regarding hospitalisation, wheezing and asthma in the first 2 years of life.

Animal experiments demonstrate that lung injury is initiated very rapidly after onset of mechanical ventilation. Obtaining consent antenatally for trials in preterm babies is difficult and significantly affects recruitment rates as was seen in the CPAP or Intubation at Birth trial (COIN-trial; Australian and New Zealand Clinical Trials Registry number, 12606000258550). Fewer babies with acute or serious complications were recruited and more mothers received antenatal corticosteroids in this trial.

We believe there is currently equipoise surrounding choice of bias gas flow with clinicians choosing a bias gas flow based on personal preference or beliefs, but without any supporting clinical evidence as to which flow is optimal and with no data on potential harm. Providing evidence is thus urgent and will improve medical care. We intend to apply for ethical approval for a waiver of consent for 24 hours.

**Statistical analyses** will be on an intention to treat basis. This study is powered to detect a 35% reduction in CXCL8 concentrations in TA. Continuous data will be analyzed using t-test or repeated measures ANOVA and presented as relative reduction with 95% Confidence Intervals (CI). Categorical outcome data will be analyzed using Chi-Square Test and will be presented as relative risk with 95% CI. Calculation of the number needed to treat to benefit (NNTB) or number needed to treat to harm will be performed when appropriate. We will require 64 babies per group. A p value less than 0.05 will be considered to indicate statistical significance for the primary outcome; a p value of less than 0.01 for the secondary outcomes. All p values will be two-sided.

If feasible, data generated by this pilot study will be used to develop a larger multicentre study powered to detect a difference in BPD.
6.2. **Background**

### 6.2.1. Incidence of preterm birth

The normal gestation for a human baby is 37-42 weeks. However, the proportion of babies born preterm is increasing, a trend that is consistent throughout the developed world (Tucker, *et al.*, 2004). In the USA about 13% of babies are born preterm (before 37 weeks’ gestation) and about 1% are born extremely preterm (before 28 weeks’ gestation) (Martin, *et al.*, 2009). In National Women’s Hospital about 11% are born before 37 weeks’ gestation and 1.5% are born before 28 weeks’ gestation (Battin, 2008). Preterm birth is responsible for 75% of neonatal deaths (Hack, *et al.*, 1999), despite a decrease in the mortality rate for preterm infants in the USA of 19% between 1993 and 2003 (March of Dimes, 2007). This significant reduction in mortality and also in morbidity is based on major advances in perinatal care over the last decades, particularly antenatal corticosteroid treatment in women at risk of preterm birth (Roberts, *et al.*, 2006; Crowther, *et al.*, 2006) and the use of surfactant (Sinn, *et al.*, 2002).

### 6.2.2. Health and economic implications of preterm birth

Respiratory disease remains the most common complication of preterm birth (Bancalari, *et al.*, 2003), with respiratory distress syndrome (RDS), caused by lung immaturity and surfactant deficiency, and development of bronchopulmonary dysplasia (BPD), defined as an ongoing requirement for supplemental oxygen at 36 weeks’ postmenstrual age (Jobe, *et al.*, 2001a), being the two main respiratory complications. Up to half of babies born at less than 30 weeks’ gestation will suffer from RDS (Ramanathan, 2006). In National Women’s Hospital, about 140 infants per year need mechanical ventilation, of which 40-50 are born at less than 28 weeks’ gestation (Battin, 2008). About 30-35% of babies born before 28 weeks’ gestation in National Women's Health will develop BPD (Battin, 2008); however, data from the Australian and New Zealand Neonatal Network report an incidence of 52% in this population (Kuschel, *et al.*, 2004), which is consistent with the international literature (Lemons, *et al.*, 2001).
Respiratory disease contributes significantly to the health costs in neonatal intensive care (Crowther, et al., 2000). In National Women’s Hospital, Auckland, New Zealand, the cost of caring for a preterm baby is between NZ$820 and NZ$2,200 per day, depending on the level of care required.

Babies who develop BPD require a higher level of care and more days on respiratory support than babies without BPD, they often require longer hospitalisation, and may be discharged home on supplemental oxygen. They also require more frequent re-admission during early childhood years and are at increased risk of developing asthma (Greenough, et al., 2004, 2002), all contributing to the health burden of preterm birth. There are limited data on the long term respiratory outcomes after preterm birth. A longitudinal study, assessing respiratory symptoms at 21 years of age in ex-preterm subjects demonstrated significantly more respiratory symptoms, such as coughing or wheezing, in ex-preterm subjects compared with their peers (odds ratio 4.2; 95% confidence interval 1.3 to 13.5; p=0.01), although spirometry and airway hyper-responsiveness were not significantly different (Narang, et al., 2008). The babies in this study had a median gestation of 31.5 weeks and median birth weight of 1,440 g (Narang, et al., 2008). Recently, the EPICure study assessed the degree of respiratory morbidity and functional impairment at 11 years in children born at or before 25 weeks’ gestation and compared this with classroom control subjects. Twice as many ex-preterm babies had asthma (25 vs. 13%; p<0.01). Ex-preterm babies had abnormal baseline spirometry in 56% and positive bronchodilator response in 27%, but less than half of those were receiving asthma medication; therefore, the actual number of ex-preterm babies with asthma may be higher than their reported 25%. Impaired lung function and respiratory morbidity were worse among those with prior BPD (Fawke, et al., 2010).
6.2.3. Mechanical ventilation

*Preterm birth and the role of mechanical ventilation*

BPD is multifactorial in origin, with factors such as preterm birth, immature development of preterm lungs, infection (antenatal and/or postnatal), oxygen treatment and mechanical ventilation all playing a role (Bancalari, *et al.*, 2003).

Mechanical ventilation can lead to ventilator-induced lung injury (VILI), giving rise to pulmonary oedema, increases in endothelial and epithelial permeability, ultra-structural damage, increases in inflammatory markers and ultimately BPD (Chu, *et al.*, 2004). The individual components of VILI, referred to as barotrauma (damage to airway epithelium and alveoli caused by pressure generated by the ventilator), volutrauma (injury caused by overdistension of the lung by the volume used to inflate the lungs), atelectotrauma (damage caused by repeated opening and closing of the airway units), biotrauma (trauma caused by infection or inflammation) and oxytrauma (injury caused by free radicals produced as a consequence of treatment with supplemental oxygen), may be interrelated and may act synergistically (Donn, *et al.*, 2006).

A thus far relatively unexplored area of research is the effect of gas flow in the airways during mechanical ventilation. During mechanical ventilation a bias gas flow is maintained continuously in the ventilator circuit, resulting in positive pressure within the circuit, which is used to administer cyclical and forcible injection of gas to the lungs during inspiration.

Most neonatal ventilators use a set bias gas flow, either based on manufacturers’ guidelines (Bhutani, 2002) or set by the clinician. Despite recommendations about flows, experimental data to support different flows are lacking. This has not stopped some authors recommending that in preterm infants a bias gas flow of 6-10 L/min is generally sufficient, in term infants 12 L/min, whereas older infants need higher flows (Gerhardt, *et al.*, 2008).
Chapter 6

Preliminary studies

It is well established for the cardiovascular system that fluids flowing through a tube create shear stress on the surrounding walls (Resnick, et al., 2003), leading to various cellular responses (Resnick, et al., 2003; Kamiya, et al., 1980; Gimbrone, et al., 2000) and ultimately increasing the risk of atherosclerosis and thrombosis in the blood vessels (Araim, et al., 2001; Chappell, et al., 1998). Gas flowing through a tube behaves in a similar fashion to liquids (Cebral, et al., 2004) and ventilator bias gas flow may therefore also contribute to VILI, especially in the delicate, immature and therefore vulnerable preterm lung.

In a recent preterm lamb paradigm, we have demonstrated a relationship between bias gas flow and lung injury in the preterm lung (Bach, et al., 2009a). Preterm lambs (131 d gestation, normal gestation 148 d) were ventilated for 2 hours at a low, medium or high bias gas flows (8, 18 or 28 L/min). These flows were based on data from studies in term lambs, which demonstrated optimal ventilator efficiency index, a parameter that integrates ventilation with respiratory support in the absence of spontaneous ventilation, at a bias gas flow of 1.5-2 L/Kg.min (Bach, et al., 2009b). Preterm lambs at a gestational age of 131 d have a birth weight of 4.5-6 Kg; thus 8 L/min was chosen as the lowest bias gas flow for this study. The highest bias gas flow was the maximum reliable flow on the Dräger Babylog8000 plus, the most commonly used neonatal ventilator in Australia and New Zealand. A medium flow (18 L/min) was chosen between low and high flows.

After ventilation, the mRNA levels of three acute early response genes CTGF, CYR61 and EGR1 were all elevated in lung tissue and the highest levels were seen after ventilation at the higher bias gas flows (~8-11 fold increase compared to age-matched non-ventilated control lambs). These genes are likely early biomarkers of VILI in the newborn (Wallace, et al., 2009) and are also known to play a role in adult human lung injury, such as chronic obstructive pulmonary disease, pulmonary fibrosis and after lobectomy (Zhang, et al., 2000; Ning, et al., 2004; Pan, et al., 2001). In the preterm lamb, up-regulation of CTGF, CYR61 and EGR1 is seen within 15 minutes of onset of ventilation and levels are still
increased after 2 hours of ventilation. Furthermore, the level of gene expression is correlated to the severity of lung injury (Wallace, et al., 2009). We reported higher mRNA levels after ventilation at the medium flow rate, which can probably be explained by a turbulent gas flow pattern in the endotracheal tube with high bias gas flows (Jarreau, et al., 1999), paradoxically leading to a lower flow in the respiratory airways, and subsequently to lower levels of the early response genes.

In addition to up-regulation of these early response genes with higher bias gas flows, we have also reported histological evidence of lung injury (Bach, et al., 2009a). BPD is characterized by poor alveolarisation (Albertine, et al., 1999; Coalson, et al., 1999), abnormal elastin deposition (Coalson, et al., 1999; Albertine, et al., 1999; Pierce, et al., 1997), fibrosis (23, 25), an increase in relative tissue space in the lung, likely resulting from cellular proliferation (Coalson, et al., 1999; Allison, et al., 2008), and abnormal capillary growth (Coalson, et al., 1999). After only 2 hours of ventilation, histological analyses of the preterm lamb lung demonstrated increased cellular proliferation, an increase in relative tissue space and abnormal elastin, collagen and differentiated myofibroblast deposition in the lambs ventilated at the higher bias gas flows (Bach, et al., 2009a).

This study demonstrated for the first time that ventilator bias gas flow is a determinant of VILI in the preterm lamb lung. It is, therefore, likely that bias gas flow may also contribute to the lung injury in the preterm human lung and potentially to development of BPD.

Evaluation of VILI in the preterm baby

Lung injury and increased gene expression in lung tissue obviously cannot be assessed clinically in the preterm baby. Biomarkers on inflammation or oxidative stress are available to predict the risk of BPD and assist in the evaluation of ventilator-induced lung injury in the preterm baby. Pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and CXCL8 (previously known as IL-8) are well-recognised biomarkers for lung injury as are markers for oxidative stress, such as protein carbonyls, myeloperoxidase and 3- chlorotyrosine (Buss, et al., 2000;

The pro-inflammatory cytokines enhance the acute inflammatory response, which leads to increased vascular permeability, oedema and recruitment of inflammatory cells, such as neutrophils and macrophages. This initiates the chronic inflammatory response, which is characterised by infiltration with inflammatory cells, tissue repair, angiogenesis and fibrosis. Cytokines can be rapidly synthesised following hyperoxia or hypoxia, infection or mechanical ventilation (Speer, 2006a). In rats, TNF-α and MIP-2 (cytokine equivalent of CXCL8 in the rat) are elevated in TA and blood samples in response to ventilation (Chiumello, *et al.*, 1999; Tremblay, *et al.*, 2002). An increase was seen as early as 30 minutes after the onset of ventilation (Chiumello, *et al.*, 1999) and concentrations were dependent on the ventilatory strategy (Tremblay, *et al.*, 2002). The role of these cytokines in the recruitment of neutrophils and in the initiation of lung injury in preterm babies is supported by clinical studies. Preterm babies who develop BPD have higher numbers of neutrophils and macrophages in their TA compared with babies who recovered from RDS (Munshi, *et al.*, 1997; Kakkera, *et al.*, 2005; Groneck, *et al.*, 1994). Concurrent with the influx of these cells into the lung a decrease in circulating neutrophils is seen (Ferreira, *et al.*, 2000; Speer, 2006a). Furthermore, the influx of inflammatory cells is preceded by an elevation of pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and CXCL8 in TA (Munshi, *et al.*, 1997; Kakkera, *et al.*, 2005). All are demonstrated to be elevated in the first 5 days of life in babies who develop BPD compared with babies who recovered from RDS (Speer, 2006a; Lista, *et al.*, 2006; Huang, *et al.*, 2005; Kakkera, *et al.*, 2005; Munshi, *et al.*, 1997). CXCL8 is a potent chemo-attractant of neutrophils (Shimotake, *et al.*, 2004) and is probably the most important chemotactic factor in the lung (Speer, 2008). Concentrations of CXCL8 in TA are considerably higher in babies who develop BPD than in babies who do not develop BPD (Huang, *et al.*, 2005; Jonsson, *et al.*, 1997). Huang *et al* demonstrated that on day 1 there is already a 5 fold increase...
in CXCL8 concentrations (445.9 ± 79.9 versus 83.2 ± 32.0 pg/mL, p 0.002) in babies less than 33 weeks’ gestation who later develop BPD (Huang, et al., 2005), and in the first week of life the CXCL8 concentrations doubled in these babies (Huang, et al., 2005; Jonsson, et al., 1997). Ventilatory strategy can also impact on the concentration of CXCL8 in TA as was demonstrated by Lista et al. They ventilated preterm babies with a tidal volume of 3 or 5 mL/Kg, with the former resulting in longer duration of ventilation, most likely based on atelectotrauma. The CXCL8 concentrations in these babies increased 8 fold in the first 7 days, whereas the group ventilated with the higher tidal volume had lower CXCL8 concentrations on day 7 than on day 1 (Lista, et al., 2006). Huang et al used the CXCL8 concentrations from day 1, with a cut-off concentration of 110 pg/mL, to calculate sensitivity (83%) and specificity (73%) for prediction of later BPD development (Huang, et al., 2005). Up-regulation of cytokines is not only seen in preterm infants; in adults with acute respiratory distress syndrome, concentrations of IL-1β and IL-6 in TA and concentrations of IL-6 and TNF-α in blood samples were higher after potentially more injurious ventilation strategies compared with lung protective strategies (Ranieri, et al., 1999).

Inflammation and hyperoxia can lead to production of oxygen free radicals, which play a role in the development of VILI in the preterm lung (Speer, 2006a). Preterm babies are more vulnerable to damage caused by oxygen free radicals, as they have an immature anti-oxidant defence system (Saugstad, 2003). Neutrophils, attracted to the lung upon injury, generate oxygen radicals and release myeloperoxidase, which in turn catalyses a reaction that produces hypochlorous acid (Buss, et al., 2003; Speer, 2006a). This acid can react with tyrosil residues in proteins to produce 3-chlorotyrosine, a minor reaction, but nevertheless the only physiological source of 3-chlorotyrosine (Buss, et al., 2003; Kettle, 1996). Thus, 3-chlorotyrosine can be used as a marker of oxytrauma and/or biotrauma caused by neutrophils attracted to injured (lung) tissue.

Increased concentrations of 3-chlorotyrosine have been demonstrated in preterm babies compared with larger babies without respiratory disease (83 compared with 13 μmol/mol tyrosine) and in preterm babies who later develop
BPD compared with those who do not (88 compared with 49 μmol/mol tyrosine) (Buss, et al., 2003).

Other potentially promising biomarkers for injury in the preterm human lung are CTGF and CYR61. These genes were demonstrated to be quantitative markers of ventilator-induced lung injury in preterm lambs in the first two hours after the onset of ventilation (Wallace, et al., 2009). CTGF and CYR61 are secreted proteins and their mRNA levels may therefore be measurable in leucocytes. This is a novel technique, which is currently in development by our collaborators at Monash University in Melbourne. Preliminary results from ventilated preterm lambs confirmed the feasibility of measurements of these early response genes in blood samples. Techniques to measure the genes in much smaller blood volumes are being developed in collaboration with Royal Women’s Hospital in Melbourne. Measurements of these genes in blood might result in much earlier detection of ventilator-induced lung injury and thus give more time to adjust ventilator settings, which could potentially reduce lung injury in the vulnerable preterm lung.

6.2.4. Proposed study

There is no evidence to support default setting of the ventilator bias gas flow and there is currently clinical equipoise regarding which flow is used: clinicians use various flows ranging from 4 to 10 L/min, based purely on clinician preference. Animal studies suggest that high bias gas flows are more injurious to the preterm lamb lung, resulting in higher levels of early response genes and histological changes consistent with the changes seen in BPD.

We propose to evaluate whether a bias gas flow of 4 L/min, compared with a bias gas flow of 10 L/min, reduces concentrations in tracheal aspirates of cytokines and markers of oxidative stress known to be predictive for BPD. These flows were chosen as 4 L/min is the lowest flow currently being used in Auckland City Hospital and 10 L/min is the standard bias gas flow to ventilate a neonate in our neonatal intensive care unit (NICU). The preferred ventilator is the Babylog 8000plus; however, any ventilator using a continuous bias gas flow, which can be adjusted to 4 or 10 L/min, is acceptable. If available, we will use
volume guarantee to prevent bias from volutrauma. Other modes of ventilation and ventilator settings are upon discretion of the neonatologist.

Primary outcomes will be CXCL8 concentrations in tracheal aspirates (TA) and number of ventilated days. CXCL8 is used as biomarker of lung injury and has the capacity to predict the risk of BPD. The number of ventilated days is an accepted parameter to measure the need for mechanical ventilation and indirectly the severity of lung injury. Secondary clinical outcomes will be BPD, defined as ongoing respiratory support and/or supplemental oxygen at 36 weeks’ postmenstrual age, duration and mode of respiratory support, average value for mean airway pressure (MAP) while ventilated, average value for FiO\textsubscript{2} and a composite outcome of neonatal death and serious neonatal morbidity. This will include pulmonary interstitial emphysema (PIE), pulmonary haemorrhage, intraventricular haemorrhage (IVH), periventricular leucomalacia (PVL), retinopathy of prematurity (ROP), necrotising enterocolitis (NEC) or persistent ductus arteriosus (PDA). Furthermore, concentrations of IL-1\textbeta, IL-6, TNF-\alpha and 3-chlorotyrosine will be measured in TA and IL-1\textbeta, IL-6, CXCL8 and TNF-\alpha in blood; blood samples will also be stored for later analysis of mRNA levels of early response genes. Data on hospitalisation, wheezing and asthma will be collected from the medical records of follow-up clinics in the first two years of life.

If feasible, the data generated from this study will be used to develop a larger multicentre study powered to detect a difference of BPD.

6.3. Study design

6.3.1. Participants

Inclusion criteria

We will include babies born at less than 28 weeks’ gestation and/or with a birth weight less than 1,000 g, who require mechanical ventilation in the first week of life. Clinicians at each of the participating centres will decide on whether mechanical ventilation is required.
We will determine gestational age from the last menstrual period, from an early pregnancy ultrasonogram or the best possible estimate in the absence of either of these.

**Exclusion criteria**

We will exclude babies diagnosed with congenital cardio-respiratory anomalies and known chromosomal or genetic abnormalities.

**6.3.2. Ethical consent**

We propose to apply for a waiver of consent for 24 hours on the following grounds:

- Currently, there is equipoise with respect to the ventilator bias gas flow that is used. There is no consensus on which bias gas flow is optimum. Most NICUs have a default setting; however, clinicians are free to alter the flow according to personal preference, although there is no evidence in preterm babies to support one flow over another. The purpose of this study is to provide evidence for an informed decision about the most effective gas flow so that practice can be evidence-based.

- Animal experiments demonstrate that lung injury is initiated very rapidly after the onset of mechanical ventilation. A systemic inflammatory response was observed within 5 minutes after the onset of resuscitation of preterm lambs (Jaarsma, et al., 2004) and within 30 minutes increased levels of pro-inflammatory cytokines and early response genes were measured in lung tissue (Wallace, et al., 2009). We have demonstrated that lung injury, and gene expression levels of genes known to be involved in acute lung injury, differ with different bias gas flows after only 2 hours of mechanical ventilation. Therefore, even a brief period of ventilation at higher bias gas flows in babies later randomized to the low bias gas flow group may bias the results. We, therefore, believe that the allocated flow should be applied from the commencement of any respiratory support, including those necessary for resuscitation at the time of delivery.
Although antenatal consent will be sought from all women who are in hospital because of the risk of preterm birth, a significant proportion of women present acutely with either preterm labour or a serious antenatal complication. Obtaining consent urgently in situations where the mother is about to deliver, may be in considerable labour-related pain or sedated, is difficult and the ethical validity of the consent given has been questioned (Vain, et al., 2004). The validity of informed consent to neonatal randomised controlled trials was evaluated as part of a study performed in 9 European countries, the Euricon study; validity was based on 4 components (competence, information, understanding and voluntariness). In 70% validity of given consent was compromised on at least one of the components and impaired consent was 2 to 3 fold more likely for research on emergency treatment compared with non-urgent standard treatment (Mason, et al., 2000).

However, babies born preterm as a result of sudden preterm labour or antenatal complications are those most likely not to have the benefit of a completed course of antenatal corticosteroids and are, therefore, most likely to be at risk of severe RDS and thus BPD. These babies might be expected to benefit most from an intervention that may reduce the risk of BPD.

The Continuous positive airway pressure (CPAP) Or INtubation trial (COIN-trial) illustrates both the difficulty of recruiting eligible babies when antenatal consent is obligatory and the higher percentage of antenatal corticosteroid treatment in the babies taking part in the trial compared with the ANZNN-data on corticosteroid treatment. Of the 2,165 babies who underwent assessment for the COIN-trial, only 28% were randomised. Consent was refused or babies were not eligible in only 25% of the 2,165 babies; thus, over 1,000 babies who were not enrolled might have been able to participate in the trial (Morley, et al., 2008). Furthermore, 94% of the babies enrolled in the COIN-trail were exposed to antenatal corticosteroids, whereas 88% of the babies born at less than 32 weeks’ gestation in 2008 in National Women’s Hospital (NWH)
received any and only 51% received a course starting between a day and
7 days before delivery (Battin, 2008), demonstrating the bias towards
babies exposed to antenatal steroids in this trial.

• Waivers of consent have been granted previously, both in New Zealand
and overseas. In neonatal populations, two large multicentre studies
examining the need for intrapartum suction to prevent meconium
aspiration syndrome (Vain, et al., 2004; Wiswell, et al., 2000) operated
under a waiver of consent. In adult populations, the very recently
published NICE trial (ClinicalTrials.gov number, NCT00220987) also
operated under a delayed consent. Auckland City Hospital contributed a
significant number of patients to this study (The NICE-SUGAR Study
Investigators, 2009).

• The requested waiver of consent fulfils the American requirements for a
waiver of informed consent (Code of Federal Regulations, Department of
Health and Human Services for the protection of human research
subjects, USA, 45 CFR 46.116(d) (Code of Federal Regulations).

• The waiver of consent for 24 hours is requested in order to best obtain a
valid consent based on all 4 components (competence, information,
understanding and voluntariness) (Mason, et al., 2000; Tyson, et al.,
2000).

Approval for the study protocol will be sought by the Multi-region Ethics
Committee of the New Zealand Health and Disability Ethics Committees.

If possible, parents will be given written information about the study before
delivery, which will be reviewed with them by a member of the study team. If
delivery occurred without time for this to happen, information will be handed to
the parents after delivery and reviewed with them by a member of the study
team. We will seek a waiver of consent for 24 hours from the time of intubation
and ventilation, but before blood or tracheal aspirate is collected, to enable
written informed consent to be obtained from the parents. Parents will be able to
withdraw their baby from the study at any time.
6.3.3. Randomization and blinding

Once the need for ventilation has been established, babies will be randomly assigned to one of the two treatment groups. Group assignments will be drawn from consecutively numbered, sealed, opaque envelopes stratified by centre. Envelopes will be prepared by an independent research nurse at the data collection centre. Each envelope will be opened by the doctor or nurse once intubation is imminent at delivery or in the first week of life. This person will adjust the flow setting of a Neopuff and/or a Babylog 8000plus to the assigned flow. The allocated flow will be used as soon as the baby is intubated and intermittent positive pressure ventilation is given, either using the Neopuff or the ventilator.

After randomisation a baby will stay in the same treatment group; the allocated flow will be used each time the baby needs to be ventilated.

6.3.4. Primary outcomes

Primary outcomes will be CXCL8 concentrations in tracheal aspirate (TA) after 24 hours of ventilation and the number of ventilated days.

TA will be done after 24, 72 and 120 hours, if the baby is still mechanically ventilated.

6.3.5. Secondary outcomes

Clinical outcomes:

- BPD (defined as continued respiratory support and/or supplemental oxygen at 36 weeks’ postmenstrual age)

- Duration of respiratory support (days) on
  - Mechanical ventilation
    - Conventional
    - Rescue HFO
Chapter 6

- CPAP
  - Low or high flow oxygen

- Average value for mean airway pressure (MAP) in cmH₂O, measured daily while ventilated

- Average value for FiO₂ (%)

- Composite outcome of neonatal death or neonatal morbidity, including pulmonary interstitial emphysema (PIE), pulmonary haemorrhage, intraventricular haemorrhage (IVH), periventricular leucomalacia (PVL), retinopathy of prematurity (ROP), necrotising enterocolitis (NEC) or persistent ductus arteriosus (PDA).

- Hospitalisation and/or episodes of wheezing and/or asthma as reported at follow-up clinics in the first two years of life

Sample analysis

1. Concentrations of CXCL8, IL-1β, IL-6 and TNF-α in TA and blood and of 3-chlorotyrosine in TA 24, 72 and 120 hours after the onset of ventilation. TA will only be performed if the baby is still mechanically ventilated.

2. mRNA levels of acute early response genes (CTGF, CYR61 and EGR1), expressed as a fold change of levels on day 1 in term, non-ventilated control babies, in blood 24, 72 and 120 hours after the onset of ventilation.

Definitions

- Pulmonary interstitial emphysema, based on radiographic changes as reported by radiologist

- Pulmonary haemorrhage: Blood or pink coloured secretions visible in the endotracheal tube and/or visible in the suction catheter, occurring at a time of respiratory deterioration (low saturation, increased supplemental oxygen)
• Death: Death of a neonate after randomisation and before 36 weeks’ corrected gestational age.

• Intraventricular haemorrhage, defined according to the grading system of Papile (Papile, et al., 1978). Cranial ultrasound is recommended towards the end of the first week of life, at 4-5 weeks and before discharge or around 36 weeks' corrected age (whichever occurs first).

• Periventricular leucomalacia, based on changes on cranial ultrasound as reported by radiologist.

• Retinopathy of prematurity, defined according to the international classification of ROP (International Committee for the Classification of Retinopathy of Prematurity, 2005). ROP requiring treatment before discharge will be recorded.

• Necrotising enterocolitis, diagnosed during surgery or autopsy, or clinical symptoms combined with occurrence of pneumatosis intestinalis, hepatobiliary gas or free peritoneal air as reported by radiologist (Walsh, et al., 1986).

• Persistent ductus arteriosus, based on an echocardiogram with a ductal lumen >1.5 mm and a reversed descending aortic diastolic flow. Echocardiogram is recommended on day 3 of life and/or on clinical suspicion of a PDA. PDAs requiring treatment will be recorded.

6.3.6. Data management and analyses

Statistical analyses

Statistical analyses will be on an intention to treat (ITT) basis i.e. patients will be analysed according to the group they were randomised into regardless of whether treatment was given according to protocol. Twins or triplets will be randomised as separate babies, with the non-independence of these pairs taken into account during analysis. Continuous outcome data will be analyzed using a Student t-test or repeated measures ANOVA. Data not normally distributed will
be transformed to near-normality before statistical analysis. The non-parametric Mann-Whitney U test will be used, if near-normality is not achieved. Continuous outcome data will be presented as relative reduction with 95% Confidence Interval (CI). Categorical outcome data will be analyzed using Chi-Square Test and will be presented as relative risk with 95% CI. Calculation of the number needed to treat to benefit (NNTB) or number needed to treat to harm will be performed when appropriate. A p value less than 0.05 will be considered to indicate statistical significance for the primary outcome; a more stringent p value of less than 0.01 will be considered to indicate statistical significance for the secondary outcomes to prevent type I errors. All analyses will be two-sided.

<table>
<thead>
<tr>
<th>Type of measure</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous</td>
<td>Concentrations of biomarkers mRNA levels of acute early response genes Duration of ventilation, respiratory support or FiO₂ Average MAP during ventilation Average FiO₂</td>
</tr>
<tr>
<td>Categorical</td>
<td>BPD Death PIE / IVH / PVL / ROP / NEC / PDA Hospitalisation / wheezing / asthma</td>
</tr>
</tbody>
</table>

Table 6.1: Characteristics of collected outcome data

Sample size calculation

The sample size calculation is based on a reduction in CXCL8 concentrations in TA of preterm babies ventilated with a bias gas flow of 4 L/min compared with babies ventilated with a bias gas flow of 10 L/min.

The expected concentration for CXCL8 in tracheal aspirate is 5,806 ± 4,923 pg/ml, which is derived from a study performed by Shimotake et al in preterm babies at day 5 of life (5.7 ± 1.1; mean ± SEM) (Shimotake, et al., 2004). CXCL8 concentrations were approximately 5 times higher in babies who developed BPD compared with babies who recovered from RDS (Huang, et al., 2005; Jonsson, et al., 1997) and higher concentrations were measured in babies who needed
longer duration of mechanical ventilation (Huang, et al., 2005; Jonsson, et al., 1997; Lista, et al., 2006).

The primary outcome is a relative reduction of 35% in CXCL8 concentrations in TA in babies ventilated with a flow of 4 L/min compared with babies ventilated with a flow of 10 L/min. A sample size of 128 babies (64 babies in each arm) is needed to detect a 35% reduction in the CXCL8 concentrations in TA with 80% power (β=0.2) and a two-sided significance level of 5% (α=0.05).

The preferred outcome would be a reduction in BPD at lower ventilator gas flows. To test this hypothesis a large number of patients would be needed. This pilot study will be used to generate data regarding the feasibility of recruiting preterm babies to ventilation at two different flows and to collect data on concentrations of inflammatory markers. If feasible, a larger multicentre study powered to detect a difference in BPD will be planned.

6.3.7. Presentation of results

The results will be presented in 5 tables i.e. baseline characteristics of mothers (Table 6.2) and enrolled babies (Table 6.3) at trial entry, concentrations of biomarkers in tracheal aspirates (Table 6.4), concentrations of biomarkers and mRNA levels of early response genes in blood (Table 6.5) and clinical outcome data (Table 6.6). Data will be presented as mean ± SD or median ± interquartile range, depending on normal or non-normal distribution or as n (%) for categorical data.
### Table 6.2: Baseline characteristics of mothers at trial entry

<table>
<thead>
<tr>
<th></th>
<th>Intervention (4 L/min) N=</th>
<th>Comparison (10 L/min) N=</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (years)</td>
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<tr>
<td>Ethnicity</td>
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<tr>
<td>Multiple pregnancy (twin or triplet)</td>
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<tr>
<td>Caesarean section</td>
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<td>Preterm labour</td>
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<tr>
<td>Preterm rupture of membranes (d)</td>
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<tr>
<td>Chorioamnionitis</td>
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<tr>
<td>Antenatal steroids:</td>
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<tr>
<td>&lt; 24 h before delivery</td>
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<tr>
<td>1-7 days before delivery</td>
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<tr>
<td>&gt;7 days before delivery</td>
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<tr>
<td>Multiple course</td>
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</table>

N: total number, n: number. Data: n/N (%)

### Table 6.3: Baseline characteristics of enrolled babies at trial entry

<table>
<thead>
<tr>
<th></th>
<th>Intervention (4 L/min) N=</th>
<th>Comparison (10 L/min) N=</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestation (weeks)</td>
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<tr>
<td>Age at entry (d)</td>
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<tr>
<td>Birth weight (g)</td>
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<tr>
<td>Male sex</td>
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<tr>
<td>Resp. support before entry:</td>
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<tr>
<td>None</td>
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<tr>
<td>CPAP</td>
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</tbody>
</table>

N: total number, n: number. Data: n/N (%)

### Table 6.4: Concentrations of biomarkers in tracheal aspirate (TA) taken at 24, 72 and 120 hours after the onset of ventilation

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>24 hours</th>
<th>72 hours</th>
<th>120 hours</th>
<th>24 hours</th>
<th>72 hours</th>
<th>120 hours</th>
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<tbody>
<tr>
<td>CXCL8</td>
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<tr>
<td>IL-1β</td>
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<td>IL-6</td>
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<td>TNF-α</td>
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<tr>
<td>3-chlorotyrosine</td>
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<tr>
<td>Biomarker</td>
<td>Intervention (4 L/min); N=</td>
<td>Comparison (10 L/min); N=</td>
<td>RR (95% CI)</td>
<td>P value</td>
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<td>24 hours</td>
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<td>CXCL8</td>
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<td>IL-1β</td>
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<td>IL-6</td>
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<td>TNF-α</td>
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<tr>
<td>CTGF</td>
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<tr>
<td>CYR61</td>
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<tr>
<td>EGR1</td>
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<td></td>
<td>24 hours</td>
<td>72 hours</td>
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</table>

Table 6.5: Concentrations of biomarkers and mRNA levels of early response genes, expressed as fold change of levels in term, non-ventilated control babies, in blood taken at 24, 72 and 120 hours after the onset of ventilation.

Table 6.6: Clinical outcome data
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Interim analysis

An independent Data Safety Monitoring Committee (DSMC) will be established to review the data every six months. They will be asked to review all cases of mortality and serious respiratory morbidity (pulmonary haemorrhage, pneumothorax, requirement for rescue ventilation with high frequency oscillatory ventilator) and determine whether these may be related to the study. If any Serious Adverse Events are thought to be due to the study intervention, the DSMC may recommend termination.

6.3.8. Recruitment strategy

Patients will be recruited from January 2011 to January 2014. We will need to recruit a total of 128 patients in order to provide 80% power to detect a reduction of 35% in CXCL8 protein concentrations in tracheal aspirate.

At National Women’s Hospital (NWH) 120 babies were inborn before 28 weeks’ gestation in 2008; however, only 58 of these infants were admitted to Newborn Services. Furthermore, 21 infants not born in NWH were admitted to Newborn Services of NWH. About half of these babies needed ventilation (Battin, 2008). Since this absolute number is small, we have chosen to approach other hospitals to collaborate in this pilot study.

The recruitment period of three years is based on the assumption that 80% of eligible patients will consent to participate to the study in order to reach a sample size of 128 babies.

The collaborating hospitals have to be able to provide the appropriate care for small babies born at less than 28 weeks’ gestation (level 3 care) at their neonatal intensive care unit. Study procedures will be conducted according to this common protocol at the collaborating hospitals.
Chapter 7. Conclusions

This thesis reports the novel finding that the rate of ventilator bias gas flow affects ventilator-induced lung injury in the immature lung. High bias gas flows increase peak inspiratory pressures, alter airway pressure waveforms, increase the velocity of gas flow in and out of the lung, increase mRNA levels of acute early response genes and also result in histological evidence of lung injury. The histological changes observed following ventilation at high bias gas flows, with altered deposition of elastin and collagen, reduced number or altered shape of septal crests, increased saccular wall thickness and non-uniform saccular size, are consistent with changes seen in VILI (Coalson, et al., 1999; Albertine, et al., 1999; Pierce, et al., 1997) and BPD (Husain, et al., 1998; Thibeault, et al., 2000; Thibeault, et al., 2003). The findings occurred after only 2 hours of ventilation; it remains to be determined whether adverse effects would increase with a longer duration of ventilation.

The experimental studies described in this thesis investigated the hypothesis that bias gas flow contributes to VILI in the immature lung. This hypothesis arose from the appreciation that vascular injury is related to blood flow and, given that gas is fluid, bias gas flow may, therefore, be related to injury in the lung. Other mechanisms contributing to VILI, which often leads to the development of BPD in preterm human infants, are known to include prematurity, infection, oxygen treatment and mechanical ventilation (Bancalari, et al., 2003). However, despite refinement of ventilatory techniques and neonatal care to minimise the effect of these factors on VILI, rates of BPD have not fallen (Donn, et al., 2003). On the other hand, certain advances in ventilatory techniques, such as controlling the tidal volume and synchronising the breaths, have been shown to lead to a shorter duration of ventilation (Singh, et al., 2006; McCallion, et al., 2005; Greenough, et al., 2008), a trend towards a reduction in BPD at 36 weeks' postmenstrual age (McCallion, et al., 2005) and a favourable outcome at follow-up at 2 years of age (Singh, et al., 2009). Therefore, ongoing research into improving ventilatory techniques used in the care of preterm babies is valuable.
and may lead to decreased morbidity and thus to a significant decrease in the health burden these vulnerable infants experience and also place on society.

Well-established animal paradigms in sheep and primates reproduce BPD-like changes in lung morphology following mechanical ventilation for prolonged periods (days to weeks) in settings similar to that provided to preterm infants in a neonatal intensive care unit (Coalson, et al., 1999; Albertine, et al., 1999). For the animals to survive the long-term experiments, PaCO₂ levels had to be maintained within acceptable levels by altering the rate of ventilation or the PIP, supplemental oxygen was given to all animals and some animals developed infections, for which antibiotics were given. Therefore, although these paradigms have increased our understanding of the pathogenesis of BPD, they are unable to unravel the independent factors leading to alterations in lung morphology. Short-term studies allow evaluation of the effects of a single variable or the immediate effects of ventilation on the outcome of interest, such as the inflammatory response. For example, ventilation of both rats and immature rabbits demonstrated that volutrauma is more important than barotrauma by applying high and low airway pressures to animals with restricted thoraco-abdominal expansion (Dreyfuss, et al., 1992; Dreyfuss, et al., 1988; Hernandez, et al., 1989). In preterm lambs, even only a few large inflation breaths during resuscitation resulted in lung injury (Bjorklund, et al., 1997). Furthermore, short-term ventilation with high (supraphysiological) tidal volumes up-regulates pro-inflammatory cytokines in rats (Tremblay, et al., 2002; Chiumello, et al., 1999), mice (von Bethmann, et al., 1998) and preterm lambs (Wallace, et al., 2009) and up-regulates mRNA levels of early response genes in preterm lambs (Wallace, et al., 2009). Some of these pro-inflammatory cytokines, such as TNF-α, IL-1β, IL-6 and CXCL8, can be used in the clinical setting as biomarkers for lung injury, as can markers of oxidative stress, such as protein carbonyls, myeloperoxidase and 3-chlorotyrosine (Buss, et al., 2000; Buss, et al., 2003). These cytokines and oxidative stress markers can be measured in blood and tracheal aspirates of preterm babies and their concentrations in tracheal secretions are associated with risk of later development of BPD (Munshi, et al.,
In addition to experiments in preterm animals after delivery, *in-utero* experiments have been developed in fetal sheep (Allison, *et al.*, 2008). This paradigm has further potential to reduce confounding factors, such as the need for altering ventilator settings to correct PaCO₂ or the need for supplemental oxygen to maintain acceptable values for saturation and survival of the animal. Furthermore, *in-utero* experiments allow examination of animals of a younger gestational age and, therefore, a more immature lung than would be otherwise viable for *ex-utero* experiments.

Three short-term experiments were undertaken for this thesis to address the hypothesis that bias gas flow affects lung injury in the immature lung. Firstly, a study in term lambs was performed to investigate the effect of different bias gas flows on ventilatory parameters. Secondly, a study in preterm neonatal lambs investigated the effect of three different levels of bias gas flow on lung injury, assessed histologically and through mRNA levels of acute early response genes. The last experimental study addressed the effect of bias gas flow at an earlier stage of gestation when the lungs are in the canalicular stage of development, and also investigated whether different modes of ventilation modified the effect of bias gas flow. The lambs in this experiment were studied whilst still connected to the placenta, enabling very immature lambs to be studied and avoiding the confounding factors associated with ventilation of extremely immature lungs.

Volutrauma is a more important determinant of VILI than barotrauma (Hernandez, *et al.*, 1989; Dreyfuss, *et al.*, 1992; Dreyfuss, *et al.*, 1988). Therefore, studies in this thesis aimed to achieve a consistent tidal volume amongst different experimental groups using the volume guarantee ventilation mode. Tidal volume measurements demonstrated that this approach was successful in preterm, neonatal and in fetal lambs. However, lambs ventilated at higher bias gas flows in the flow-cycled mode required significantly higher peak inspiratory pressures to achieve similar tidal volumes. Preterm neonatal lambs ventilated at high flows tended to develop pneumothoraces more often, a characteristic
described for barotrauma (Moylan, *et al*., 1978). The fact that only one pneumothorax was seen following ventilation of fetal lambs was unexpected. These fetal lambs were exposed to a prolonged inflation breath at the onset of ventilation, a procedure not performed in the preterm lambs in chapter 4. This change followed the appreciation that several ventilator breaths were required to achieve the set tidal volume in SIPPV mode, whereas this volume could not be achieved at all in the PSV mode in the animals used to establish the experiment. Following the implementation of a prolonged inflation, set tidal volume could be achieved in all fetal lambs. Prolonging the inflation will have helped the fluid filled lungs to establish an FRC (te Pas, *et al*., 2009). Fluid filled lungs inevitably have a longer time constant (Milner, 2001) than air filled lungs, based on the higher resistance of moving liquid compared with air through the airways. Observations in spontaneously breathing preterm babies in the first minutes of life demonstrated that nearly 80% of the breaths had a prolonged expiration, mainly characterised by an expiratory breath hold (te Pas, *et al*., 2008b). This pattern was also observed in the first breaths in term babies and is recognised as a mechanism to maintain elevated intra-pulmonary pressure, possibly leading to enhanced fluid clearance from the lung, distribution of gas within the lung and splinting of the alveoli and airways (te Pas, *et al*., 2008b; Mortola, *et al*., 1982). Studies in preterm rabbits demonstrated that an inflation time of 20 s, compared with 1, 5 and 10 s, was the optimal duration for achieving full recruitment of FRC and uniform inflation of the lung at 20 s, without leading to overdistension, as evaluated by phase contrast x-ray imaging (te Pas, *et al*., 2009). Therefore, the prolonged inflation likely led to a more uniform inflation of the lung in the fetal lambs at the start of ventilation, thereby likely reducing atelectotrauma and making them less vulnerable to pneumothoraces during the 2-hour study.

A more uniform inflation can also be achieved by the application of PEEP, which is part of ‘lung protective ventilation’ (Cools, *et al*., 2009). The animal studies described in this thesis all used ‘lung protective ventilation’ with physiological tidal volumes, application of PEEP and acceptance of mild hypercapnia. In the acutely injured lung an unequal distribution of ventilation is normally seen (Attar, *et al*., 2002), which leads to counteracting forces in an attempt to open the
collapsed areas of the lung (Mead, et al., 1970). Application of PEEP is thought to reduce the tidal collapse and reopening, or atelectotrauma, in the injured non-uniform lung (Gattinoni, et al., 1987; Falke, et al., 1972), likely resulting in less endothelial damage and microvascular permeability (Muscedere, et al., 1994; Dreyfuss, et al., 1998). Furthermore, ventilator rates were increased only for PaCO₂ levels above a pre-established threshold in the preterm lambs, thereby accepting mild hypercapnia, since this led to a trend towards reduction of BPD and shorter duration of mechanical ventilation in neonates (Mariani, et al., 1999; Thome, et al., 2002). Despite 'lung protective ventilation', the experiments performed in this thesis clearly demonstrate that different levels of bias gas flow affect ventilator dynamics and early markers of lung injury differently after only 2 hours of ventilation. Therefore, it can be concluded that bias gas flow is an important determinant of VILI in the immature lung.

Thus far, it has only been suggested that inappropriate levels of bias gas flow during ventilation may be one of the determinants leading to VILI, a mechanism described as rheotrauma (Donn, et al., 2006). This thesis demonstrates, for the first time, that levels of bias gas flow affect VILI in the immature lung. Shear stress is the term used for the frictional forces of liquid or gas flowing through a tube (Resnick, et al., 2003; Tarran, et al., 2005). The responses to shear stress, both from fluid dynamics and cellular point of view, are well described for the cardiovascular system (Resnick, et al., 2003; Fisher, et al., 2001; Lu, et al., 2002; Chen, et al., 2004). The level of shear stress in the cardiovascular system is affected not only by the characteristics, but also the rate of blood flow (Ali, et al., 2002). Much less is known about the effect of shear stress in the respiratory system, although gas behaves like a liquid, albeit with lower frictional forces (Cebral, et al., 2004). An increase in bias gas flow increases the rate of pressure rise in the ventilator circuit, which leads to a higher pressure gradient for gas to enter the lung during the dynamic inflation phase. This results in higher gas flows into the lung, which potentially can create shear stress on the respiratory epithelial cells, possibly leading to lung injury, or rheotrauma. If similar mechanisms occur in the respiratory system as in the cardiovascular system then the rate of gas flow may determine the level of shear stress. The data
presented in this thesis demonstrating increased lung injury with higher flows suggest that the mechanism may be shear stress injury. However, shear stress was not directly measured in these experiments; future work could involve measurement of shear stress at the various levels of the branching airways.

Traditionally, shear stress in the airways is described as being roughly equal in all generations of the airways, based on calculations using the Horsfield-model (Horsfield, et al., 1968). However, this calculation assumes that air flow has a laminar pattern, that flow dynamics do not change during breathing and that downstream compliance is not heterogeneous, whereas dynamic changes in both flow patterns and shear stress were calculated in adults with known narrowing of the airways using visualisation with CT (Cebral, et al., 2004). At a cellular level, it is known that the alveolar epithelium is responsive to mechanical forces and it has been suggested that these forces can lead to a rearrangement of the cytoskeleton in order to adapt to these mechanical forces (dos Santos, et al., 2006). The altered deposition of collagen and elastin and the reabsorption of septal crests in animal paradigms of VILI and infants who died of BPD might be a reflection of this (Allison, et al., 2008; Coalson, et al., 1999; Albertine, et al., 1999; Pierce, et al., 1997; Husain, et al., 1998; Thibeault, et al., 2000; Thibeault, et al., 2003).

Although this thesis has demonstrated that bias gas flow affects lung injury at an alveolar level, the injurious effect of high bias gas flow may also affect the airways. Branching points or areas of complex flow are vulnerable to shear stress related injury in the cardiovascular system (Ali, et al., 2002; Lu, et al., 2002) and it is likely that similar injurious mechanisms can be expected for gas flow through the branching airways. Computer models support this hypothesis; these models indicate that the effect of shear stress is greatest at the level of the terminal bronchioles during non-uniform inflation, as opposed to the trachea or at respiratory bronchiole and alveolar level (Gillis, et al., 1999; Nucci, et al., 2003). Asthma is a disease of the smaller, conducting bronchioles and bronchial hyper-responsiveness and reduced airflow on respiratory function monitoring are a reflection of disease of these airways (Sly, 2000). BPD survivors generally are reported to have higher rates of asthma, bronchial hyper-responsiveness
(Doyle, et al., 2008) or wheezing (Fawke, et al., 2010) and to have reduced airflow on respiratory function tests at long-term follow-up (Doyle, et al., 2008; Fawke, et al., 2010). The effect of bias gas flow on lung injury, both at alveolar level and the airways, is, therefore, an important area of research to pursue. Reducing bias gas flow has the potential to reduce injury in different areas of the lung, which could reduce not only neonatal, but also long-term, respiratory morbidity of many preterm infants. Respiratory disease is the most common complication following preterm birth, with RDS and BPD being the two most important respiratory complications (Bancalari, et al., 2003). Babies developing BPD require a higher level of care, frequently have a longer duration of hospitalisation and require more frequent re-admission to hospital in the first few years of life than babies without BPD (Greenough, et al., 2002). Therefore, respiratory morbidity in the preterm infant places a huge burden on the cost of health care. Reducing lung injury through a simple intervention such as reducing the bias gas flow would incur no additional cost and potentially may lead to improved long-term outcomes and decreased costs for preterm babies. Further research needs to investigate the long-term outcomes of reduced bias gas flows during ventilation of the smallest, and therefore most vulnerable, preterm babies.

Recognition of the role bias gas flow plays on ventilator dynamics and VILI is important, since the velocity of the bias gas flow is adjustable on most continuous flow ventilators. A minimum circulating bias gas flow in the circuit is necessary to maintain PEEP; additional bias gas flow is necessary to generate a pressure rise in the circuit. The rate of pressure rise is related to the bias gas flow, as shown in chapter 5. Higher levels of bias gas flow resulted in higher inspiratory flows and, therefore, a steeper rise of the airway pressure wave. Furthermore, a higher bias flow in the flow-cycled pressure support ventilation (PSV) mode led to shorter inspiratory times, higher PIP values and a steeper sawtooth pressure wave. Traditionally, time-cycled pressure-limited ventilation (TCPLV) is used for neonatal ventilation (Claure, et al., 2007). During TCPLV a square pressure wave is often desired by the clinician, since this has been associated with increased oxygenation (Herman, et al., 1973; Reynolds, 1971).
Two components are important during creation of the airway pressure wave. Firstly, the rate of rise of pressure is related to the bias gas flow and the lung mechanics, which together determine the velocity of the inspiratory flow (Gerhardt, et al., 2008). The inspiratory flow determines the time it takes to reach PIP. Secondly, during TCPLV the duration of the plateau phase depends on the inspiratory time set by the clinician. Both a high inspiratory flow and a long inspiratory time, or an inspiratory plateau phase, have been related to an increased risk of pneumothorax, since they can trigger an active expiratory reflex (Greenough, 1988; Kamlin, et al., 2003; Gerhardt, et al., 2008).

All our animals were sedated and, therefore, did not have spontaneous respiration or an active expiratory reflex. Despite this, many preterm lambs developed a pneumothorax, with a trend towards more pneumothoraces following ventilation at 18 L/min. PIP and MAP values were not significantly different between groups ventilated at 18 or 28 L/min, thus barotrauma is less likely responsible for this effect. Furthermore, these animals did not have a long inspiratory time. The greatest rate of rise for inspiratory flow was seen during ventilation with 18 L/min, and not 28 L/min, the latter possibly explained by a turbulent flow pattern in the endotracheal tube at a bias gas flow of 28 L/min (Jarreau, et al., 1999). Therefore, the role of bias gas flow and the speed at which the immature lung inflates during mechanical ventilation on development of lung injury has been confirmed.

Both the up-regulation of early response genes and histological changes in the extracellular matrix seem to support the more damaging effect of high bias gas flows, leading to the highest inspiratory flows, during flow-cycled ventilation. The early response gene *EGR1* is a growth hormone, which has been demonstrated to up-regulate rapidly after acute tissue injury, hypoxia or pneumonectomy (Zhang, et al., 2000; Yan, et al., 2000; Landesberg, et al., 2001). Both preterm and fetal lambs had higher mRNA levels of *EGR1* following ventilation at high compared with low bias gas flows. Furthermore, the other two genes investigated, *CTGF* and *CYR61*, which play a role in tissue regeneration, cell proliferation and synthesis of extracellular components (Brigstock, 2003; Kubota, et al., 2007; Chen, et al., 2001), have been suggested to be related
quantitatively to lung injury, with higher levels following ventilation at high compared with physiological tidal volumes (Wallace, et al., 2009). This is consistent with the finding that higher mRNA levels of these genes tended to occur in lung tissue with more severe injurious changes at histological analysis.

The mRNA levels of CTGF and CYR61 were much lower in the fetal lambs than in the preterm lambs. An explanation for this can possibly be found in the fact that fetuses were connected to the placental circulation. Although the cytokines IL-1β, IL-6 and TNF-α are not able to cross the placenta (Aaltonen, et al., 2005), it has been suggested that the placenta has a down-regulatory effect on the fetal inflammatory response (Hillman, et al., 2007). The mRNA levels of IL-6 and CXCL8 in lung tissue of fetal lambs, ventilated for 3 hours while the placental circulation remained intact, were significantly lower when compared with lambs of a similar age ventilated ex-utero (Hillman, et al., 2007). CYR61 is involved in the early inflammatory response and has been demonstrated to up-regulate IL-1β (Chen, et al., 2001). Therefore, lower levels of pro-inflammatory cytokines in the study described above may be a reflection of lower expression of early response genes while the placental circulation remained intact. Despite the relatively low levels of the early response genes in the fetal lambs, the highest values for CTGF and CYR61 corresponded with a lower septal crest density and an increased percentage of space taken in by tissue, which is consistent with more severe lung injury.

In the fetal lambs, there was an interaction effect between mode and flow for two of the early response genes, for septal crest density and for percentage of space occupied by tissue during TCPLV, all indicative of more severe injury at the low, and not the high bias gas flow. This was an unexpected finding. However, the fetuses ventilated at a low bias gas flow had significantly lower values for relative humidity, equal to the measured humidity in the room. Cold and dry air has been demonstrated to lead to ciliar dysfunction in the trachea (Chalon, et al., 1979) and to a trend towards up-regulation of IL-1β in alveolar epithelium (Pillow, et al., 2009). Therefore, the combination of ventilation with a long inspiratory plateau phase using relatively drier air may have acted synergistically and increased the lung injury in the vulnerable immature lung.
Histological analyses in the preterm lambs demonstrated an abnormal deposition of the extracellular matrix proteins elastin and collagen following ventilation at higher flows. In both preterm and fetal lambs, the appearance of septal crests was altered following ventilation at higher bias gas flows compared with low flows, with blunter crests and altered proportions of myofibroblasts. Therefore, a faster rate of inflation of the lung, either measured by the rate of rise of the inspiratory flow or the rate of rise for pressure and volume, seemed to lead to more severe lung injury on histological analyses, suggesting a role for rheotrauma in the development of VILI.

In conclusion, the experiments in this thesis demonstrate that in the immature lamb lung, high bias gas flows lead to a deterioration in ventilator efficiency, increased histological evidence of lung injury and increased mRNA levels of key genes known to be activated early in lung injury. This thesis has, therefore, demonstrated for the first time that ventilator bias gas flow is a determinant of VILI in the immature lamb lung. Furthermore, this thesis demonstrated an interaction between bias gas flow and the mode of ventilation during the first two hours of ventilation. It is likely that bias gas flow also may contribute to the lung injury in the preterm human lung and potentially to development of BPD but there are no data from clinical studies. BPD is multifactorial in origin (Bancalari, et al., 2003), with the activation of inflammatory pathways in the immature lung being an important pathway that leads to lung injury (Sosenko, et al., 2008). Mechanical ventilation rapidly leads to an inflammatory response and increased concentrations of pro-inflammatory cytokines or markers for oxidative stress in blood or tracheal secretions are associated with risk of later BPD (Munshi, et al., 1997; Kakkera, et al., 2005; Huang, et al., 2005; Kotecha, et al., 1995; Buss, et al., 2003). Chapter 6 describes the design of a randomised controlled trial, in which it is hypothesised that ventilation at low bias gas flows will result in decreased levels of markers of inflammation and oxidative stress in the lungs known to be associated with risk of BPD. This study aims to (i) determine whether low bias gas flows decrease concentrations of cytokines and oxidative stress markers in tracheal aspirates and blood in ventilated extremely preterm infants and affect short-term clinical outcomes including ventilator
parameters and duration of ventilation, and (ii) obtain preliminary data on effect size and feasibility of the proposed approach in order to plan a multicentre RCT with BPD as the primary outcome. The ethics application for this trial is attached in the appendix of this thesis and a full application for funding has been submitted to the Health Research Council of New Zealand following a successful expression of interest. Two neonatal intensive care units in New Zealand and one in Australia have agreed to take part in this trial. It is anticipated that recruitment will commence later this year. This trial will provide the first data on the effect of bias gas flows in the preterm human neonate.
Appendix I. Application form for Ethical Approval

NATIONAL APPLICATION FORM FOR ETHICAL APPROVAL OF A RESEARCH PROJECT

NAF-2009-v1

The application guidelines (NAFG-2009-v1) are to be read before completing this form to ensure that the questions are answered appropriately.

The electronic version of this form is formatted the same way as the paper version so that, for example, where an answer needs six lines, six lines are formatted, but where an answer only needs one line, one line is formatted. Please note the number of lines allowed for a question before answering it and make sure that you do not use extra lines.

You may find it helpful to print out the application form before completing it to help you to keep to the page limits allowed. **No extra pages should be added**, except where specified, as appendices.

The relevant paragraphs of the Operational Standard for Ethics Committees (Ministry of Health document) have been included in subject headings for reference.

The page breaks are not to be deleted as this will affect the formatting of the form.

When collating your application, please ensure that the information sheet, consent form and any attachments are placed behind the application form before copying. Applications not correctly collated, ie not in complete sets ready to be sent to committee members will be returned.

**Do not include this page with your application.**
# Checklist for Applicants – attach to front of application

Before sending your application form, please check to make sure that all relevant information has been attached. If not applicable to the application write N/A. Protocols, information sheets, consent forms, questionnaires, advertisements, letters of invitation, data collection or other study forms must have a version number and date (marked *).

Please note: Incomplete applications will not be considered. Pending is an option only for written confirmation of Maori consultation, SCOTT approval, and Locality assessment by organisation. For multi-region studies, the documentation for one site must be complete.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Item</th>
<th>Yes, pending or N/A</th>
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<tr>
<td>Observational Studies Guidelines 5.11</td>
<td>* Study protocol – must be supplied with all applications</td>
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<td>Page 21 of NAFG, QE on NAF</td>
<td>* Consent form</td>
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<td>Page 23 of NAFG, QE on NAF</td>
<td>* Information sheet</td>
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<td>QA 5.4 on NAF</td>
<td>* Questionnaire/interview guidelines</td>
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<td>Page B of NAFG, QA 2 of NAF</td>
<td>Scientific assessment</td>
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<td>QA 5.3 of NAF</td>
<td>Statistical report</td>
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<td>QD2, NAFG page 8 Q A4</td>
<td>* Advertisement, letter of invitation</td>
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<td>Section F of NAFG, Page 15</td>
<td>Evidence of Māori consultation</td>
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<td>Part 4 of NAF</td>
<td>Declaration signed by principal investigator, Head of Department or Dean (for each site)</td>
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<td>Part 4, Form A or B of NAF</td>
<td>Accident compensation declaration correctly witnessed</td>
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<td>NAFG page 20, NAF page 28</td>
<td>Form/s for registered and unregistered medicines</td>
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<td>Pages 11 and 35 (Appendix 1) of NAFG, QB17 on NAF</td>
<td>Standing Committee on Therapeutic Trials (SCOTT) approval attached if drug is unregistered in New Zealand</td>
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<td>Locality assessments form(s) NAFG pages 18-20</td>
<td>Completed by ethics committee if required, or completed by locality organisation(s) if received at time of submission</td>
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<td>NAFG pages 30-32</td>
<td>Part 5: If there any use of tissue (includes blood, saliva, skin)</td>
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<td>Appendix 2 of NAFG</td>
<td>Part 6: If the research involves any gene or genetic studies</td>
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<td>Appendix 2 of NAFG</td>
<td>Part 7 if the study involves xenotransplantation</td>
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<td>NAFG pages 33-34</td>
<td>Part 8 if any participants are unable to consent themselves including children</td>
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<td>Parts 6 and 7, Appendix 2 of NAFG</td>
<td>GTAC approval if required</td>
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<td>National Radiation Laboratory risk assessment if required</td>
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<td>Company sponsored studies</td>
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<td>Evidence of Investigator indemnity insurance to cover C.6</td>
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### NATIONAL APPLICATION FORM FOR ETHICAL APPROVAL OF A RESEARCH PROJECT

| Ethics reference number and date received (for office use only) |

### Part 1: Basic Information

1. **Full project title (include protocol number if applicable)**

   Does bias gas flow in ventilated preterm babies affect lung injury or the risk of BPD? (the FLORA study)

2. **Short project title (lay title)**

   Flow at lower rates; the FLORA study

3. **Principal investigator's name and position**

   K.P. Bach  
   Paediatric neonatologist

4. **Contact address of principal investigator**

   Newborn Services, Auckland City Hospital  
   Support Building, level 9  
   PO Box 92024  
   Auckland Work phone no. 021-2284802  
   Emergency no.* 09-3074949, ext 24920  
   Fax 09-3754373  
   Email kittyb@adhb.govt.nz

5. **Principal investigator's qualifications and experience in the past five years (relevant to proposed research)**

   Qualified as paediatrician and neonatologist. Provisional vocational scope of practice with the Medical Council of New Zealand and awaiting vocational registration. Working as a neonatologist in Auckland City Hospital (ACH) since 1 November 2010. Completed PhD on effect of ventilator bias gas flow rate on injury in the preterm lung in March 2011. Worked as locum consultant and clinical neonatal fellow on the NICU in both ACH and VU Medical Center, Amsterdam, the Netherlands. Active participation in clinical (multicentre) trials.
6. Co-investigator’s name(s), qualifications and position(s) and, if more than one locality; principal investigator at each locality

| A | Frank Bloomfield, BSc(Hons), MBChB, MRCP(UK), FRACP, PhD; Associate Professor of Neonatology |
| B | Elza Cloete, MBChB (Pretoria), DCH (SA), Clinical Neonatal Fellow |
| C | Carl Kuschel, BHB, MBChB, FRACP, Clinical Associate Professor, Medical Director |

7.1 Address of A above

Liggins Institute, University of Auckland
Private Bag 92019
Auckland Work phone no. 09-3737599
Emergency no.* 021-497598
Fax
Email f.bloomfield@auckland.ac.nz

7.2 Address of B above

Newborn Services, Auckland City Hospital
Support Building, level 9
PO Box 92024
Auckland Work phone no. 09-3074949, ext 24920
Emergency no.* 021-305575
Fax 09-3754373
Email

7.3 Address of C above

Neonatal Services, The Royal Women's Hospital
Locked Bag 300
Parkville
VIC 3052 Australia Work phone no. +61 3 8345 2041
Emergency no.*
Fax +61 3 8345 2660
Email carl.kuschel@thewomens.org.au

7.4 Address of D above

Work phone no.
Emergency no.*
Fax
Email
Appendices

7.5 Address of E above

- Work phone no.
- Emergency no.*
- Fax
- Email

7.6 Address of F above

- Work phone no.
- Emergency no.*
- Fax
- Email

7.7 Address of G above

- Work phone no.
- Emergency no.*
- Fax
- Email
  (* option for ethics committee's information only)

8. Where this is supervised work

8.1 Supervisor's name
- Position
- Daytime phone number

8.2 Signature of supervisor (where relevant)
- Declaration: I take responsibility for all ethical aspects of the project

9. List locality organisation/s involved, including contact address, and complete the locality assessment in Part 4: Declarations (refer to the Guidelines (NAFG-2009-v1))

  Royal Women's Hospital, Melbourne, Australia
  For full contact details, see 7.1 to 7.3

10. I wish the protocol to be heard in a closed meeting. [ ] Yes [x] No

If the answer is yes, please provide a reason why you wish the protocol to be heard in a closed meeting in accordance with the Official Information Act 1982.
11. If the study is based, in part or in full, overseas, which countries are involved?

New Zealand and Australia

12. Has this application been reviewed by another ethics committee in New Zealand or overseas

[ ] Yes  [x] No

(If yes, advise which country, the name of the committee/s and the decision/s of the committee/s)

Please note a copy of the report/s may be requested.

13. Human tissue – Does the project involve collection or use of human tissue?

[x] Yes  [ ] No

If yes, complete Part 5.

14. Gene studies – Does this research involve any gene or genetic studies?

[ ] Yes  [x] No

If yes, complete Part 6.

15. Xenotransplantation – Does this research involve the transplantation of living biological material from one species to another?

[ ] Yes  [x] No

If yes, complete Part 7.

16. Consent – Are all participants able to provide consent for themselves?

[ ] Yes  [x] No

If no, complete Part 8.

17. Lay summary – give a brief lay (non-technical) summary of the study (not more than 200 words) such as you would give as an explanation to participants.

Mechanical ventilation of preterm babies has increased their survival over the last decades; however, ventilation also results in injury to the delicate immature lung. Several aspects of ventilation have been identified as contributing to this injury, including the pressure generated by the ventilator and the volume of gas delivered, which have led to advances in ventilatory strategies. To generate the pressure and tidal volume necessary to ventilate a patient, a ventilator applies a continuous bias gas flow running through the ventilator circuit. During ventilation of the preterm baby, this bias gas flow is normally set at 8-10 L/min, independent of babies’ weight and without evidence for this to be the correct setting. However, high compared to low ventilator bias gas flows have been proven to be more injurious on the immature lamb lung. This study will compare preterm babies ventilated at either the standard gas flow of 10 L/min, or a low gas flow of 4 L/min. Outcomes will be measurement of levels of inflammatory markers and duration and intensity of respiratory support.

18. Proposed starting date (dd/mm/yy)
19. Proposed finishing date (dd/mm/yy)

01/06/14

20. Duration of project in New Zealand (mm/yy)

36 months

21. Proposed final report date (mm/yy)

10/14

22. Has the clinical trial been registered? Yes No

If yes, name the register.

Comment: Will apply before submitting ethical application form
A. Validity of research  (Operational standard paragraphs 53–59)

SCIENTIFIC BASIS

A1. Aims of the project

A1.1 What is the hypothesis/research question(s) and/or the specific aims of the project? (State briefly.)

Comment: To determine if ventilation at a lower ventilation bias gas flow of 4 L/min results in lower concentrations of the inflammatory marker CXCL8 and in shorter duration of ventilation in preterm babies compared to ventilation at the standard bias gas flow of 10 L/min.

References:
Bach KP, Flecknoe SJ, Zahra VA, Kuschel CA, McKnight S, Peachey S, et al., editors. Acute lung injury in the ventilated preterm lamb is reduced at low ventilator gas flow rates #752211. PAS Annual Meeting; 2009; Baltimore, USA.

A2. Scientific background of the research

A2.1 Has this project been scientifically assessed by independent review?

[x] Yes    [ ] No

If yes, describe the process, for example, HRC funding assessment process. A copy of the report should also be attached. The researcher’s response may also be included.

If no, do you intend to have the project scientifically assessed and by whom?

The study design was presented at annual meeting of the Interdisciplinary Maternal Perinatal Australasian Clinical Trials Network in Wellington in 2010, where (inter)national clinicians have expressed their interest in collaboration. Furthermore, a full application for funding was submitted to the HRC following a successful expression of interest.

A2.2 Describe the scientific basis of the project (300 words maximum). Where this space is inadequate, continue on a separate sheet of paper. Do not delete page breaks or renumber pages.

Rates of preterm delivery are increasing worldwide with about 1-2% of the babies being born before 28 weeks’ gestation. Half of these babies will develop bronchopulmonary dysplasia (BPD), which means they have an ongoing need for oxygen or respiratory support once they are 36 weeks’
corrected gestation. These babies generally require longer hospitalisation, more frequent re-admission to hospital and they are at a higher risk of developing asthma. Respiratory disease / BPD contribute significantly to the neonatal health care costs.

BPD is multifactorial in origin, with factors such as prematurity, infection and mechanical ventilation all playing a role. Several factors in artificial ventilation have been clarified as contributing to lung injury; however, the role of ventilator bias gas flow has not been investigated in the human lung as yet. Ventilator bias gas flow is applied by the ventilator to generate a positive pressure in the ventilator circuit, resulting in the delivery of a volume of air on inspiration. During ventilation of the newborn, the flow is typically set at 8-10 L/min, independent of the size of the baby, which can vary between 500 g and 5 Kg. This contrasts with fluid intake, medication and inspiratory volume per breath during mechanical ventilation which are all dependent on weight of the baby.

We have recently demonstrated in preterm lambs that higher bias gas flows are more injurious to the immature lung than lower flows. Some genes, known to play a role in acute and chronic lung disease in the human, were up-regulated after only 2 hours of ventilation. Furthermore, we reported histological evidence of lung injury, comparable to changes seen in babies with BPD. It is, therefore, likely that bias gas flow may also contribute to lung injury in the preterm baby and, potentially, to the development of BPD.

A3. Study design

A3.1 Describe the study design. Where this space is inadequate, continue on a separate sheet of paper. Do not delete page breaks or renumber pages.

This is a randomised multicentre pilot study to determine whether cytokine concentrations in tracheal aspirates, which are markers for bronchopulmonary dysplasia, are decreased with lower flows and this study will also generate pilot data for a future multicentre RCT with BPD as the primary outcome. In addition, this pilot will confirm the feasibility of randomising preterm babies to such a trial and the acceptability of a waiver of consent for 24 h. 128 babies <28 weeks’ gestation and/or <1,000 g and requiring mechanical ventilation during the first week of life will be randomised to ventilation at a low (4 L/min) or standard (10 L/min) ventilator bias gas flow. Ventilator settings, other than the flow, are upon the discretion of the neonatologist. Primary outcomes will be concentration of CXCL8 in tracheal aspirate and the number of ventilated days. Secondary outcomes will be other respiratory outcomes and major neonatal outcomes, as an indicator of safety. Other markers of inflammation and oxidative stress will be measured in tracheal aspirate and blood after 24, 72 and 120 hours of ventilation (if still ventilated). Blood samples will be stored in anticipation of the validation of an assay for mRNA levels of acute early response genes.

Animal experiments demonstrate that lung injury is initiated very rapidly after onset of mechanical ventilation. Obtaining consent antenatally for
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trials in preterm babies is difficult and significantly affects recruitment rates, as seen in the COIN trial. We believe there is currently equipoise surrounding choice of bias gas flow, and clinicians are already altering bias gas flows based on no clinical evidence and with no data on potential harm. Providing evidence is thus urgent. We are applying for ethical approval for a waiver of consent for 24 hours, which is explained in more detail in the attached protocol (page 213-215).

A4. Participants

A4.1 How many participants do you intend to recruit? (Include details for each locality organisation.)

The preferred outcome would be a reduction in BPD at lower ventilator gas flow rates. To prove this hypothesis a large number of patients would be needed. This pilot study will be used to generate data on concentrations of inflammatory and oxidative stress markers and to collect recruitment data. The data generated from this study will be used to develop a larger multicentre study powered to detect a difference of BPD.

We intend to study 128 babies in this pilot study. In 2008 79 babies born at less than 28 weeks’ gestation (58 inborn, 21 not born in ACH) were admitted to the NICU of Auckland City Hospital. Only about half of these babies needed ventilation. We expect to obtain consent for the study in 80% of the eligible babies, thus 90-95 babies will be enrolled in ACH and a further 33-38 babies will be enrolled in the collaborating hospitals over a 3 year study period.

A4.2 Give a justification for the number of research participants proposed, giving the details of power calculations when appropriate.

CXCL8, an inflammatory marker, is used as biomarker of lung injury and concentrations of CXCL8 in tracheal aspirates in the first 5 days of life in preterm babies have been used to predict BPD. In tracheal aspirates of a preterm baby, the concentration for CXCL8 was 5,806 ± 4,923 pg/ml on day 5 of life (Shimotake 2004). The primary outcome in our study is a relative reduction of 35% in CXCL8 concentrations in tracheal aspirate in babies ventilated at a flow of 4 L/min compared with babies ventilated at 10 L/min. A sample size of 128 babies (64 babies in each arm) is needed to detect a 35% reduction in the CXCL8 concentrations in TA with 80% power (β=0.2) at a two-sided significance level of 5% (α=0.05).

A4.3 If randomisation is used, explain how this will be done.

Babies will be randomly assigned to one of the two treatment groups, once the need for ventilation has been established by a doctor or neonatal nurse / nurse practitioner. Group assignments will be drawn from consecutively numbered, sealed, opaque envelopes stratified by centre.
Each envelope will be opened by the doctor or nurse, once intubation is imminent at delivery or in the first week of life. This person will adjust the flow setting of a Neopuff and/or a Babylog 8000 plus to the assigned flow. The allocated flow will be used as soon as the baby is intubated and mechanical ventilation is given, either using the Neopuff or the ventilator. The baby will be ventilated at the same flow each time ventilation is needed.

A5. Statistical method

A5.1 Is the method of analysis quantitative? [x] Yes  [ ] No
Or qualitative?  [ ] Yes  [x] No

If the method of analysis is wholly qualitative, go to question A5.4.
If the method of analysis is wholly or partly quantitative, complete the following:

A5.2 Describe the statistical method that will be used to analyse the data.

Statistical analyses will be on an intention to treat (ITT) basis. Twins or triplets will be randomised as separate babies, with the non-independence of these pairs taken into account during analysis. Continuous outcome data will be analyzed using a Student t-test or repeated measures ANOVA. Data not normally distributed will be transformed to near-normality before statistical analysis. The non-parametric Mann-Whitney U test will be used, if near-normality is not achieved. Continuous outcome data will be presented as relative reduction with 95% Confidence Interval (CI). Categorical outcome data will be analyzed using Chi-Square Test and will be presented as relative risk with 95% CI. Calculation of the number needed to treat to benefit (NNTB) or number needed to treat to harm will be performed when appropriate. A p value less than 0.05 will be considered to indicate statistical significance for the primary outcome; a more stringent p value of less than 0.01 will be considered to indicate statistical significance for the secondary outcomes to prevent type I errors. All analyses will be two-sided.

A5.3 Has specialist statistical advice been obtained about this study?  [ ] Yes  [x] No

If yes, from whom? (A brief statistical report should be included if appropriate.)

A5.4 If the method of analysis is wholly or partly qualitative, specify the method. Why is this method appropriate? If interviews are to be used, include the general areas around which they will be based and a copy of the interview
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guide, if one is to be used. Copies of any questionnaires that will be used must be included.

A6. Expected outcomes or impacts of research

A6.1 What is the potential significance of this project for improved health outcomes?

This multicentre randomised controlled pilot trial will provide preliminary data on the recruitment rates to such a study. We will also generate data on cytokine and oxidative stress marker concentrations, and rates of bronchopulmonary dysplasia (BPD) in this group of babies in a contemporary, national cohort, that will allow us to calculate an accurate sample size for a multicentre definitive study with BPD as the primary outcome. If data from this pilot trial demonstrate that lower bias gas flow result in lower concentrations of CXCL8 in tracheal aspirates and fewer days on the ventilator, results from this study will be used in the design of a larger multicentre trial powered to detect a difference in BPD. BPD is an expensive and time-consuming condition that has a high social cost; if ventilation at a lower ventilator bias gas flow results in a reduction in BPD, this will potentially benefit thousands of babies worldwide and will potentially reduce the emotional and physical costs of their care.

A6.2 What is the potential significance of this project for the advancement of knowledge?

There is no evidence to support default setting of the ventilator bias gas flow and there is currently clinical equipoise regarding which flow is used: clinicians use various flows ranging from 4 to 10 L/min, based purely on clinician preference. Animal studies suggest that high bias gas flows are more injurious to the preterm lamb lung, resulting in higher levels of early response genes and histological changes consistent with the changes seen in BPD. Besides generating data on the concentrations of inflammatory and oxidative stress markers and on the number of ventilated days, this pilot study will generate data on recruitment. These data will be used in the power calculation of a large randomised controlled trial conducted on the comparison of two different ventilator gas flow rates with as endpoint BPD.

A6.3 What steps will be taken to disseminate the research results?

Results of this study will be presented at local meetings in each of the collaborating hospitals, at national and international meetings and in peer-reviewed journals. The data from this pilot study will be used to
develop a large multicentre trial, which will occur in close collaboration with peers in the field. Results of the study will be provided to the parents or caregivers of a preterm baby if they so wish.

**A7. Publication of results**

Will any restriction be placed on publication of results? [ ] Yes [x] No

If yes, please supply details.

**A8. Funding**

**A8.1 How will the project be funded?**

The pilot study and a part-time research nurse will be funded via a generous private donation of a philanthropist, specifically donated for research on the neonatal intensive care unit. We will seek additional funds from the HRC and other external research funds.

**A8.2 Does** the researcher, the host department, the host institution or the locality organisation have any conflict of interest, eg, financial interest, in the outcome of this research? If yes, please give details. [ ] Yes [x] No

**A9. Incentive payments**

**A9.1 Have you read and understood the description of incentive payments in the Guidelines?** [x] Yes [ ] No

**Note: Details about any payment (in money or kind) or reward made to participants recruited into the project are to be provided in question E10.**

**A9.2 Does** the funding available to the project depend upon the number of participants recruited, eg, is the funding on a per participant basis? [ ] Yes [x] No

If yes, give details of the amount per participant. Where there is a significant difference between these, this incentive to recruit should be declared in the information sheet.

**A9.3 Does** the funding available to the project include any form of incentive (in money or kind) for the early or complete recruitment of a specified number of
participants, eg, bonus payments to the researcher, host department or host institution?  

[ ] Yes  [x] No

If yes, give details.

A9.4 Will all funding available to the project be passed through an audited research account or cost centre?  

[x] Yes  [ ] No

If yes, give details. If no, specify why not.

Liggins Institute, University of Auckland

B. Minimisation of harm  

(Operational standard paragraphs 60–68)

B1. How many visits/admissions of participants will this study involve? Clarify what is in addition to standard treatment. Give also an estimate of total time involved for participants.

This study will be performed whilst the baby is an inpatient of the NICU. No additional hospitalisation is necessary.

B2. Who will carry out the research procedures?

Staff of the neonatal intensive care unit in Auckland City Hospital and in collaborating hospitals.

B3. What other research studies is the lead investigator currently involved with?

Completion of PhD studies, looking into the effects of using lower ventilator bias gas flows on lung injury in the immature lung.

B4. Where will the research procedures take place?

Neonatal intensive care unit, Auckland City Hospital and collaborating hospitals.

B5. How do the research procedures differ from standard treatment procedures?

The flow on the Neopuff is normally set at 8 L/min, the flow on the ventilator at 10 L/min, although in reality the latter is set between 4 and 10 L/min. During the study, both flows will be set according to the allocated flow.

1 mL of blood will be obtained at the time of routine blood sampling at 24, 72 and 120 hours after the onset of ventilation. Saline (1.0 mL/Kg) will be instilled in the endotracheal tube before suctioning to obtain a tracheal aspirate at 24, 72 and 120 hours after the onset of ventilation; normally, suctioning is done on indication and performed dry or with 0.2-0.3 mL of
saline. Blood and aspirates will be processed by the hospital laboratory and stored until further analyses.

B6. What are the benefits to research participants of taking part in the project?

There are no direct benefits to a patient in this project. If the lower ventilator bias gas flow is found to reduce the number of days on the ventilator then both infants in this trial and future infants will benefit.


(If National Health Index (NHI) information is used, see the Guidelines (NAFG-2009-v1).)

Information will be obtained by direct observation of the (potential) participants. Furthermore, laboratory results, radiology results, medical records and downloaded information from routinely used equipment will be used to collect information about participants in the study.

B8. Briefly describe the inclusion/exclusion criteria and include the relevant page number(s) of the protocol or investigator’s brochure.

We will include babies born at less than 28 weeks’ gestation and/or with a birth weight less than 1,000 g, who require mechanical ventilation in the first week of life. Clinicians at each of the participating centres will decide on whether mechanical ventilation is required. We will exclude babies diagnosed with congenital cardio-respiratory anomalies and known chromosomal or genetic abnormalities. (Page 213)

B9. What are the physical or psychological risks or side effects to participants or third parties? Describe what action will be taken to minimise any such risks or side effects.

Suction of the endotracheal tube is an accepted routine procedure on the NICU to maintain a patent tube. For study purposes, suction will be done at predetermined times, thus might not necessarily coincide with a clinical indication and 1.0 mL/Kg instead of a maximum of 0.3 mL of saline will be used. Suction may not be comfortable for the baby, but is performed by trained neonatal nurses.

B10. What facilities/procedures and personnel are there for dealing with emergencies?

All babies will be admitted to the neonatal intensive care unit, where they will be looked after by trained personnel. Adequate equipment for the care, ventilation and resuscitation of these babies is available.
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B11. What arrangements will be made for monitoring and detecting adverse outcomes?

A data safety monitoring committee will be established.

B12. If the study is a clinical trial, are participants to be provided with a card confirming their participation, medication and the contact phone number of the principal investigator?  [ ] Yes  [x] No

B12.1 Do you intend to inform the participant’s GP that their patient is a participant in this study? (If yes, consent from the participant is required.)  [ ] Yes  [x] No

B12.2 Do you intend to inform the GP of all clinically significant abnormal results obtained during study conduct?  [ ] Yes  [x] No

B13. Is the trial being reviewed by a data and safety monitoring board (DSMB)?  [x] Yes  [ ] No

If yes, who is the funder of the DSMB?  [ ] Yes  [ ] No  [x] Other

If ‘Other’, please specify.

The DSMB will consist of neonatologists from Australia and New Zealand, who will not be involved in the study but who volunteered to review the data.

B14. What are the criteria for terminating the study?

The data safety monitoring committee will review all cases of mortality and serious respiratory morbidity (pulmonary haemorrhage, pneumothorax, requirement for rescue ventilation with high frequency oscillatory ventilator) and determine whether these may be related to the study. If any Serious Adverse Events are thought to be due to the study intervention, the DSMC may recommend termination.

B15. Will participants be exposed to any potential toxins, mutagens or teratogens?  [ ] Yes  [x] No

If yes, specify and outline the justification for their use.
B16. Will any radiation or radioactive substances be used?  [ ] Yes  [x] No

Note: If any form of radiation is being used, please answer B16.1–B16.2. If no, go to question B17.

B16.1 How many x-rays or other procedures are planned for the purposes of this study, ie, that are not part of standard treatment?

B16.2 Under whose licence is the radiation being used?

B16.3 Has the National Radiation Laboratory (NRL) risk assessment been completed?  [ ] Yes  [ ] No

If yes, please enclose a copy of the risk assessment and a contact name and phone number.
If no, please explain why not.

B17. Will any medicines be administered for the purposes of this study?  [x] Yes  [ ] No

B17.1 If yes, is Standing Committee on Therapeutic Trials (SCOTT) approval required?  [ ] Yes  [ ] No

B17.2 Has SCOTT approval been given? (Please attach.)  [ ] Yes  [ ] No

B18. Does the study involve the use of health care resources?  [x] Yes  [ ] No
If yes, please specify:

The babies will be under the care of the neonatal intensive care unit of either Auckland City Hospital or any of the collaborating hospitals. Blood samples and tracheal aspirates will be processed by the hospital’s own laboratory. Final analyses will be performed in the laboratory of Auckland City Hospital, but additional costs for processing and analysing will be paid for by the study.

B19. What effect will this use of resources have on waiting list times for patients, that is, for diagnostic tests or for standard treatments?

Nil.
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C. Compensation for harm suffered by participants

(Operational standard paragraphs 87–95)

(Refer also to Appendix 3 of the Guidelines (NAFG-2009-v1).)

C1. Will participants be treated by, or at the direction of, a registered health professional as part of the research? (Treatment includes screening, diagnosis, for definitions see the Guidelines (NAFG-2009-v1) pages 11-13.)

[ ] Yes [ ] No

If no, go to section D. If yes, please answer questions C2–C5.4.

C2. Is the research being carried out principally for the benefit of a manufacturer or distributor of the drug or item in respect of which the research is taking place?

[ ] Yes [x] No

C2.1 If the answer to C2 is yes, please complete Statutory Declaration Form B and answer questions C3–C5.4.

C2.2 If the answer to C2 is no, please complete Statutory Declaration Form A and go to section D.

Depending on all the circumstances, the minimum cover that is likely to be acceptable to the ethics committee is that provided under ACC. In any case, all exclusions to compensation must be clearly and explicitly set out in the participant information sheet, including those that may be described in C5.

C3. Is the manufacturer/distributor’s agreement to provide compensation in accordance with the RMI attached?

[ ] Yes [ ] No

C4. Has the manufacturer or distributor agreed to cover any injury/adverse consequence resulting from participation in this research?

[ ] Yes [ ] No

C4.1 If no, what qualifications have been specified for cover?

C4.2 Limiting the type of compensation

C4.2.1 Has the manufacturer or distributor excluded any type of compensation, for example, pain and suffering, loss of earnings, loss of earning capacity, funeral costs, dependents’ allowances or any other financial loss or expenses?

[ ] Yes [ ] No

C4.2.2 If yes, please state what is excluded. (Include in the compensation statement on the information sheet)
C5. Limiting liability – exclusion clauses

C5.1 Has the manufacturer or distributor limited or excluded liability if the injury is attributable to the negligence of someone other than the manufacturer or distributor (such as negligence by the investigator, research staff, the hospital or institution, or the participant)?  
[ ] Yes  [ ] No

C5.2 Has the manufacturer or distributor limited or excluded liability if the injury resulted from a significant deviation from the study protocol by someone other than the manufacturer or distributor?  
[ ] Yes  [ ] No

C5.3 Is evidence of the following indemnity insurance attached?

Sponsor  
[ ] Yes  [ ] No

If yes to either C5.1 or C5.2;

Hospital/institution  
[ ] Yes  [ ] No
Investigator  
[ ] Yes  [ ] No

C5.4 Is company liability limited in any other way?  
[ ] Yes  [ ] No

If yes, please specify.

D. Privacy and confidentiality  
(Operational standard paragraphs 48–56)

D1. How will potential participants be identified?

Any baby born at less than 28 weeks’ gestation and/or with a birth weight of < 1,000 g and who needs ventilatory support in the first week of life, based on a clinical decision by the doctor or neonatal nurse / nurse practitioner caring for the baby, is eligible.

D2. How will participants be recruited (for example, advertisements, notices)?

If a baby as outlined in D1 needs ventilation, the baby will be randomised and ventilated at the allocated flow rate. If the parent(s) or caregiver(s) does not give consent 24 hours after the onset of ventilation, ventilation will be continued as per normal protocol.

D3. Where will potential participants be approached (for example, outpatient clinic)? If appropriate, describe by type (for example, students).

See D1
D4. Who will make the initial approach to potential participants?

Neonatologist or neonatal nurse / nurse practitioner

NB: Do not include information on storage and use of tissue samples and related information in the following questions. That is covered separately under Part 5.

D5. How will data, including audio- and videotapes, be handled and stored to safeguard confidentiality (both during and after completion of the research project)?

Consent forms and identifying data will be stored in a locked file in the Newborn Services. Computerised data will be password protected on a computer at the NICU.

D6. What will be done with the raw data when the study is finished?

Raw data will be kept for at least 26 years after last enrolment. After this time, all data will be destroyed by shredding or burning. Computerised data will be wiped from the computer.

D7. How long will the data from the study be kept, and who will be responsible for their safe keeping? (Health information relating to an identifiable individual must be retained for at least 10 years, or in the case of a child, 10 years from the age of 16.)

Data will be kept till at least 10 years after the infant has turned 16 years, i.e. data will be stored for at least 26 years after last enrolment into the trial. The principal investigator, Dr. KP Bach, will be responsible for the safe keeping of the data.

D8. Name those who will have access to the raw data, participant information and/or clinical records during, or after, the study?

The research team will have sole access to the data.

D9. Describe any arrangements to make results available to participants, including whether they will be offered their audio- or videotapes.

Parents or caregivers will be provided with a lay summary at the conclusion of the study, if they so require.
E. Informed consent

A participant's informed consent should be obtained in writing, unless the procedures are not experimental and there are good reasons for not requiring written consent. If consent is not to be obtained in writing, the justification should be given and the circumstances under which consent is obtained should be recorded. Attach a copy of the information sheet and consent form provided to participants.

E1. By whom, and how, will the project be explained to potential participants?

Verbal explanation and information sheet will be given by a member of the research team or another staff member working on the neonatal intensive care unit.

E2. When and where will the explanation be given?

Preferably before birth on the antenatal ward or delivery unit, alternatively as soon as is appropriate after admission of the baby on the neonatal intensive care unit.

E3. Will a competent interpreter be available, if required? [x] Yes [ ] No

If no, why not?

E4. How much time will be allowed for the potential participant to decide about taking part in the project?

We are applying for a waiver of consent for 24 hours, thus randomisation of the baby can occur, after which the parents / caregiver can decide to withdraw from the study in the first 24 hours of ventilation, before blood samples or tracheal aspirates are taken (see protocol page 213-215 for more detailed information).

E5. In what form (written, or oral) will consent be obtained? If oral consent only, state reasons.

Written consent will be obtained.

E6. If recordings are made, will participants be offered the opportunity to edit the transcripts of the recordings? [ ] Yes [ ] No
E7. Will data or other information be stored for use in a different study for which ethics committee approval would be required?  

[ ] Yes  [x] No

E7.1 If yes, please explain how.

____________________________________________________________________________________

E8. Is there any special relationship between the participants and the researchers (for example, doctor/patient, student/teacher)?

No.

E9. Will there be any financial cost to the participant, for example, travel and parking costs? If so, will such cost be reimbursed? (Refer to the Guidelines (NAFG-2009-v1).)

No.

E10. Will any payments be made to participants, or will they gain materially in other ways from participating in this project?  

[ ] Yes  [x] No

E10.1 If yes, please supply details.

____________________________________________________________________________________

F. Cultural and social responsibility

(Operational standard paragraphs 73–82)

Section F enshrines two fundamental principles. They are:

i. Culturally safe research practice: Research involving participants from specific ethnic or socially identified groups (even when small numbers from each group are involved) must involve those participant groups in the research process as full participants. Where a particular ethnic or socially identified group is the principal subject of the research, there must be engagement with appropriate parties, and this process must be outlined in the application.

ii. If the research is in an area of health inequalities, then the researcher must demonstrate how the research will contribute to achieving equity of outcomes for those population groups most in need within the public good health system.

F1. Have you read the HRC booklet *Guidelines for Researchers on Health Research Involving Māori*?  

[ ] Yes  [ ] No

Relevance and responsiveness to Māori

F2. All health research conducted in Aotearoa New Zealand is of relevance to Māori. How relevant is a decision to be made by Māori. The researcher must
be able to articulate the context and the relevance of the proposed research to Māori and the possible consequences for Māori health outcomes, and generally, the greater the degree of relevance to Māori, the greater the expectation of participation of Māori and hence consultation expectations.

F2.1 Given your approach to sampling, what are the anticipated numbers of Māori participants?

All babies born before 28 weeks’ gestation and/or born with a birth weight < 1,000 g are eligible to enrol in the study. Of the total population born preterm in ACH, Māori babies make up 16%, thus we expect 20 babies to be of Māori descent in our study.

F2.2 What is the incidence among Māori of the health issue/disability relevant to the study?

See F2.1

F3. Please explain how this research will contribute to improving Māori health outcomes and reducing health inequalities for Māori.

Maori babies and babies of other ethnicity are eligible to enter the study, as long as they fit the inclusion criteria. If this study confirms the hypothesis that lower ventilator bias gas flows lead to lower concentrations of inflammatory markers and reduced number of days on a ventilator, this is equally beneficial for Māori babies and babies of other ethnicity. Māori have a higher incidence of preterm birth, and thus this study may have the potential to provide additional benefit for Māori.

F4. Describe the process by which Māori have been engaged in the conception and design of the proposed research. Please identify the group(s) with which consultation has taken place and outline their stated view about the proposed research. Please attach their letter(s) of support for this specific research project.

We have consulted with the Auckland District Health Board Maori Health Services advisor Mata Forbes RGON, who has provided suggestions for improving the protocol so that it upholds the Treaty obligation and does not disadvantage Māori. These suggestions have been implemented into the study design.

F4.1 Describe any ongoing involvement the group(s) consulted have in the project.

We will seek further advice from the Auckland District Health Board Maori Health Services advisor should any cultural issues arise.
F4.2 Describe how information will be disseminated to participants and the group(s) consulted during and at the conclusion of the research project.

Parents or caregivers will be provided with a lay summary at the conclusion of the study, if they so require.

**Responsiveness to ethnic peoples**

F5. What other ethnic groups will be participating in this research based on your sampling frame (for example, Pacific peoples or Asian peoples)?

This study represents the overall population of New Zealand. In NWH's annual report of 2008 39.1% of the preterm babies were New Zealand-European, 16% Maori, 17.4% Pacific, 10.8% Asian, 8.7% Indian, 5% other European and other ethnicity in 3%. We expect a similar representation in our study.

F5.1 Are there any aspects of the research based on participation or the relevance of the research to specific ethnic groups that might raise specific cultural issues?  
[ ] Yes  
[x] No 
If yes, please outline. 
If no, go to F6.

F5.2 How can this research contribute to reducing inequalities for ethnic peoples in the New Zealand health system?

F5.3 Describe what consultation has taken place with specific ethnic group(s) prior to the project's development and attach evidence of their support.

F5.4 Describe any ongoing involvement the group(s) consulted have in the project.

F5.5 Describe how you intend to disseminate information to participants and the group(s) consulted at the end of the project.
Responsiveness to other peoples of interest

F6. Are there any aspects of the research based on participation or the relevance of the research to specific peoples of interest that might raise specific issues for such communities (for example, for prisoners, people with disabilities, people with diverse sexual identities)?

[ ] Yes  [x] No

If yes, please outline.
If no, go to F7.

F6.1 How can this research contribute to reducing inequalities for other peoples of interest in the New Zealand health system?

F6.2 Describe what consultation has taken place with specific peoples of interest group(s) prior to the project’s development and attach evidence of their support.

F6.3 Describe any ongoing involvement the group(s) consulted have in the project.

F6.4 Describe how you intend to disseminate information to participants and the group(s) consulted at the end of the project.

F7. Will the study drug/treatment continue to be available to the participant after the study ends?

[x] Yes  [ ] No

F7.1 If yes, will there be a cost, and how will this be met?

Ventilation is part of the standard care on a neonatal intensive care unit and is thus available to preterm babies admitted to a NICU.

F7.2 If no, why not?

F7.3 If there was a placebo arm, what will happen to these participants at the end of the study?

Note: This information needs to be included in the information sheet.
Describe and discuss any ethical issues arising from this project, other than those already dealt with in your answers above.

None

Thank you for your assistance in helping us assess your project fully.

Please now complete:
- the declarations (Part 4). If there is more than one site, include a declaration for each site.

If applicable complete:
- a Registered Drug Form
- Form A or B
- Part 5
- Part 6
- Part 7
- Part 8

Attach:
- Checklist to ensure all relevant documents are attached. Incomplete applications will not be reviewed.
Part 4: Declarations

Full project title:
Does bias gas flow rate in ventilated preterm babies affect lung injury or the risk of BPD?

Short project title:
Flow at lower rates; the FLORA study

1. Declaration by principal investigator

The information supplied in this application is, to the best of my knowledge and belief, accurate. I have considered the ethical issues involved in this research and believe that I have adequately addressed them in this application. I understand that if the protocol for this research changes in any way, I must inform the ethics committee.

Name of Principal Investigator (please print): Katinka P. Bach

Signature of Principal Investigator:

Date:

2. Declaration by Head of Department in which the Principal Investigator is located or appropriate Dean or other Senior Manager

I have read the application, and it is appropriate for this research to be conducted in this department. I give my consent for the application to be forwarded to the ethics committee.

Name (please print):

Signature: Institution:

Date: Designation:

- Where the Head of Department is also one of the investigators, the Head of Department declaration must be signed by the appropriate Dean, or other senior manager.
- If the application is for a student project, the supervisor should sign the Head of Department declaration.
- Submit a declaration by the principal investigator for each site.

3. Locality organisation approval

Locality organisation approval is being sought/is attached from the following locations:

Royal Women’s Hospital, Melbourne, Australia
Form A: Declaration of Eligibility of a Clinical Trial for Consideration of Coverage under Accident Compensation Legislation

**Instructions**: This form is to be completed and the statutory declaration signed by the most senior registered health professional providing or directing the provision of treatment as part of the research. It should be forwarded to the appropriate ethics committee together with the documents seeking ethical approval for the proposed study. The information provided must be sufficiently detailed to enable the ethics committee to be satisfied that the proposed research is not conducted principally for the benefit of the manufacturer or distributor of the medicine or item in respect of which the research is carried out.

The provision of this information will enable the ethics committee to be satisfied that participants in the clinical trial will be considered for coverage under accident compensation legislation for injury caused as a result of their participation in the research.

### Details of proposed research study

**Title of research project:**

| Does bias gas flow rate in ventilated preterm babies affect lung injury or the risk of BPD? |

**Name of research director/investigator:**

| Katinka P Bach |

**Location/s of proposed study:**

| Auckland, Melbourne |

**Number of participants:**

| 128 |
Organisations providing support (in money or kind) for the direct and indirect costs of the research *(please provide names of organisations and details of the type of support provided)*:

| Philanthropic donation to Friends of the Liggins, University of Auckland (donation specifically for clinical research at the neonatal intensive care unit) |

Relationship of proposed research to the pharmaceutical industry or other company involved in health research *(please describe the involvement of industry in your proposed research and provide details of support to be received from them)*:

| N/A |

**Statutory declaration**

I Katinka Bach (name) of Auckland (town/city) solemnly and sincerely declare that as the most senior registered health professional providing or directing the provision of treatment as part of the research, the proposed study is not conducted principally for the benefit of the manufacturer or distributor of the medicine or item in respect of which the trial is carried out. I make this solemn declaration conscientiously believing the same to be true and by virtue of the Oaths and Declarations Act 1957.

| Katinka Bach | Name *(please print)* | Signature | this day of |

before me

<table>
<thead>
<tr>
<th>Name of witness (please print)</th>
<th>Signature of witness</th>
</tr>
</thead>
</table>

| a Justice of the Peace, or a Solicitor of the High Court or other person authorised to take a statutory declaration |

**Warning**: Please note that it is an offence under part VI subsection 111 of the Crimes Act 1961 to make a false statutory declaration. **Note**: Applicants conducting a research study that is conducted principally for the benefit of the manufacturer or distributor of the medicine or item in respect of which the trial is carried out should complete Form B.
**Form B: Declaration of Provision of Compensation for Injury for Participants in a Research Study for a Pharmaceutical Company or any Other Company Involved in Health Research**

**Instructions:** This form is to be completed and the statutory declaration signed by the applicant. It should be forwarded to the appropriate ethics committee together with the documents seeking ethical approval for the proposed study and appropriate assurance from the pharmaceutical company or any other company involved in health research. The information provided must be sufficiently detailed to enable the ethics committee to be satisfied that:

- the proposed research is conducted principally for the benefit of the manufacturer or distributor of the medicine or item in respect of which the research is carried out;
- participants in the proposed research project will receive an acceptable level of compensation from a pharmaceutical company or any other company involved in health research in the event of injury to participants resulting from their involvement in the proposed research project;
- researchers and institutions have indemnity cover to provide an acceptable level of compensation in the event of injury to participants resulting from any researcher or research staff deviating substantially from the trial protocol.

**Details of proposed research project**

<table>
<thead>
<tr>
<th>Title of research project:</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Name of research director/investigator:</td>
<td></td>
</tr>
<tr>
<td>Location of proposed study:</td>
<td></td>
</tr>
<tr>
<td>Number of participants:</td>
<td></td>
</tr>
</tbody>
</table>

Organisations providing support (in money or kind) for the direct and indirect costs of the research *(please provide names of organisations and details of the type of support provided)*:

Relationship of proposed research to the pharmaceutical industry or other company involved in health research *(please describe the involvement of industry in your proposed research and provide details of support to be received from them)*:

Details of compensation to be provided to participants in the event of injury *(documents signed by the sponsoring pharmaceutical company or other company involved in health research must be attached)*:

**Statutory declaration**

I **(name)** of **(town/city)** solemnly and sincerely declare that as director of the proposed research, the proposed study is conducted principally for the benefit of the manufacturer or distributor of the medicine or item in respect of which the trial is carried out and that in the event of injury arising from their participation in the research, an appropriate level of compensation, in line with the *New Zealand Researched Medicines Industry Guidelines on Clinical Trials – Compensation for Injury Resulting from Participation in Industry Sponsored Clinical Trials*, will be provided by **(name of pharmaceutical company or...**
another company involved in the research project) as detailed in the attached
documents, unless the injury is a result of a significant deviation from the study
protocol. I confirm that I, my research staff and the host institution have
indemnity insurance that covers injury as a result of significant deviation from
the study protocol. I make this solemn declaration conscientiously believing the
same to be true and by virtue of the Oaths and Declarations Act 1957.

Name (please print)  Signature  this day of

before me

Name of witness (please print)  Signature of witness

a Justice of the Peace, or
a Solicitor of the High Court
or other person authorised to take a
statutory declaration

Warning: Please note that it is an offence under part VI subsection 111 of the
Crimes Act 1961 to make a false statutory declaration. Note: Applicants
conducting a research study that is not conducted principally for the benefit of
the manufacturer or distributor of the medicine or item in respect of which the
trial is carried out should complete Form A.
Form for Registered and Unregistered Medicines
*(Refer Question B19)*

**Information required for trials involving administration of medicines currently registered in New Zealand**

This form is to be completed for all medicines including non-registered medicines except where the medicine will be given regardless of entry into the trial (e.g., anaesthetic) and that medicine is not being studied in any way.

<table>
<thead>
<tr>
<th>Trade name of medicines</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Generic name of medicines</td>
<td></td>
</tr>
<tr>
<td>Pharmacological class</td>
<td></td>
</tr>
<tr>
<td>Form of administration used in the study</td>
<td></td>
</tr>
<tr>
<td>Recommended dose range</td>
<td></td>
</tr>
<tr>
<td>Contraindications</td>
<td></td>
</tr>
<tr>
<td>Known or possible interactions with non-trial medicines the participants may be taking</td>
<td></td>
</tr>
<tr>
<td>Common side effects and serious adverse reactions</td>
<td></td>
</tr>
<tr>
<td>Additional information, e.g., long half-life, immunosuppression</td>
<td></td>
</tr>
</tbody>
</table>
Appendices

Appendix II. Use of Human Tissue

To be completed if the research involves collection of human tissue. (See guidelines page 28 for definition.)

1.1 Provide details, eg, type, number of samples, total volume to be obtained.

Three extra blood samples will be taken (1 mL each), taken 24, 72 and 120 hours after the onset of ventilation. These samples can be taken from an arterial line, if one is available, or from a venipuncture. In most cases this will be combined with blood sampling for other clinically necessary tests. At the same time points (24, 72 and 120 hours) a tracheal aspirate will be collected by instillation of saline (1 mL/Kg) in the endotracheal tube, if the baby is still ventilated, followed by suctioning of the endotracheal tube according to the standard NICU protocol.

1.2 Will consent, or has consent, been obtained?  [x] Yes  [ ] No

If yes, proceed to 1.6

1.3 If consent is not able to be obtained for use of tissue, how consistent is the proposed use of tissue with the original consent for the use of the tissue?

1.4 State reasons why informed consent cannot be obtained or why it would not be desirable or possible to do so.

1.5 State the public good associated with continuing the research without the consent of the individual.

1.6 How was or will the tissue be obtained (including frequency and scope of consent that was or will be given)?

Blood samples will be obtained from an arterial line, if present, or venipuncture and tracheal aspirate will be collected by suctioning of the endotracheal tube at 24, 72 and 120 hours after the onset of ventilation. Each baby will have 3 blood samples collected and 3 tracheal aspirates, if they are still ventilated at the time of sampling. Informed consent will be given on the consent form for the trial.
1.7 What are the current use(s) of the tissue and any intended or foreseeable future uses of that tissue (including the scope of consent that was or will be given) and why is it necessary?

Blood samples will be analysed for CXCL8, IL-6, IL-1β and TNF-α and the rest of the sample will be frozen and stored till the method for analysis of early response genes in blood of preterm babies is completely established. Currently, measurement is possible in a larger volume of blood (5 mL), which is obviously a too large sample for a preterm baby. Early response genes are up-regulated in lung tissue of adults with acute and chronic lung disease and higher expression levels were seen after ventilation with larger volumes or at higher gas flow rates, i.e. more injurious ventilation, in preterm lambs after only 2 hours of ventilation. Therefore, altered levels of these genes in blood might reflect the level of lung injury and predict the risk of BPD development in the preterm baby. The tracheal aspirates will be analysed for concentrations of CXCL8, IL-6, IL-1β, TNF-α and 3-chlorotyrosine, which are all known to be up-regulated during ventilator-induced lung injury. The blood samples and tracheal aspirates will be destroyed after the assays are done.

1.8 If access is to be granted to third parties, how will that be done?

No access will be granted to third parties.

1.9 How will tissue be stored, eg, identified/de identified/anonymised, length of time, means of storage and labelling, security, the responsible individual or organisation?

Blood samples and tracheal aspirates will be labelled with the baby’s name, date of birth and hospital number. The samples will be frozen and stored in the hospital laboratory in the usual fashion, but identified as being part of a research study. Samples collected in hospitals other than NWH, will be frozen and sent to NWH in batches by courier, where samples will be analysed in batches to reduce variability and for economy. The samples will be destroyed once the analysis is completed.

1.10 How will the tissue be disposed or returned?

The samples will be disposed of by the laboratory after testing.

1.11 Is genetic analysis to be carried out (see also Part 6)?

No.

1.12 Can the participant request the tissue to be withdrawn from the research? If so, how and at what point?
The parents of the baby can withdraw from the trial at any stage. If they withdraw, they will be asked whether they would like any extant samples to be destroyed or whether they are happy for these samples to be analysed. If the former, the lab will be contacted and requested to destroy any samples that have not yet been analysed. If the latter, the samples will be destroyed once analyses have been completed.

1.13 Will personal and health information or sensitive information be linked to the tissue? If yes, are procedures in place to recontact participants or their clinician to provide clinically relevant information if it arises.

The baby’s name, hospital number and date of birth will be on the sample. There will be no health or sensitive information linked to the sample. At the time of analysis the babies involved in the study are presumed to be off ventilatory support, thus clinically relevant increase in early response genes or inflammatory markers will not aid their care; however, this information might potentially benefit future preterm babies.

1.14 What safeguards will be in place to ensure that the tissue will not be vulnerable to unethical use.

Informed consent will be obtained before blood samples and tracheal aspirates are taken. Only tests for which there has been informed consent obtained will be performed, and only a volume of blood sufficient for the analyses will be taken. All samples will be destroyed after they are analysed.

2. If there any additional safeguards in place not covered in 1.1–1.14 above please give details.

3. Will the human tissue involved in the research project be stored for later use in a future study? [ ] Yes [x] No
If yes, please give details.

Is this covered by distinct informed consent? [ ] Yes [ ] No

4. Will any human tissue samples or the information derived from them go out of New Zealand? [ ] Yes [x] No
If yes, complete the following questions.

4.1 If so to what organisation/s and how will they be transferred?
Appendices

4.2 What governance structures, procedures and processes does this organisation have to ensure the participant's choices are respected ie storage facilities, control of access, appropriate disposal methods?

4.3 How will tissue be stored, eg, identified/de identified/anonymised, length of time, means of storage and labelling, security, the responsible individual or organisation? (If different from 1.9.)

4.4 How long will the tissue and/or data be stored and what will happen at the end of this time?

4.5 If identified tissue and/or data is stored, will participants be able to request that their tissue be returned to New Zealand or request confirmation that their tissue and related information has been destroyed?

4.6 What appropriate ethical safeguards will be in place?

4.7 Will all future use of the tissue and the information derived from it be subject to review by an ethics committee approved by the New Zealand Health Research Council of New Zealand? [ ] Yes [x] N/A

If no, provide confirmation from the organisation storing the tissue or the information derived from it, that it has protocols in place to ensure that any future use will have ethical and scientific review by a committee or institutional review board that conforms to the International Ethical Guidelines for Biomedical Research Involving Human Subjects (Council for International Organizations of Medical Sciences and World Health Organization 2002).
## Appendix III. Participant Unable to Make Informed Choice

### Part 8: When a Participant is Unable to Make an Informed Choice

To be completed when one or more participants in a project will likely not be able to make an informed choice about whether to take part. **Do not complete this section if all participants in the study are competent to make an informed choice and give informed consent themselves.** Refer to the Guidelines for information about children in research.

1. Will any of the participants have a person with them who is available and entitled to make an informed choice on their behalf if they themselves are unable to do so?  
   [ ] Yes  [ ] No

   **If yes,** that person can make a proxy informed choice for the potential participant. Include an appropriate consent form for that person legally entitled. (Note: Where possible the incompetent person should also orally consent to the level of his or her understanding.)

   **If no,** complete sections 1.1 and 1.2.

   1.1 Is there any person interested in the potential participant’s welfare who knows the participant (eg, family member/friend/whānau) and is willing and available to express a view as to what the potential participant would choose were he or she competent and fully informed about the study?  
      [ ] Yes  [ ] No

   **If yes,** include an information sheet for the family member/friend/whānau statement as per page 24.

      Please note: if it is appropriate that there be wider consultation with family, then this should be encouraged.

   1.2 Explain why it is not possible for a potential participant to make an informed choice and why it is not possible for a proxy choice to be made or for a person interested in the potential participant’s welfare to state what the participant would choose if he or she was competent and fully informed.
2. What would be the risks to the participants of taking part in this study?

There is a risk of ventilator-induced lung injury for anyone on ventilation; however, these preterm babies need artificial ventilation to survive. Based on the results in preterm lambs, ventilation at lower gas flows might lead to less severe lung injury, thus babies in the treatment arm might have better outcomes. Babies enrolled in the study will undergo tracheal suction at a pre-determined time, thus at a time where suction might not be clinically indicated. Suction can be uncomfortable for a baby; however, all babies on a ventilator are exposed to tracheal suctioning to clear airway secretions and maintain a patent airway. Suction is a standard procedure on the neonatal unit and carried out by experienced nurses.

3. Could the research be carried out on people who are able to consent?

[ ] Yes  [x] No

4. Explain why approval is being sought to use this participant/population/patient group.

A study in preterm lambs has demonstrated that lower bias gas flow rates lead to significantly less lung injury and lower expression levels of early response genes in lung tissue, even after only 2 hours of ventilation. The only way to test if lower ventilator gas flow rates are beneficial in preterm babies is to do a trial in this group of patients, who are vulnerable to ventilator-induced lung injury, because of their immature lungs and increased need of ventilation.

5. What is the potential health interest for the group of patients/population of which the participant would be a member?

Mechanical ventilation is a standard treatment for preterm babies; however, the optimum rate for the ventilator bias gas flow is currently unknown and different clinicians use different values. Research in preterm lamb has demonstrated that lower bias gas flow rates are less injurious on the immature lung. If lower gas flow rates are also better during ventilation of the preterm baby, this may potentially lead to shorter duration of mechanical ventilation and reduced incidence of BPD, which will in turn lead to a shorter stay in hospital, less re-hospitalisation, reduced risk of asthma development and reduced health care costs.
STATEMENT BY RELATIVE/FRIEND/WHANAU

Lay title: Flow at lower rates; the FLORA study
Principal investigator: Kitty Bach
Participant’s name: 

I have read and I understand the information sheet dated ___ for people taking part in the study designed to compare ventilation at lower flow rates to ventilation at the standard rate. I have had the opportunity to discuss this study. I am satisfied with the answers I have been given.

I believe that ___ (participant’s name) would have chosen and consented to participate in this study if he/she had been able to understand the information that I have received and understood.

I understand that taking part in this study is voluntary and that my relative/friend may withdraw from the study at any time if he/she wishes. This will not affect his/her continuing health care.

I understand that his/her participation in this study is confidential and that no material which could identify him/her will be used in any reports on this study.

I understand that the treatment will be stopped if it should appear to be harmful (if applicable).

I understand the compensation provisions for this study (if applicable).

I know whom to contact if my relative/friend has any side effects to the study or if anything occurs which I think he/she would consider a reason to withdraw from the study.

I know whom to contact if I have any questions about the medication of the study.

This study has been given ethical approval by the Multi-region Ethics Committee. This means that the Committee may check at any time that the study is following appropriate ethical procedures.

I believe my relative/friend would agree to an auditor appointed by the sponsoring pharmaceutical company and approved by the Ethics Committee reviewing my relative’s/friend’s relevant medical records for the sole purpose of checking the accuracy of the information recorded for the study (if applicable).

I/my relative/friend would like a copy of the results of the study.

[ ] Yes [ ] No
I believe my relative/friend would agree to his/her GP being informed of his/her participation in this study.  [] Yes  [] No

Signed: ___________________________ Date: ___________________________

Printed name: ___________________________

Relationship to participant: ___________________________

Address for results: ___________________________
STATEMENT BY PRINCIPAL INVESTIGATOR

I ___ (name of investigator) declare that this study is in the potential health interest of the group of patients of which ___ (name of participant) is a member and that participation in this study is not adverse to ___ (name of participant)’s interests.

(If applicable)

I confirm that if the participant becomes competent to make an informed choice and give an informed consent, full information will be given to him/her as soon as possible, and his/her participation will be explained. If the participant makes an informed choice to continue in the study, written consent will be requested and if the participant does not wish to continue in the study, he/she will be withdrawn.

Signed: ____________________________  Date: ________________
(Principal Investigator)

(If applicable at a later stage)

I (participant) having been fully informed about this study agree to continue taking part in it.

Signed: ____________________________  Date: ________________
(Participant)

STATEMENT BY INDEPENDENT CLINICIAN

I confirm that participation in the study is not adverse to ___ (participant)’s interests.

Signed: ____________________________  Date: ________________
(Clinician)

Printed name: ____________________________
Appendix IV. FLORA Information Sheet

INFORMATION SHEET
Flow at lower rates; the FLORA study

Investigators:
Dr Kitty Bach, Neonatal Paediatrician. Ext. 25361, mobile 021 228 4802
A/Prof Frank Bloomfield, Neonatal Paediatrician. Ext. 25363

Newborn Services
Auckland City Hospital
Private Bag 92 024
Auckland

While your baby is in the NICU, he or she may need the support of a ventilator to help with breathing. We are doing research to see if babies do better when we reduce the air flow from the ventilator, because ventilation, and also air flow, can lead to some injury of immature lungs. You are invited to enrol your baby in this study. This document gives you more information.

Background to this study
Many babies who are born preterm need mechanical ventilation to support their breathing. Ventilation in these babies is essential for the survival of the baby. However, we also know that ventilation can lead to some injury in the immature lung. About 40% of the preterm babies needing ventilation will develop bronchopulmonary dysplasia, which means that these babies need prolonged respiratory support, i.e. mechanical ventilation or supplemental oxygen. To prevent lung injury or bronchopulmonary dysplasia, several lung protective strategies have been developed, including using the lowest possible pressure and the smallest possible volume during ventilation. To generate pressure and volume, a ventilator applies a gas flow running through the ventilator. During ventilation of a preterm baby, this flow is normally set at a rate of 8-10 L/min, independent of the weight of the baby. There is evidence to suggest that lower flows create less severe lung injury to the immature lung, whilst still supporting the breathing. Furthermore, there is evidence that injury can already occur in the first 5 minutes of mechanical ventilation.
What we propose
We propose to compare a low ventilator gas flow of 4 L/min to a ventilator gas flow of 10 L/min, which is the default setting. We hope to include 128 babies in total, who are either admitted to this unit or other units in New Zealand and Australia. Your baby is eligible for this study, because your baby was born at less than 28 weeks’ gestation or weighs less than 1,000 g at birth. Since injury can occur quickly, it is very important to start the designed flow as soon as your baby needs mechanical ventilation, either after delivery or in the first week of life.

We will discuss this study with you before your baby is born and ask if you agree for your baby to take part in this study. However, if your baby was delivered unexpectedly and if your baby needed mechanical ventilation, we will have randomised your baby to the low or standard gas flow. The importance of starting ventilation at the flow of either 4 L/min or 10 L/min has been discussed with the ethical committee. They approved randomisation of your baby without your initial consent, on the condition that the study is discussed with you, the parent(s), within 24 hours. You are then asked if you allow your baby to continue to take part in the study, or if you do not want your baby to continue in the study.

How will this affect your baby
If you agree your baby is to take part or to continue to take part in this study, we will measure the levels of several inflammatory markers in the fluid collected during suctioning of the endotracheal tube, we will collect clinical data, such as the number of days your baby will be on a ventilator, the number of days your baby needs extra oxygen treatment and how long your baby will be in hospital for and we will collect follow-up data from the medical records made of your baby at follow-up clinics. We will also take 3 blood samples, taken 24, 72 and 120 hours after the start of ventilation, to measure markers that are elevated quickly in response to lung injury. This blood will be collected from an arterial line, if your baby has one, or this blood sample will be combined with blood collection necessary for the clinical care of your baby. Suctioning of the endotracheal tube is a standard procedure on the neonatal unit, if a baby is ventilated. Normally this is done on indication, for example when there are a lot of secretions in the tube. For this study, we will instil 1 mL of saline in the tube, which is immediately retrieved by suctioning. This procedure has to be done at 24, 72 and 120 hours after the start of ventilation, and might thus happen at a time that your baby would normally not have had suction.

If you do not agree for your baby to take part in the study, or if you are not able to make a decision within 24 hours after the onset of mechanical ventilation of your baby, we will start or will continue mechanical ventilation at a flow determined by the clinician caring for your baby and no blood samples or endotracheal suction samples will be collected for research purposes. You are free to withdraw from the study at any time, without having to give a reason, and this will not in any way affect the continuing care to your baby.

Information gained in this study may not be of any direct benefit to you or your baby, but it may help us to best decide what the optimum flow is during ventilation.
Further information
If you would like any further information about the study, you can contact one of the investigators whose contact details are at the top of the previous page, or the nurse or doctor looking after your baby. If you need an interpreter, we will be pleased to provide one.

If you have any queries or concerns about your rights as a participant in this study, you may contact the Health Advocates Trust (Phone 0800 555 050 Northland to Franklin).

You may also contact the Auckland District Health Board Maori Health Services, Mata Forbes RGON, Co-ordinator/ Advisor, Auckland City Hospital, Grafton. Mobile 021 348 432, tel. 307 4949 extn. 23939.

No material which could personally identify you or your baby will be used in any reports in this study. Any information about your baby will be kept strictly confidential. If you wish to be sent a summary of the results of the study, please indicate this by marking the appropriate box on the consent form. We do not expect the results of this study to be available before October 2014.

Compensation
In the unlikely event of a physical injury as a result of your baby's participation in this study, he/she may be covered by ACC under the Injury Prevention, Rehabilitation and Compensation Act 2001. ACC cover is not automatic and your baby's case will need to be assessed by ACC according to the provisions of the 2002 Injury Prevention Rehabilitation and Compensation Act 2001. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses and there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators.

If you have any questions about ACC, contact your nearest ACC office or the investigator.

Ethical approval
This study has received ethical approval from the Multi-region Ethics Committee. The Manager and the Clinical Director of Newborn Services, Auckland City Hospital have given permission for this study to be carried out.

Please feel free to contact the researchers if you have any questions about this study
Appendix V. FLORA Consent Form

CONSENT FORM

Flow at lower rates: the FLORA study

<table>
<thead>
<tr>
<th>English</th>
<th>I wish to have an interpreter</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deaf</td>
<td>I wish to have a NZ sign language interpreter</td>
<td>Yes</td>
<td>No</td>
</tr>
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<td>Māori</td>
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<td>Kao</td>
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<td>Fijian</td>
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<td>Io</td>
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<tr>
<td>Tokelaun</td>
<td>Ko au e sofou ki he tino ke fakalili ike gagana Peletania ki na gagana o na motu o te Pahefika</td>
<td>Ioe</td>
<td>Leai</td>
</tr>
<tr>
<td>Tongan</td>
<td>Oku ou fiema’u ha fakatonulea</td>
<td>Io</td>
<td>Ikai</td>
</tr>
</tbody>
</table>

Consent form

- I have read and understand the information sheet dated 25 May 2010 for my baby to take part in this study comparing ventilation of preterm babies at two different gas flows. I have had the opportunity to discuss this study. I am satisfied with the answers I have been given.
- I have had the opportunity to use whanau support or a friend to help me ask questions and understand the study.
- I understand that my agreeing to my baby taking part in this study is voluntary (my choice) and that I may withdraw him/her from the study at any time and that this will in no way affect his/her continuing care.
- I understand that my baby’s participation in this study is confidential and that no material that could identify my baby or me will be used in any reports on this study.
- I have had time to consider my baby’s participation in this study.
- I know whom to contact if I have any questions about this study.
- I consent to blood samples and tracheal aspirates being taken from my baby for the purpose of this study.
- I consent to medical information to be collected from the medical records made at follow-up clinics after discharge of my baby from the NICU.

This study has been given ethical approval by the Multi-region Ethics Committee. This means that the committee may check at any time that the study is following appropriate ethical procedures.
Appendices

I wish to receive a copy of the results: YES/NO
I .............................................................................................(full name) hereby consent to my
Baby ......................................................................................(name) taking part in this study.
Date
Signature:
Names of researchers: Dr Kitty Bach, Dr Frank Bloomfield
Contact phone numbers of researchers:
(09) 307 4949, Ext 24920 (NICU)
Ext 25361 (Dr. Bach)
Ext 25363 (Dr. Bloomfield)
Project explained by:
Project role:
Signature:
Date:
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