SURFACTANT METABOLISM IN THE NEWBORN; THE IMPACT OF VENTILATION STRATEGY AND LUNG DISEASE

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To my beautiful children, Oscar and Elisa
ABSTRACT

Developmental deficiency in pulmonary surfactant leads to respiratory distress syndrome (RDS) in preterm infants, but all newborns may have impaired surfactant metabolism secondary to lung disease or ventilator induced lung injury. Exogenous surfactant treatment is usually administered in conjunction with mechanical ventilation. If instead surfactant administration is followed by nasal continuous positive airway pressure (nCPAP), the treatment response appears to be more sustained.

The aims of the thesis were to (1) distinguish normal and abnormal surfactant turnover in term and preterm infants using a novel stable isotope technique, (2) determine if high frequency oscillatory ventilation (HFOV) decreases surfactant production in preterm infants with RDS, (3) systematically examine stable isotope methodology for in vivo studies of surfactant metabolism (4) follow-up the implementation of INSURE, i.e. surfactant administration during a brief intubation, and (5) experimentally test the hypothesis, that surfactant administration followed by spontaneous breathing improves the treatment response.

After an intravenous infusion of stable isotope (13C) labeled precursors for surfactant phospholipid, the 13C-enrichment over time was measured in serial tracheal aspirates using gas chromatography/mass spectrometry. Term infants without lung disease had significantly faster endogenous surfactant turnover compared to preterm infants with RDS. Term infants with severe respiratory failure exhibited disrupted surfactant metabolism and decreased amounts of surfactant phospholipids in tracheal aspirates, suggesting delayed maturity of the surfactant system or impairment from the underlying disease. HFOV versus conventional ventilation did not affect the surfactant metabolic indices in preterm infants with RDS. The method yielded reproducible data and similar surfactant metabolic indices regardless of mass spectrometry instrumentation and the surfactant phospholipid pool being analysed. Fractional catabolic rate, which is tracer independent, is suggested to be the primary measure of surfactant turnover.

A retrospective, 10-year follow-up of all inborn infants with RDS (n=420, gestational age ≥27 to <34 weeks) at two Stockholm neonatal units showed that after the implementation of INSURE, the number of infants requiring mechanical ventilation was reduced by 50%, with no adverse effects on outcome. Surfactant treatment by INSURE resulted in a sustained improvement in oxygenation and a significant reduction in additional surfactant doses. In a preterm rabbit model, animals received radiolabeled surfactant and were randomized to spontaneous breathing or mechanical ventilation. The mechanical ventilation group exhibited impaired tissue association of labeled surfactant, lower dynamic compliance and evidence of surfactant inactivation, consistent with a poorer treatment response.

In conclusion, this investigation is one of the first to describe normal surfactant turnover in vivo in term infants. Severe lung disease in term infants disrupts endogenous surfactant metabolism similar to that of infants with developmental surfactant deficiency. Mode of mechanical ventilation has minimal impact on endogenous surfactant turnover in preterm infants with RDS. However, the treatment response to exogenous surfactant is significantly impaired by mechanical ventilation, both clinically and experimentally. The INSURE strategy for surfactant treatment is a powerful approach to improve the treatment response and reduce the need for mechanical ventilation in moderately preterm infants.
This thesis is based on the following papers, listed in chronological order. Papers will be referred to by their Roman numerals:


III. Bohlin K, Bouhafs RKL, Jarstrand C, Curstedt T, Blennow M, Robertson B. Spontaneous breathing or mechanical ventilation alters lung compliance and tissue association of exogenous surfactant in premature newborn rabbits, Pediatric Research, May 2005, in press.


V. Bohlin K, Gudmundsdottir T, Katz-Salomon M, Jonsson B, Blennow M. The implementation of surfactant treatment during continuous positive airway pressure in moderately preterm infants - a population-based follow-up, manuscript.
LIST OF ABBREVIATIONS

a/A ratio  arterial to alveolar ratio
APE    atom percent excess
BAL    bronchoalveolar lavage
BPD    bronchopulmonary dysplasia
CLD    chronic lung disease
CV    conventional ventilation
DPPC   dipalmitoylphosphatidylcholine
DSPC   disaturated phosphatidylcholine
DSPL   disaturated phospholipid
ECMO   extra corporeal membrane oxygenation
Emax   maximum enrichment
FCR    fractional catabolic rate
FiO2   fraction of inspired oxygen
FSR    fractional synthetic rate
GC/C/IRMS gas chromatography/combustion/isotope ratio mass spectrometry
GC/MS  gas chromatography/mass spectrometry
HFOV   high frequency oscillatory ventilation
HMD    hyaline membrane disease
IVH    intraventricular haemorrhage
KH    Karolinska Huddinge
KS    Karolinska Solna
MAP    mean airway pressure
MAS    mechonium aspiration syndrome
MIDA   mass isotopomer distribution analysis
MST    microbubble stability test
nCPAP  nasal continuous positive airway pressure
NICU   neonatal intensive care unit
PC     phosphatidylcholine
PDA    patent ductus arteriousus
PE     Phosphatidylethanolamine
PEEP   positive end expiratory pressure
PG     phosphatidylglycerol
PI     phosphatidylinositol
PL     phospholipid
PS     phosphatidylserine
RDS    respiratory distress syndrome
ROP    retinopathy of prematurity
SP     surfactant protein
T1/2   half-life
TA     tracheal aspirate
Tapp   time of appearance
Tmax   time of maximum enrichment
TTR    tracer to tracee ratio
My interest in neonatology sparked off when I, in the last year of medical school, got the chance to do my pediatric rotation at Vanderbilt University, Tennessee. The rapid pace in the NICU and the everyday drama attracted me. The tiny size of the patients never frightened me, being on the small side myself. And the sheer happiness when a baby so sick you didn’t even know if he was going to survive miraculously recovered, convinced me forever. Surfactant is a medicine that does just that, works like magic in the right setting. So choosing the focus of my research was not difficult.

With my mind set, I continued through my internship in Skövde, a small town in southern Sweden, by a short detour at the Albert Schweitzer Hospital in Gabon, to Stockholm. Here, I met Mats Blennow who said: “Welcome to the best part of medicine – the beginning of life!” And my choice of supervisor was done.

Like most people, we started off with a project that never mounted to anything. At the time, Mats had just started implementing INSURE at our unit and was struck by how well these babies were doing. Together with the true experts, Bengt Robertson and Tore Curstedt, we set out to test INSURE in a rabbit model. It was agony for many months trying to get the premature rabbit pups to survive breathing spontaneously. We tried everything, including lots of TLC (tender loving care) and finally we got the model working. Almost done, my work at the surfactant lab was interrupted for a couple of years, when the family moved to St Louis. There, my husband did his post-doctoral fellowship and I got the fantastic opportunity to work with Aaron Hamvas and FS Cole at Washington University. Now my focus shifted towards the metabolism of endogenous instead of exogenous surfactant. In an incredibly stimulating scientific atmosphere and together with some of the most brilliant people I will probably ever work with, I got to explore the mysterious ways of pulmonary surfactant even further. It was a great project, involving both patient recruitment in the large and always busy NICU and quiet pipetting by the radio in the lab.

After more than two years and another baby of my own, we were back home in Stockholm and I decided to find out what had happened since we had started with the INSURE treatment five years earlier. The results from that and the other studies have become this thesis, which I hope elucidates some important aspects of both exogenous and endogenous surfactant metabolism. My research has been hard work and dark despair sometimes, but also intensely rewarding and fun. I have enjoyed writing this book (most of the time) and I hope you will enjoy reading it.
INTRODUCTION

PULMONARY SURFACTANT

History

The story of surfactant research begins in 1929 with the publication by von Neergaard stating that lowering the surface tension of the air/liquid interface stabilized the alveoli. This observation remained un-pursued for several years until 1955 when Pattle described an insoluble layer that could abolish the tension of the alveolar surface. A couple of years later Clements demonstrated that compression of surface films from animal lung extracts lowered surface tension, which provided for the first definition of surface active material from the lung. Already in 1903, Hochheim reported on hyaline membranes in the lungs of infants with respiratory distress. In the late 1940s and 1950s, hyaline membrane disease (HMD) was recognized as the most common cause of death in premature infants. The hallmark of the disease, the histological finding of hyaline membranes, was not seen at birth but was formed soon after as a result of atelectasis and lung injury. Gruenwald first proposed the linkage between elevated surface tension and hyaline membrane formation in 1947. It was confirmed in 1959 when Avery and Mead showed that lung extracts from premature infants dying of HMD were unable to lower surface tension and associated this with deficiency of surface active material. In the 1960s, pulmonary surfactant underwent further biochemical and functional characterization. The high content and functional importance of disaturated phosphatidylcholine was described. Gluck et al. demonstrated that surfactant deficiency was linked to the immaturity of the fetal lung and could be predicted by the lecithin/sphingomyelin ratio in amniotic fluid. During the 1970s, ground-breaking experimental work of surfactant replacement in animal models performed by Robertson and Enhörning led up to the first successful trial of endotracheal surfactant administration to preterm infants with respiratory distress syndrome (RDS) in 1980 by Fujiwara et al. During this time, proteins were also recognized as important constituents of surfactant and prenatal steroid treatment was first reported to decrease the incidence and severity of HMD in premature infants. The efficacy and safety of surfactant therapy was further established by several multi-centre trials and proven to dramatically decrease neonatal mortality and serious pulmonary air-leak syndromes. In 1990 the American Food and Drug Administration approved the clinical use of exogenous surfactant that has since become one of the cornerstones in the care of preterm infants with respiratory distress syndrome.
Composition

Surfactant lipids

Pulmonary surfactant is a complex mixture composed of two main fractions: lipids and surfactant-specific proteins (Fig. 1). The lipids constitute the major part, approximately 90%, of which the largest portion are phospholipids (80-90%) and the remaining portion are neutral lipids, primarily cholesterol. The phospholipids are to 70-80% made up by phosphatidylcholine (PC), of which around 60% contain two saturated fatty acids, mainly palmitic acid, i.e. dipalmitoylphosphatidylcholine (DPPC) \(^{25}\). Saturated PC is predominantly present in surfactant and to a much lesser extent in lipid fractions of the lung not associated with surfactant. DPPC is the principal surface-active component of pulmonary surfactant and can therefore be used as a relatively specific marker of surfactant metabolism. The second major phospholipid in the mature lung is phosphatidylglycerol (PG), constituting around 10% of surfactant phospholipids. Another 10% of the phospholipids are made up of phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE). Surfactant from immature lungs contains relatively large amounts of PI instead of PG and the ratio of PG/PI can serve as a marker for lung maturity \(^{26}\). The lipid composition of surfactant recovered from lungs of a large number of mammalian species exhibits a highly consistent pattern \(^{25}\).

![Figure 1. Composition of pulmonary surfactant](image)

DPPC (dipalmitoylphosphatidylcholine) is the predominant lipid and the principal surface-active component of the pulmonary surfactant complex. Unsaturated PC (phosphatidylcholine), other phospholipids e.g. PG (phosphatidylglycerol), PI (phosphatidylinositol), PS (phosphatidylserine), PE (phosphatidylethanolamine) and neutral lipids constitute the remaining lipid fraction. The surfactant proteins (SP-A, SP-B, SP-C and SP-D) constitute approximately 10% of pulmonary surfactant.
Surfactant proteins

Pulmonary surfactant contains four specific proteins, termed surfactant protein (SP)-A, SP-B, SP-C and SP-D, comprising approximately 10% of surfactant. SP-A and SP-D are hydrophilic and SP-B and SP-C are extremely hydrophobic. SP-A is a lectin-like peptide encoded on human chromosome 10. It is required for tubular myelin formation and plays a role in phospholipid uptake and secretion. SP-A also functions as an important non-immune host defence protein. The SP-B gene is located on human chromosome 2. The peptide is found in the lamellar bodies and is secreted together with the phospholipids. The most crucial function of SP-B is to facilitate the adsorption and spreading of lipids at the air-liquid interface, thereby greatly enhancing the formation of a stable surface film. SP-B also interacts with SP-A in the formation of tubular myelin. SP-C is a small peptide encoded on chromosome 8 that in conjunction with SP-B promotes insertion of phospholipid into the air-liquid monolayer. SP-D is not exclusively produced in the lung and less than 10% is associated with surfactant phospholipids. Like SP-A, SP-D is a lectin-like peptide with immunomodulatory properties. The role of SP-D in surfactant function is not clear, but it may be involved in phospholipid homeostasis.

Metabolism

Synthesis and secretion

The phospholipids and proteins of pulmonary surfactant are synthesized in the alveolar type II cells, assembled in lamellar bodies and extruded into the alveolar lumen by exocytosis. The lamellar bodies unravel to form loose lipid arrays and tubular myelin, a lattice-like structure serving as a precursor, or a reservoir, for the surface film at the air-liquid interface. The phospholipids and proteins are subsequently degraded and recycled back into the type II cells or phagocytized by macrophages. This review will focus primarily on phospholipid metabolism. The phospholipids are composed of a glycerol backbone, two fatty acids and a polar phosphorylated moiety. The fatty acids required for surfactant lipid synthesis may be recruited from the circulation as free fatty acids or as triacylglycerols in lipoproteins. Fatty acids may also be synthesized de novo by the type II cells, from several precursors e.g. glucose, lactate and acetate. The relative contribution of de novo synthesized fatty acid and preformed fatty acid from the circulation is only partly elucidated and probably varies with development and nutritional status. In adult pigs, preformed fatty acids constitute the primary source for surfactant PC. Experimental data suggest that de novo synthesis may be of greater importance for phospholipid formation in the perinatal period. Lactate has been proposed as the preferred substrate in vitro, however in the late fetal period and in the neonate, glycogen and acetate are reported to be important fatty acid precursors. PC is synthesized in the endoplasmatic reticulum through a series of biochemical events beginning with the formation of phosphatidic acid, which is hydrolysed to diacylglycerol and together with CDP-choline form PC. A deacylation-reacylation process to yield DPPC remodels the PC-molecule.

The regulation of phospholipid synthesis in the lung is influenced by developmental and hormonal factors affecting various rate-limiting metabolic steps. Corticosteroids and thyroid hormones enhance the activity of several enzymes within the...
phospholipid synthetic pathway and insulin, epidermal growth factor and beta-adrenergic drugs influence lung maturation and surfactant production 36,39.

After synthesis in the endoplasmatic reticulum, pulmonary surfactant is transferred from the Golgi system by vesicular transport into the lamellar bodies. The lamellar bodies are the storage granules of surfactant and are a characteristic feature of the alveolar type II cell 40. A mature lamellar body consists of a limiting membrane surrounding about 20-70 tightly packed phospholipid bi-layers, or lamellae, arranged in a hemisphere 41,42(Fig. 2). They develop from small multi-vesicular bodies and enlarge by accumulating lamellae. The largest lamellar bodies are found near the alveolar surface.

Surfactant is secreted into the alveoli by exocytosis of the lamellar bodies. Cholinergic and beta-sympathomimetic agents, calcium ionophores, purine agonists and arachidonic acid metabolites stimulate this process 43-46. However, the most important stimulus for surfactant release is the onset of breathing, causing repetitive alveolar stretching 47-49. In the alveolus, the lamellar bodies unravel and form tubular myelin. The tubular myelin is a highly surface-active lipoprotein array of phospholipid bi-layers with SP-A at the corners of the lattice. The unique structure also requires SP-B and calcium. From the tubular myelin, the surface film at the air-liquid interface is formed. In addition, bi-layer complexes are formed serving as a surface
surfactant reservoir, directly participating in the transfer of surfactant material in and out of the monolayer at the interface. Compression of the surface film during expiration forces molecules of the surfactant monolayer to be squeezed out. Consequently, the monolayer is refined and the molecules retained in the surface film are predominantly DPPC. The shape and orientation of the DPPC molecules with the long, straight, saturated fatty acid residues generates a very stable layer with a surface tension close to zero when compressed, thus preventing the alveoli from collapse.

Recycling and turnover

During respiration, the cyclic changes in surface area generate small vesicular forms of surfactant representing worn-out, inactive surfactant. This subfraction of small aggregates can be separated by centrifugation of lung lavage fluid from the subfraction of heavy, large aggregate forms containing more surface active material, i.e. tubular myelin, lamellar bodies and proteins. Measurement of aggregate conversion is used experimentally to estimate surfactant inactivation. Small aggregates represent used surfactant destined for clearance and re-uptake into the alveolar type II cell and an increasing fraction of small aggregates correlates with lung injury. In the newborn, only small amounts are degraded by macrophages or lost via the airway. The surfactant lipids that are recycled back into the type II cell can either be catabolized in lysosomes or reutilized intact in the lamellar bodies. The lysosomal degradation products are reused for de novo lipogenesis or lost from the type II cell. In the adult lung, macrophages or lysosomal pathways catabolize approximately 50% of the surfactant phospholipids and 50% is recycled back into lamellar bodies for resecretion into the alveoli. In contrast, animal data suggest that, in the newborn lung the efficiency of recycling is greater than 90% and the catabolism is minimal. Knowledge on surfactant recycling in humans is limited.

Surfactant pool sizes

The amount of surfactant lipid in lung tissue and airspaces changes dramatically with development. In 1970, the endogenous surfactant pool measured in alveolar lavage fluid from infants who died of RDS was found to be on average 5 mg/kg. However, direct measurements are difficult to perform in humans. Instead, surfactant pool size can be estimated using the Fick principle, i.e. after intratracheal administration of a marker, the distribution at time zero is calculated by extrapolating the line describing the exponential dilution of the marker, assuming complete mixing of the alveolar and intracellular surfactant pools. By administering exogenous surfactant containing phosphatidylglycerol (PG) to preterm infants with RDS having no PG in their endogenous surfactant, the pool size was estimated to about 9 mg/kg. Assuming that approximately 50% of the exogenous surfactant is rapidly tissue associated, the alveolar pool size in preterm infants with RDS would be close to 4 to 5 mg/kg. By this approach, eight preterm infants with RDS receiving stable isotope labeled PC in surfactant treatment doses were found to have an apparent pool size of 5.8 mg/kg. The validity of the apparent pools size using the Fick principle has been demonstrated in preterm ventilated baboons by comparison with direct measurements at autopsy. In preterm monkeys the alveolar pools size was measured to about 5 mg/kg by lavage, increasing to about 100 mg/kg in 3-day old term monkeys. Preterm rabbits and lambs are also reported to have surfactant pool sizes of 3-10 mg/kg; hence the pools size estimates appear to be fairly
consistent across species. There is a progressive increase in surfactant pools with gestation to about 100 mg/kg at term, which then subsequently decreases to about 60 mg/kg in lung tissue and 4 mg/kg in airspaces of the adult human. After preterm birth and during the recovery phase of RDS the surfactant pool sizes increase towards normal values over a 4 to 5-day period. An increasing concentration of DPPC in airway suction samples from infants recovering from RDS, reaching values comparable to those of normal infants, has been reported. Therefore, the PC concentration in airway samples are sometimes used as a reflection of pool size, at least for comparative purposes (paper II). Components of surfactant may also be quantified on the basis of a reference compound. The ratio of saturated PC (lecithin) to sphingomyelin, i.e. the L/S ratio, is the most widely used. Additional markers are PG and SP-A levels in airway samples, as these surfactant components are decreased in RDS. In kinetic studies of surfactant metabolism, the surfactant system is most often assumed to be at steady state. This assumption is probably not entirely correct. Apart from developmental changes in surfactant pool size, ventilatory management, lung injury and lung disease can alter the pool size and thereby affect the measurements. Very preterm baboons showed a 4.5 fold increase in total surfactant pool size over 6 days of mechanical ventilation and thermally injured pigs exhibited a significantly reduced PC-pool size in alveolar washes. Infants developing BPD showed higher apparent surfactant pool sizes compared to those not developing BPD and the saturated PC pool size of infants with CDH was lower, about 37 mg/kg, compared to about 59 mg/kg in term control infants at 4-5 days of age. Hence, it is essential to always consider possible differences in pool size when interpreting data on surfactant metabolism in the newborn.

**Kinetics**

The development of stable isotope technique made possible in vivo studies of surfactant metabolism in newborn infants. Previously, in vitro studies in fetal and neonatal lung sections had shown increasing surfactant synthesis towards the end of gestation. In animal models, the synthesis and clearance of surfactant was measured using radioactively labeled surfactant precursors as tracers, an approach not ethically and medically acceptable in humans. Following intravascular injection of a radiolabeled precursor in preterm lambs, the accumulation in alveolar lavage PC was slow, reaching maximum enrichment at about 40 hours post-injection. In rabbits, labeled endogenous surfactant was cleared very slowly with a $T_{1/2}$ of more than 150 hours in preterm animals, 50 hours in 3-day old newborn rabbits and only 20 hours in adult rabbits. In term lambs, the $T_{1/2}$ was about 50 hours after i.v. administration of radiolabeled palmitate and in preterm ventilated lambs, intratracheal trace doses of radiolabeled surfactant was cleared from the alveolar space but virtually no loss from total lung (i.e. including lung tissue) was detected, consistent with minimal catabolism and surfactant recycling. In the preterm and the term rabbits, the efficacy of recycling exceeded 90% and surfactant turnover times were longer compared to adult animals. In summary, animal studies using radioactively labeled tracers show that surfactant metabolism (the end result of synthesis, secretion, recycling and clearance) is a slow process in the newborn. Stable isotope technique to study surfactant metabolism has been applied in adult pigs receiving 4-8 hours of intravenous infusion with $^{13}$C-labeled acetate, as a substrate for de novo fatty acid synthesis and uniformly labeled $^{13}$C-palmitate to trace incorporation of preformed fatty acids. The results showed that plasma free fatty acid was the primary source of palmitate for...
Surfactant metabolism in the newborn

Figure 3. Tracer incorporation into surfactant phospholipid.
Stable isotope labeled precursors of DPPC (disaturated phosphatidylcholine) are administered as 24-h intravenous infusions. [U-13C6] glucose and [1-13C1] acetate are incorporated into palmitate by de novo fatty acid synthesis in the alveolar type II cell. [1,2,3,4-13C4] palmitate is taken up from plasma as a circulating free fatty acid and directly incorporated into DPPC.

Surfactant PC synthesis. The fractional synthetic rate from plasma palmitate was approximately 36% per day compared to 3% per day for acetate. About 90% of the secreted PC was recycled back into the lamellar bodies. In 1998, Bunt et al. performed the first human study using this technique. To measure de novo synthesis of endogenous surfactant PC, six preterm infants with a mean gestational age of 28 weeks received a 24-hour infusion of uniformly labeled 13C-glucose. The maximum 13C-enrichment in palmitate from serial tracheal aspirate samples was found to be 48-96 hours after the start of infusion and the T1/2 ranged from 87-144 hours, consistent with the values of preterm lambs and rabbits. The mean FSR from glucose was 2.7% per day (similar to that found for acetate in pigs). In 1999, Cogo et al. used uniformly labeled 13C-palmitate and linoleic acid to study a group of critically ill infants and found FSR values ranging from 10-82% per day and T1/2 values of 17-178 hours. The somewhat faster synthesis rate from plasma palmitate compared to glucose was in line with the findings of Martini et al. in pigs. The wide range of values is likely due to variations in gestational age, age at the start of the study, mechanism of respiratory failure and disease severity, which were not assessed at the time. The power of this technique to provide information about synthesis and clearance rates and relative contributions of specific precursors to pulmonary surfactant metabolism in newborn infants generated several hypotheses, some of which are addressed in this thesis.

Complete interpretation of the data requires normal values of surfactant turnover. A limitation of the technique in newborn infants is that the only realistic samples are tracheal aspirates. Thus, airway access is needed, requiring intubation. The long infusion time...
for the tracer and the slow nature of surfactant turnover also require that the infant remains intubated for at least 3-4 days for adequate data collection. Hence, studying infants with normal lung function is difficult. The impact of ventilation strategies and severity of lung disease on surfactant metabolism are additional factors that are important to evaluate. Since the first study using stable isotope labeled tracers in 1998, data on the kinetics of surfactant metabolism is available from studies in newborn infants 59,68,74,75,83-90, pigs 35,73,91 and baboons 60,92. To date, three distinct tracers have been used to label endogenously synthesized surfactant phospholipid; glucose and acetate to label acetyl-CoA for de novo lipogenesis, or palmitate that is directly incorporated into DPPC (Fig. 3). Via intravenous administration of an endogenous tracer we have learned that prenatal corticosteroid treatment stimulates surfactant synthesis 86, that exogenous surfactant treatment does not impair endogenous surfactant synthesis 85,90 and that endogenous surfactant synthesis is preserved in infants with congenital diaphragmatic hernia 68,75. Intratracheal administration of tracer has allowed estimation of surfactant pools size 59,74,75,87,88, and the recently described dual tracer infusion protocol provides a mean to more fully interpret the surfactant kinetic indices 75. However, in the above mentioned studies several methodological approaches have been used. There are variations regarding the mass spectrometry instrumentation, the precursor pool for FSR-calculations, the sample processing and the subsequent subfraction of surfactant analyzed. To what extent these variations affect the kinetic measurements is important to determine for accurate comparison of results.

**NEONATAL LUNG DISEASE**

**Epidemiology**

Pulmonary disorders represent one of the most common diagnoses in infants admitted to a neonatal unit. The overall incidence of any form of acute lung disease in the newborn is reported to be between 2.1 and 3.3% 93-96. RDS and transient tachypnoea of the newborn (TTN) are the most common specific diagnoses, followed by infection/pneumonia 93,95. As expected, the incidence of both unspecified respiratory disorders and RDS increases with decreasing gestational age and birth weight 93,97. In infants with birth weight between 501 and 1500 g more than 50% have signs of RDS, increasing to almost 90% in infants below 750 g 98,99. Over the last three decades neonatal care has changed dramatically. Improvement in ventilatory support, including CPAP, antenatal corticosteroid treatment and the introduction of exogenous surfactant replacement are major contributors to the greatly reduced morbidity and mortality from neonatal lung disease. Antenatal corticosteroid treatment clearly reduces the incidence of RDS in randomized control trials 100,101. However, this is not reflected in the few population-based, epidemiological trials available. Data from the late 1970s and the 1990s report a similar overall incidence of RDS of about 1% 95,96. In the Swedish study by Hjalmarson et al. from 1976-77, no significant difference was found in the RDS incidence at any gestational age in hospitals with and without a maternal corticosteroid program 93. However, the impact on disease severity is not addressed in these studies and the increasing numbers of viable, extremely premature infants may affect the incidence numbers. In a recent study from northern Finland the overall incidence of RDS did not change significantly during 1990-95 compared to 1996-99, although a shift towards the more immature infants was noted 102. Surfactant
replacement significantly reduces mortality in treated infants with RDS. The introduction of surfactant therapy in the United States was reflected in an accelerated reduction in mortality from RDS and found to be the single most important factor for reduction in overall neonatal mortality rate in the early 1990s.

**Respiratory Distress Syndrome (RDS)**

*Respiratory Distress Syndrome (RDS)*

**Lung development**

The normal development of the lung is divided into three periods: embryonic, fetal and postnatal. During the embryonic period the lungs first appear as a protrusion from the foregut at approximately 26 days of gestation. The lung bud branches and by 42 days the airways to the lobar and initial segmental bronchi have formed. The fetal period can be subdivided into the pseudoglandular, canalicular and saccular stages. The pseudoglandular stage, lasting from about the 7th to the 17th week of gestation, is characterized by further airway branching to the level of future alveolar duct and epithelial lining of simple, glycogen-containing cuboidal cells. During the canalicular stage, between the 16th and 25th week, epithelial differentiation with the development of the potential air-blood barrier and the start of surfactant synthesis take place. Many of the cells at this stage are intermediary cells with characteristics of both mature type I and type II epithelial cells. After about 20 weeks characteristic osmiophilic lamellar bodies appear in the cytoplasm together with smaller multivesicular forms that are precursors to lamellar bodies. As the number of lamellar bodies increases in these type II cells the glycogen content decreases as it provides a substrate for surfactant synthesis. The saccular stage, from about 25 weeks to term, exhibits the final branching of the airspaces and the initiation of alveolarization. This results in a progressive increase in surface area and lung volume, providing the capacity of sufficient gas exchange and viability after birth. The saccular stage overlaps with the alveolar stage, which continues into the postnatal period. However, many factors can delay or interfere with alveolarization, such as mechanical ventilation, both antenatal and postnatal glucocorticoid treatment, pro-inflammatory cytokines and poor nutrition. Lung development continues to 1 to 2 years of age. Thereafter subsequent lung growth occurs by increase in airway and alveolar size.

*Pathophysiology*

RDS is caused by a developmental deficiency in pulmonary surfactant. In addition, RDS is associated with delayed absorption of fetal lung water due to defective sodium transport mechanisms. Although the synthetic pathways for surfactant are present, the number of type II cells and the surfactant stores are insufficient for adequate respiration until approximately 32 weeks of gestation. In consequence, the greatest risk factor for RDS is prematurity, but additional factors include maternal diabetes and perinatal asphyxia. The elevated surface tension resulting from surfactant deficiency leads to alveolar collapse at the end of expiration. Atelectasis leads to uneven inflation and regional alveolar overdistension, producing epithelial injury and pulmonary edema. The leakage of plasma proteins into the alveolar space further aggravates surfactant deficiency by inactivation. The characteristic eosinophilic hyaline membranes seen lining the airways histologically, are derived from fibrinous material from blood and cell debris. Ventilation-perfusion mismatch gives rise to
intrapulmonary shunting and hypoxemia-hypercapnia producing respiratory acidosis that further aggravates pulmonary hypoperfusion and hypoxemia. Superimposed lung injury from mechanical ventilation and high concentrations of inspired oxygen may trigger the release of pro-inflammatory cytokines and further impair surfactant synthesis and function, as well as predispose to the development of chronic lung injury 115.

**Clinical features**

The onset of breathing at birth stimulates a surge in surfactant secretion 48. Typically, the preterm infant with RDS exhibits only mild respiratory distress immediately after birth. Because of the reduced surfactant pool size, and partly due to secondary surfactant inactivation, the symptoms worsen within the first few hours after birth. The classic features of RDS are tachypnoea, expiratory grunting, subcostal and intercostal retractions, nasal flaring and cyanosis without supplemental oxygen 116. The chest radiograph exhibits low inflation volumes and generalized atelectasis producing a symmetric, diffuse reticulogranular pattern known as “ground-glass appearance” with superimposed air-bronchograms 117. Even in the very preterm infants the type II cells mature rapidly after birth with increasing synthesis of endogenous surfactant. In the uncomplicated course of RDS the severity of symptoms usually peaks by day 2 or 3, with onset of recovery by 72 hours of age if untreated 118. Before modern neonatal care mortality was high. Treatment with early CPAP and mechanical ventilation with PEEP improves oxygenation and prevent atelectasis, thereby alleviating the symptoms 119,120. Surfactant therapy shortens the disease course and significantly increases survival 20.

**Other respiratory disorders affecting the surfactant system**

**Meconium aspiration syndrome (MAS)**

MAS is predominately seen in term or post-mature infants 121. Fetal distress is believed to trigger the passage of meconium into the amniotic fluid and may also induce fetal gasping. Meconium present in the large airways at birth rapidly migrates distally after the onset of breathing and causes plugging and air-trapping. It acts as an irritant resulting in a chemical pneumonitis that predisposes to bacterial lung infections. Meconium also directly inhibits pulmonary surfactant function by increasing the minimum and maximum surface tension and lowering the surface-spreading rate in a concentration dependent way 122,123. Whether surfactant composition is altered in MAS remains a matter of controversy. Studies using animal models of MAS have found decreased levels of SP-A and SP-B, but unaltered levels of phospholipids 124. In infants with MAS, phospholipid and SP-A levels have been reported to be both increased and similar to controls subjects 125,126. Jansen et al. measured PC pool sizes by endotracheal administration of isotopically labeled surfactant and found no differences between infants with MAS and congenital diaphragmatic hernia (CDH) on ECMO (extra corporeal membrane oxygenation), but lower values compared to non-ECMO patients without significant lung disease 88. Data on surfactant kinetics in the presence of meconium is very limited. In vitro, low concentrations of meconium were reported to increase surfactant secretion but not synthesis, and high concentrations were shown to be toxic to the cultured type II cells 127. In vivo, the damaged cells may release inflammatory cytokines contributing to the inhibitory effect on surfactant 128. The only study to date of endogenous surfactant metabolism in human infants...
Surfactant metabolism in the newborn

with MAS was performed with stable isotope technique and showed lower fractional synthetic rate from glucose in MAS patients compared to controls, which was also reflected in lower PC concentration in tracheal aspirate fluid, suggesting that disturbances in surfactant kinetics may contribute to the pathophysiology of MAS 129. 

Surfactant therapy has beneficial effects in MAS, but higher dosing is often required 130. The ability of the exogenous surfactant to resist inactivation determines the clinical response in MAS and natural or protein-containing surfactants are therefore superior to synthetic surfactant preparations 131. No clear consensus has yet been reached regarding the mode of administration. Repeated boluses as tracheal instillations are the standard method. Surfactant lavage to remove meconium and other debris from the lungs has shown promising results in animal models and limited clinical studies. However, a recent multicenter randomized controlled trial of diluted surfactant lavage followed by a more concentrated lavage found no significant improvements compared to infants receiving standard care 132.

Congenital diaphragmatic hernia (CDH)

CDH results in pulmonary hypoplasia due to an overall reduction in bronchial and vascular branching and in alveolar development 133. Pulmonary hypertension and severe respiratory insufficiency are the hallmarks of the disease. CDH-lungs are immature and morphologically resemble RDS-lungs 134. Whether primary surfactant deficiency is present remains unclear. Surfactant PC pool size was not different in CDH-infants requiring ECMO compared to a mixed non-ECMO group 88. However, the control group had severe lung disease, which might have prevented detection of significant differences. Cogo et al. showed reduced concentrations of disaturated PC (DSPC) in epithelial lining fluid from infants with CDH compared to controls without significant lung disease and demonstrated a smaller apparent pool size in mechanically ventilated CDH infants after endotracheal administration of trace doses of stable isotope labeled DPPC 68,75,87. The smaller surfactant pool size is thought to be a consequence of the reduced surface area in hypoplastic lungs. However, available data does not suggest an impaired surfactant synthesis. Surfactant kinetic studies in CDH have revealed unaltered fractional synthetic rate from plasma palmitate 68 and, using a dual tracer method with simultaneous administration of intratracheal and intravenous label, no differences in the net-DSPC synthesis were found in CDH infants compared to age-matched controls 75. Instead, the T1/2 and surfactant turnover rate were reported to be significantly faster in CDH infants. A high percentage DSPC catabolism/recycling was associated with severe disease and longer duration of mechanical ventilation 75. Whether this phenomenon is a primary feature of CDH-lungs or secondary to treatment interventions and whether increased catabolism is responsible for the reduced surfactant pool size remains to be further investigated. Several recent studies have failed to show any benefits of surfactant replacement therapy to infants with CDH; hence surfactant treatment in its current form remains controversial in CDH 48,135.

Neonatal pneumonia

Congenital bacterial pneumonia is a common cause of respiratory distress in newborn infants. The inflammatory reaction evoked by the lung infection leads to cytokine release and formation of reactive oxygen metabolites, resulting in damage to the alveolar-capillary barrier. This in turn leads to leakage of plasma proteins into the alveoli that directly inhibit surfactant 114. Neutrophils stimulated by group B streptococcus (GBS), one of the most common pathogens for congenital pneumonia, cause lipid peroxidation and impaired surfactant
The notion that surfactant inhibition has an important role in respiratory failure caused by congenital pneumonia is also demonstrated by the fact that minimal surface tension was elevated in tracheal aspirates from preterm infants with pneumonia. Exogenous surfactant treatment in neonatal pneumonia has been evaluated both experimentally and clinically. Surfactant treatment of adult rats with experimental E. Coli pneumonia also resulted in improved oxygenation. In newborn rabbits with experimental GBS pneumonia, exogenous surfactant reduced bacterial proliferation and improved lung function. Inactivation of SP-A, believed to be important in the pulmonary antimicrobial defense, did not influence the bacterial proliferation in the rabbit model. Other studies have suggested that exogenous surfactant may promote bacterial growth by reducing the capacity of alveolar macrophages to eliminate GBS. These slightly conflicting data needs further study and the clinical significance remains to be evaluated. In human neonates with severe respiratory failure due to GBS pneumonia, surfactant instillation improved gas exchange, but the response was slower than in non-infected infants with RDS and multiple surfactant doses often required. In accordance with the findings in MAS, this may suggest that surfactant inhibition is an important contributor to the respiratory distress in neonatal pneumonia. In experimental models of endotoxin induced lung injury there are evidence of decreased extracellular phospholipid levels. Human data on surfactant synthesis and turnover in neonatal pneumonia are limited.

**Genetic mechanisms of surfactant dysfunction**

Mounting evidence supports the idea that genetic variations together with environmental factors influence the susceptibility to RDS and to other neonatal lung diseases. Specific alleles on the SP-A gene are linked to high risk for RDS, SP-A and SP-B gene variants interact, also leading to increased susceptibility to RDS, and polymorphisms of collectins (SP-A and SP-D) may be associated with post-natal lung infections. Since several hundreds of genes are involved in the pulmonary development, adaptation to air breathing, oxygen uptake and lung defense, understanding of the genetic regulation of surfactant function is a great challenge for the future. To date, three single gene disorders resulting in surfactant deficiency have been described.

**Surfactant protein B deficiency**

Surfactant protein B deficiency was the first inherited disorder of surfactant metabolism to be described. The clinical presentation is typically a full-term infant with symptoms and radiographic signs resembling those of preterm infants with RDS. The family history is often notable for previous neonatal deaths. The disease is progressive and refractory despite maximum ventilatory support and surfactant treatment. Death usually occurs within 3-6 months. The only effective treatment is lung transplantation. Histopathology often reveals alveolar proteinosis representing an accumulation of abnormal pulmonary surfactant in the alveolar space, but can also show non-specific interstitial fibrosis and type II cell hyperplasia. Immunohistochemically, absence of SP-B and abundant amounts of SP-A and pro-SP-C are characteristic features. The incomplete processing of pro-SP-C peptides may lead to additional deficiency in mature SP-C. Ultrastructural changes include markedly abnormal lamellar bodies. Sp-B deficiency is inherited in an autosomal-recessive fashion and is most commonly caused by a frame-shift mutation in the SP-B gene (codon 121). However,
several other, often family-unique, SP-B mutations have been identified, some of which may result in only partial deficiency. The carrier frequency of the common mutation has been estimated to 1 per 1,000, but the exact incidence is not known. Adult carriers appear to have normal lung function, although the heterozygous state may be a risk factor for lung disease. Heterozygous mice, expressing approximately 50% of normal SP-B, are more susceptible to hyperoxic lung injury. SP-B knockout mice unable to produce SP-B die within minutes after birth. However, despite grossly abnormal lung structure, the phospholipid content of the lungs and the incorporation of labeled choline and palmitic acid into saturated PC was unaltered compared to normal mice. In vitro studies of lung explants from SP-B deficient infants showed normal synthesis rates for phospholipids. The only in vivo study of surfactant metabolism in SP-B deficient infants reported similar FSR and half-life after 24-hour infusions of labeled glucose. Thus, to date there is no clear evidence of disturbed surfactant lipid turnover related to SP-B deficiency.

Surfactant protein C deficiency

Abnormalities in the SP-C gene have only recently been described and linked to lung disease. In contrast to SP-B deficiency, mutations in the SP-C gene are associated with wide variations in phenotype and result in chronic lung disease rather than in acute respiratory failure. Characteristically there is a family history of interstitial lung disease. Different mutations exhibit considerable variability in lung pathology and disease severity within the affected families. The age of onset range from infancy to the 6th decade of life but some individuals may be asymptomatic. Inheritance in an autosomal-dominant fashion as well as de novo mutations have been described. The incidence and prevalence are unknown. The pathophysiology of the disease remains incompletely elucidated but likely results from either lack of mature SP-C or abnormal pro-SP-C, exposing hydrophobic epitopes that are directly toxic to alveolar epithelial cells. SP-C deficient mice have an altered stability of surfactant that predisposes to atelectasis and development of chronic lung disease. In human disease, SP-C expression in lung tissue is reduced or undetectable with decreased or undetectable levels of SP-C in bronchoalveolar lavage. This can also be the case in familial interstitial lung disease without identifiable mutations in the SP-C gene, suggesting that other mechanisms than genetic may affect SP-C processing. There are no studies of surfactant lipid turnover in individuals with genetic abnormalities in the SP-C gene.

ABCA3 deficiency

Many infants with signs and symptoms of severe neonatal surfactant deficiency and alveolar proteinosis have been considered idiopathic after ruling out SP-B and SP-C gene abnormalities. In a recent study of 21 infants with mostly fatal respiratory failure resembling SP-B deficiency, 16 were found to have mutations in the ABCA3 gene. ABCA3 is a member of the ATP-binding cassette family, a group of transmembrane proteins that transport substances across biologic membranes. The ABCA subclass has a role in lipid transport and ABCA3 has been localized within the alveolar type II cell on the limiting membrane of lamellar bodies. Therefore, it is hypothesized that ABCA3 transports essential surfactant lipids in or out of the lamellar bodies. Abnormal lamellar body formation has been observed in infants with mutations in the ABCA3 gene, however the importance of ABCA3, and possibly also other ABC transporters, in neonatal lung disease and surfactant function remains to be further
clarified. Although genetic surfactant disorders appear to be rare, ABCA3 deficiency may well prove to be one of the most common inborn errors of surfactant metabolism.\(^{158}\)

**SURFACTANT THERAPY**

**Evaluation of surfactant treatment**

Animal models have been crucial for experimental evaluation of surfactant therapy. Satisfactory surface properties *in vitro* does not necessarily reflect positive physiological effects *in vivo*.\(^{163}\) For example, early protein-free surfactant preparations failed to improve lung function when instilled into immature alveoli, flooded with proteinaceous edema fluid inhibiting surfactant function.\(^{113,114}\) Immature newborn rabbits have been commonly used for experimental evaluation of surfactant therapy. At approximately 85% of the normal gestation, the rabbits have very little endogenous surfactant and require mechanical ventilation for survival. Surfactant replacement is evaluated by lung mechanics, i.e. effective treatment improves dynamic lung-thorax compliance. Analysis of alveolar lavage fluid provides information about phospholipid composition and quantities. Lung tissue allows for histopathological evaluation. The vascular to alveolar protein leak can be assessed by intravenous administration of radiolabeled albumin.\(^{164}\) The ventilated preterm lamb is another important model of neonatal RDS\(^{165}\) and many comparative studies of different surfactant preparations have been performed in rabbits or lambs. Repeated lung lavage of adult animals is another model of experimental surfactant depletion.\(^{166}\)

Since the first report of successful surfactant replacement in preterm infants by Fujiwara et al.\(^{17}\) more than 35 randomized controlled clinical trials, including over 7000 infants, have been performed.\(^{71,103}\) Most studies favor the more rapid response of natural surfactant extracts over synthetic surfactant preparations. Surfactant treatment has universally been proven to reduce the need for supplemental oxygen and ventilatory support in the early course of RDS. All regimens of surfactant therapy also appear to decrease the incidence of air leaks. Most importantly, there is a significant reduction in mortality from RDS\(^{104,167}\) as well as an approximately 40% reduction in the odds ratio of neonatal death after surfactant treatment. In contrast to the great impact on mortality, the incidence of chronic lung disease (CLD/bronchopulmonary dysplasia (BPD) has not been consistently shown to decrease.\(^{168}\) Although individual trials have demonstrated a reduced risk of BPD, a compiled analysis of 10 trials was not consistent with an effect of surfactant treatment on the development of BPD (Odds ratio1.01, 95% confidence interval 0.81-1.27)\(^{103}\). A change in the clinical pattern of BPD has been seen in the surfactant era as the smaller and more immature infants have come to constitute the majority of the BPD cases.\(^{170}\) The term “new BPD has been coined to indicate this change in pathophysiology. However, there are evidence that the incidence of BPD is reduced after surfactant treatment in the more mature infants with a birth weight over 1250 g.\(^{171}\) This may imply that barotrauma and volutrauma are more important risk factors for BPD in the more mature infants whereas factors such as developmentally impaired alveolarization and vascularization, poor nutrition and recurrent infections are likely to have a greater impact in the extremely premature infants. Genetic aberrations may also increase susceptibility to develop BPD in certain individuals.
The notion has been raised that cerebral blood flow alterations in conjunction with surfactant replacement would increase the incidence of intraventricular haemorrhage \(^{172}\), but meta-analysis have not supported this finding \(^{169}\). In the European Multicenter study group \(^{20}\) an increased incidence in grade 1 and 2 IVH was associated with high arterial oxygen tension peaks within 30 minutes after surfactant administration demonstrating the importance of rapid adjustments of the ventilatory setting to prevent complications. Other possible side-effects to surfactant replacement is increased left to right shunting through the patent ductus arteriosus (PDA), with subsequent increased pulmonary blood flow and risk for pulmonary haemorrhage. Controversy regarding this subject still exists in the literature. Data from a meta-analysis suggest that pulmonary haemorrhage may occur more frequently after surfactant treatment, particularly in the most immature infants, but that PDA is not an independent risk factor \(^{173}\). Although there is a theoretical risk of transmission of infectious agents with natural surfactant preparations and potential immunogenicity of surfactant proteins \(^{174}\), no adverse effects have been reported. With the characterization of the surfactant protein genes and recombinant DNA technology the production of modified human surfactant proteins is now possible and protein-containing artificial surfactant preparations are likely to be widely available soon.

### Timing, dosing and method of administration

Prophylactic versus early or late rescue treatment with surfactant has been much debated. Administration of exogenous surfactant to the immature, surfactant deficient lung at birth is theoretically appealing. Especially if early ventilatory support is needed, there is an increased risk for lung injury and a compromised therapeutic response to later surfactant treatment \(^{175}\). Several previous studies as well as a recent meta-analysis have shown that prophylactic surfactant administration in the delivery room decrease mortality and prevents air leaks more effectively than surfactant treatment given when clinical RDS is fully established \(^{169,176-178}\). However, with unselective prophylactic surfactant administration up to 40% of preterm infants will be treated unnecessarily, carrying a risk for possible adverse effects, unnecessary intubation, increased costs and disturbing the vulnerable period of immediate postnatal adaptation. There are even studies suggesting poorer developmental outcome after prophylactic surfactant treatment \(^{179}\). Early rescue treatment offers an alternative, shown to be more effective than late treatment \(^{180,181}\), and is now the predominant approach, except for maybe in the extremely immature babies. However, a simple diagnostic test that accurately predicts RDS would allow for even earlier rescue treatment or selective prophylactic surfactant replacement with possible benefits for the outcome. No such rapid tests are yet available in general practice, but a few different approaches have been recently proposed. Gastric aspirate at birth represents mainly swallowed amniotic fluid or lung effluent from the airways and provides an accessible sampling pool, although deep-suction is not routinely performed at birth. The microbubble stability test (MST) evaluates surfactant maturity and inhibition in BAL fluid \(^{166}\). In tracheal aspirates \(^{182}\) and gastric aspirates from newborn infants \(^{183-185}\), MST predicted moderately to severe RDS with a specificity of 78-99%. The click test was reported to perform well on tracheal fluid in preterm infants \(^{186}\), but in pharyngeal aspirates from more mature infants a high number of false-positive values were seen \(^{187}\). Lamellar body count (LBC) in gastric aspirate is yet another test exhibiting promising results in recent pilot studies \(^{188,189}\).
Lamellar bodies have approximately the same size as platelets and can be counted in an automated blood counter. The test therefore has a potential advantage in being very simple and fast, since a gastric aspirate sample could be sent off to the hospital laboratory soon after birth, yielding a result within a few minutes.

In ventilated infants with severe RDS, the oxygenation and outcome were improved by additional doses of surfactant. Multiple doses may be needed both in mechanically ventilated infants with RDS and in cases of MAS or lung infections to overcome functional inhibition of surfactant caused by leakage of plasma proteins into the alveoli. If the early rescue treatment is followed by rapid extubation to nasal CPAP the need for mechanical ventilation can be significantly reduced. The meta-analysis by Stevens et al. reported an increased number of surfactant doses per patients in the early treatment group. However, in the experience of us and others the early administration of surfactant followed by immediate extubation to nasal CPAP leads to a more sustained treatment response and a reduced need for repeated dosing, which is one of the hypotheses being investigated in the present thesis.

Factors affecting the treatment response

Surfactant needs to be administered via tracheal instillation in order to be effective. Trials with nebulized surfactant have not been successful. After instillation into the airway there is a rapid tissue association of the exogenous surfactant that has been described in several experimental settings. Up to approximately 50% of the surfactant cannot be recovered by bronchoalveolar lavage immediately after administration, presumably the result of a first step towards entering the metabolic pathways of endogenous surfactant. The significance of the initial tissue association and possible consequences of disturbing it remains unclear.

Other factors that can affect the response to surfactant treatment or result in early relapse include perinatal asphyxia, acidosis, hypothermia, anaemia, fluid intake, systemic hypotension pulmonary hypertension and PDA, all of which should be attempted to correct before administration of additional surfactant doses. Charon et al. reported that infants with absent response had lower initial a/A ratio, suggesting that disease severity represents a risk factor for diminished effect of surfactant treatment. A poor response to surfactant treatment can also identify a group of infants at risk of dying. Relapse after the initial response may be due to pulmonary edema and subsequent surfactant inactivation, but other factors such as prenatal steroid treatment, timing of initial dose, ventilatory management and underlying genetic disorders may also affect the response to surfactant treatment.

VENTILATION STRATEGIES AND SURFACTANT REPLACEMENT

Lung mechanics

As a direct consequence of surfactant deficiency in RDS the terminal air spaces are unstable, difficult to inflate and have a tendency to collapse during expiration, which is aggravated by the extremely compliant chest wall of the premature infant. The epithelial lining
Surfactant metabolism in the newborn may be injured by assisted ventilation required to inflate the lung or as a result of a primary ischemic event in association with perinatal asphyxia. The epithelial disruption leads to flooding of the air spaces by protein-rich edema fluid and hyaline membrane formation from epithelial cell debris. Derecruitment (decreased number) of ventilated air spaces occurs as a result of alveolar collapse, pulmonary edema and obstruction of the airways by hyaline membrane formation, with subsequent loss of lung volume. The remaining ventilated air spaces exhibit increased elastic recoil due to increased surfaces forces, secondary to surfactant deficiency or inhibition. These two factors, derecruitment and increased elastic recoil are the major determinants of the decrease in lung-thorax compliance, one of the hallmarks of RDS. In addition, lung resistance appears to be increased in RDS, even during spontaneous breathing. Mechanically ventilated infants with RDS have 3-6 times greater resistance than normal infants during spontaneous breathing, some of which can be attributed to the endotracheal tube.

Animal studies have clearly established that surfactant treatment improves gas exchange and compliance. Dynamic compliance reflects surfactant function and activity and has long been used to evaluate treatment response. In surfactant treated preterm rabbits ventilated with standardized insufflation pressure, the tidal volume decreases with increasing time of mechanical ventilation. This is also associated with an elevation of surface tension in lung lavage fluid suggesting surfactant inactivation. Compliance measurements in surfactant treated, spontaneous breathing animals are sparse. In neonates with RDS, dynamic compliance increased with 29% after surfactant replacement during spontaneous breathing in nasal CPAP, whereas no improvement in compliance could be detected during mechanical ventilation. Others have shown an increased compliance in ventilated infants but it appears later than the immediate, dramatic improvement in oxygenation generally seen after surfactant replacement.

**Mechanical ventilation and ventilator induced lung injury**

Mechanical ventilation is one of the cornerstones in the treatment of neonatal lung disease. In the late 1960s and 1970s the use of ventilators in newborns became more widespread, contributing to the marked reduction in perinatal mortality over the following years. Since, significant advancements in both technology and knowledge about ventilation strategies have taken place, although the final word on which approach is most efficient and least damaging in a given situation has not yet been said. Positive pressure ventilators are most commonly used and several modifications have aided in the effort to accomplish more physiologic ventilation (synchronized intermittent mandatory ventilation, assist control and proportional assist ventilation). With new evidence that the magnitude of the tidal volume rather than the peak inspiratory pressure correlates with lung injury, modern ventilators can measure and even guarantee specific volume delivery. Barotrauma was previously considered to be the major cause of ventilator induced lung injury. However, over the recent years the role of volutrauma emerged as the most important issue and avoiding alveolar overdistension has become recognized as a crucial factor in lung protective ventilation strategies. Experimentally, mechanical ventilation induces various pathophysiological changes such as altered lung fluid balance, endothelial and epithelial leakage, tissue damage and inflammatory response, all of which interact with the pulmonary surfactant system. In rabbits,
mechanical ventilation increases surfactant aggregate conversion. High tidal volumes result in higher ratios of small, inactive surfactant aggregates and correlate with severity of lung injury. Mechanical ventilation has been reported to impair the normal increase in alveolar pool of surfactant during the first 24 hours after birth and reduce the alveolar secretion of saturated PC from radio-labeled palmitate in moderately preterm rabbits. In contrast, phospholipid composition and amounts in alveolar lavage and lung tissue from adult rabbits was not affected by mechanical ventilation, although mechanically ventilated animals exhibited a decreased dynamic compliance, presumably due to surfactant inactivation, compared to spontaneously breathing animals. In preterm lambs, mechanical ventilation was associated with a shift in lamellar body distribution to smaller size and a decrease in glycogen content of the type II cells. These severe morphometric disturbances after mechanical ventilation were almost completely prevented by surfactant treatment. The susceptibility of the surfactant deficient lung to injury is also demonstrated by the impaired treatment response after only a brief period of mechanical ventilation in immature lambs. The spreading of surfactant treatment doses was impaired resulting in an uneven and patchy distribution, however this could be alleviated by prophylactic surfactant treatment. Wada et al. reported similar findings in preterm lambs with a decreased treatment response following an initial tidal volume of 20 ml/kg, an effect that was prevented by surfactant treatment at birth. Others have also shown that surfactant sufficient lungs tolerate mechanical ventilation well. Although surfactant treatment appears to protect from ventilator induced lung injury and more gentle ventilation strategies may prevent substantial damage, the significance of more subtle physiologic and metabolic changes in the lungs possibly occurring during mechanical ventilation remains to be elucidated. A recent clinical trial identified mechanical ventilation as the major risk factor for BPD, one of the most important complications in surfactant treated preterm infants.

Furthermore, the physiological effect of exogenous surfactant depends on ventilation strategy. The use of positive end expiratory pressure (PEEP) keeps the lung open at end-expiration, thereby avoiding harmful mechanical stress caused by repeated collapse and re-expansion of the airspaces and facilitating an even surfactant distribution. PEEP decreased the recovery of intravascular labeled albumin in the lungs of surfactant treated preterm rabbits as an indicator of less lung injury and improved compliance in surfactant treated preterm lambs. High frequency oscillatory ventilation (HFOV) delivers small volumes of gas at very high frequencies and a comparatively high continuous distending airway pressure aiming at improved oxygenation, gas exchange and lung mechanics, as a consequence of increased functional residual capacity. HFOV has also been thought to be less injurious to the lungs by limiting the pressure swings and decrease barotrauma. Two recent large clinical trials have provided reassuring data about the safety of HFOV and suggested a modest reduction in CLD and death in very-low-birth-weight infants. The effects of HFOV on the surfactant system are controversial and not well established. The diminished repetitive alveolar stretch in HFOV could theoretically decrease surfactant secretion, which is supported by some animal studies and not verified in other. Phospholipid composition and quantities have been reported to be unchanged after HFOV in non-human primates. In contrast, a possibly decreased surfactant production is suggested by lower concentrations of SP-A in preterm infants receiving HFOV and increasing PC concentrations after switching from HFOV to conventional ventilation. Knowledge regarding the impact of ventilation strategy on surfactant synthesis, clearance and pool size in humans is still limited.
Gregory et al. first introduced continuous positive airway pressure (CPAP) via the endotracheal tube in newborns in 1971 [240]. After the initial interest, the method did not gain widespread use, possibly because the focus was on the rapid development of infant ventilators. In Scandinavia however, the tradition of early CPAP has been maintained since the late 1970s. A variety of devices and strategies to apply CPAP have been used, including mask, nasal prongs, nasopharyngeal tube and endotracheal tube. Nasal CPAP with short nasal prongs are advantageous because it is relatively atraumatic, intubation is avoided and access to the baby is allowed, as opposed to CPAP with facemask. With improved nasal prongs, increased work of breathing is no longer a significant obstacle [241]. Nasal CPAP has been the predominant mode in Scandinavia and two devices, the Benveniste valve and the Infant Flow Driver are mainly used. With increasing appreciation of the open lung concept in RDS and the development of lung injury and chronic lung disease, the use of early nasal (n) CPAP as a primary respiratory support in preterm infants is again gaining interest worldwide. Already in 1987, Avery et al. performed a survey of 8 North American neonatal units and found the lowest incidence of BPD in the one centre practicing early nCPAP instead of initial mechanical ventilation [242]. Horbar et al. later confirmed these results [243]. A recent study showed that CPAP-treated preterm baboons did not develop BPD and exhibited normal lung histology compared to near-term controls [244]. Knowing the benefits on surfactant function by adding PEEP to the ventilatory settings (as discussed above), CPAP is likely to have similar effects on the surfactant system. Although surfactant metabolism in relation to ventilation strategy has been extensively studied, there are few comparative experimental studies of mechanical ventilation versus spontaneous breathing and CPAP. One recent report found fewer neutrophils and lower levels of hydrogen peroxide in alveolar washes from CPAP-treated lambs compared to mechanically ventilated animals, indicating less lung injury in the CPAP group [245].

In Scandinavia a vast clinical experience with nCPAP exists, but unfortunately randomised trials of CPAP compared to mechanical ventilation are sparse [246]. Early nCPAP has been shown to be beneficial as initial treatment of RDS also in the very-low-birth-weight infant and thereby reduce the need for mechanical ventilation [119,247,248]. Among the few randomised studies, Tooley et al. recently confirmed that even very premature infants could be successfully managed with nCPAP after surfactant treatment [249]. More evidence is needed to appropriately evaluate this method, however in Scandinavia the long-standing practice of early nCPAP make randomised trials ethically difficult and the hope is that ongoing studies form other parts of the world will provide answers to the remaining questions regarding the pro’s and con’s of nCPAP treatment in preterm infants.

Over the last decade, a new treatment approach with administration of exogenous surfactant during a brief intubation, followed by immediate extubation to nCPAP, has been implemented. Victorin et al performed the first study of surfactant treatment in spontaneously breathing infants in Kuwait, at a center where mechanical ventilation was not available [250]. Fourteen newborns with a mean gestational age of 32 weeks and severe RDS were treated with tracheal bolus doses of surfactant and immediately extubated. Twelve responded with a rapid improvement in oxygenation that was sustained over the 72 hours observation period. In 1994, Verder et al published the first randomized controlled trial of surfactant instillation during nCPAP and showed that the subsequent need for mechanical ventilation could be reduced by half, from 85% without surfactant to 43% with surfactant treatment [193].
The effect was even more pronounced when the treatment was given early during the course of the disease \(^{192}\). Dani et al. recently reported the results of a prospective randomized study and showed that immediate reinstitution of nCPAP after surfactant administration reduced the duration of oxygen therapy, need for mechanical ventilation and need for a second dose of surfactant \(^{194}\). In Stockholm, we have adopted a treatment protocol, modified from the Danish strategy, and proposed the term INSURE (i.e. INtubation, SURfactant Extubation).

The INSURE protocol was implemented in 1998. We observed that repeated doses of surfactant were rarely indicated, an observation also noted in the Danish and Italian studies \(^{192,193}\). This generated the hypothesis being tested experimentally in paper III, that surfactant treatment followed by spontaneous breathing results in a more sustained treatment response, and warranted a retrospective, descriptive follow-up study (paper V).
AIMS OF THE PRESENT INVESTIGATION

GENERAL AIM

The general purpose was to study the metabolism of endogenous and exogenous surfactant in the newborn lung in relation to maturity, ventilation strategy and lung disease.

SPECIFIC AIMS

- To distinguish normal and abnormal endogenous surfactant turnover in term and preterm infants with and without lung disease using a novel stable isotope technique labeling precursors for endogenous surfactant synthesis.
- To determine with stable isotope technique if high frequency oscillatory ventilation decreases endogenous surfactant production in preterm infants with RDS.
- To systematically examine stable isotope methodology for studies of endogenous surfactant metabolism by comparing different tracers, means of processing lung surfactant phospholipid and mass spectrometry instrumentation.
- To perform a population-based follow-up of the implementation of a new treatment strategy (INSURE), involving surfactant administration during a brief intubation followed by spontaneous breathing in nasal continuous positive airway pressure (nCPAP).
- To test in an experimental animal model, the hypothesis generated from INSURE treatment of newborn infants that surfactant followed by spontaneous breathing results in a more sustained treatment effect compared to treatment followed by mechanical ventilation.
MATERIALS AND METHODS

STUDY POPULATIONS

Stable isotope studies (I, II, IV)

The studies of endogenous surfactant metabolism using stable isotope methodology were all performed at St Louis Children’s Hospital, Washington University, St Louis, USA. Infants admitted to the Neonatal or Pediatric Intensive Care Units were eligible. The main inclusion criterion in all studies was airway access and anticipated time requiring mechanical ventilation more than 3 days. Exclusion criteria included known chromosomal abnormalities and imminent death. A total of 70 infants were studied. Nine infants formed heterogeneous group of preterm infants with a gestational age <34 weeks and RDS or chronic lung disease, whose samples in the present work has served solely as their own controls in the comparison of phospholipid processing methods (IV). The remaining 61 infants, in which surfactant kinetics was studied, can be divided into 2 groups:

1. Preterm infants with RDS and gestational age <28 weeks, studied before 72 hours of age (n=44).
2. Term infants with and without lung disease and gestational age >37 weeks, studied before 6 months of age (n=17).

The basic characteristics of these groups are shown in Table 1.

Nineteen preterm infants with RDS were randomly assigned to either of two mechanical ventilation modes, conventional ventilation (CV) or HFOV at 24 h of age (paper I). Infants without lung disease were recruited as a control group to assess normal surfactant turnover at

<p>| Table 1. Population characteristics of surfactant kinetic studies using stable isotopes |
|-----------------------------------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Preterm infants (n=44)</th>
<th>Term infants (n=17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age (weeks)</td>
<td>25.8±0.2</td>
</tr>
<tr>
<td>Weight at study start (g)</td>
<td>781±24</td>
</tr>
<tr>
<td>Age at study start</td>
<td>34±3 hours</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>25/19</td>
</tr>
<tr>
<td>Race (white/black/other)</td>
<td>12/30/2</td>
</tr>
<tr>
<td>Survival at discharge n (%)</td>
<td>35 (80)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM
term (paper II). Since airway access was a prerequisite for the study, these infants were all in need of mechanical ventilation for non-respiratory causes. Lack of significant lung disease at inclusion was defined as a clear lung parenchyma on chest radiograph and less than 0.3 FiO2. The underlying diagnoses for the control infants were hypoxic ischemic encephalopathy, micrognathia, congenital hypotonia, subdural hematoma, central hypoventilation syndrome, choanal atresia and severe tracheomalacia. The study group of term infants with lung disease all exhibited respiratory failure from birth (paper II). The underlying diagnoses in this group were acute RDS (a clinical picture similar to that of preterm infants with bilateral granular infiltrates on chest radiograph and poor oxygenation requiring mechanical ventilation), congenital pneumonia, alveolar proteinosis, respiratory failure of unknown origin and refractory respiratory failure after congenital diaphragmatic hernia repair. The latter three patients were all studied while awaiting lung transplantation. None of these infants had an inherited disorder of surfactant protein B or C metabolism, however one (alveolar proteinosis) was later found to have a mutation in the ABCA3 gene. In all studies, the severity of lung disease was assessed by a score derived from the integrated area under the curve for average daily FiO2 x mean airway pressure over time, divided by number of days studied 251. In the term infants with respiratory failure the disease severity score showed a dichotomous distribution pattern that permitted stratifying the infants into two groups; those with mild disease, respiratory score <5 and those with severe disease, respiratory score >8.

The infant’s medical team determined all care, including ventilatory and nutritional management. Exogenous surfactant treatment (Survanta®, Abbott laboratories, Chicago, IL) was given according to current medical protocol at a dose of 4 ml/kg/dose. All preterm infants with RDS and two of the term infants with respiratory failure received 1-3 doses of surfactant, however none of the infants were treated after the initiation of the tracer infusion.

INSURE (V)

Stockholm County has approximately 24,800 deliveries per year (2004). There are five delivery centers, four of which are associated with neonatal units (Fig. 4). Karolinska Solna (KS) is the only level III neonatal intensive care unit and was the only centre providing mechanical ventilation during the study period (1993-2002). Karolinska Huddinge (KH) is one of three level II neonatal units, staffed with accredited neonatologists and neonatal nurses skilled in nCPAP care, caring primarily for infants born with a gestational age of ≥27 weeks. Women in preterm labour <27 weeks of gestation are transferred, if their medical condition so allows, to KS before the delivery. During the study period, all infants requiring mechanical ventilation were transferred to KS.

The INSURE strategy for surfactant treatment was implemented at KH in 1998. Briefly, the INSURE protocol includes: Early nCPAP started soon after birth in all preterm infants with respiratory distress. Initial pressures are set to 3-5 cm H2O using the Infant Flow system (EME, UK) or a Benveniste jet system 252. The fraction of inspired oxygen (FiO2) is adjusted to give an arterial or transcutaneous partial pressure of oxygen (PO2) value of 8-10 kPa. The oxygenation is assessed by the arterialto alveolar ratio (a/A ratio), calculated as:
Figure 4. The organization of neonatal care in Stockholm County.
Approximately 24,800 deliveries/year (2004) are divided on five delivery centers, four of which are associated with neonatal units (grey). During the study period (1993-2002), all infants requiring mechanical ventilation were transferred to one regional level III neonatal unit.

$$a/A = \frac{P_aO_2}{(FiO_2 \times 95) - P_aCO_2}$$

$P_aO_2$ is the arterial partial pressure of oxygen (kPa); $FiO_2$ the fraction of inspired oxygen and $P_aCO_2$ the arterial partial pressure of carbon dioxide (kPa).

Infants are eligible for surfactant replacement when the a/A ratio is 0.22 or less. Intravenous access is demanded for drug administration and an arterial line required for therapeutic control. A loading dose of theophyllamin (10 mg/kg i.v.) is given at start of the procedure as apnoea prophylaxis. Infants are sedated with tiopentsalsodium (Pentothal®), 2 mg/kg i.v., repeated as needed up to 5 mg/kg, and anaesthetized with morphine 0.2 mg/kg i.v.

Intubation is performed orally, preferably with the nCPAP in place. The tube position is evaluated by auscultation of the chest. Surfactant, 100-200 mg/kg of Curosurf® (Chiesi Farmaceutici, Parma, Italy), is administered as a bolus tracheal instillation. The infants are briefly ventilated manually with controlled inspiratory pressure (Neopuff Infant Resucitator™, Fisher & Paykel Healthcare Ltd, Auckland, New Zealand), while naloxone 0.1 mg i.v. is given to reverse the opiate induced respiratory depression. Extubation to continued nCPAP is then performed immediately. The whole procedure from intubation to extubation is usually completed within 4-6 minutes. Mechanical ventilation is initiated when $P_aCO_2$ exceeds 8.5 kPa and $FiO_2$ is >0.6, or when signs of severe respiratory distress or apnoeas are present.

A total of 420 preterm infants with RDS born between 1993 and 2002 at KS and KH were included. Charts were reviewed retrospectively for all inborn infants during the time period. Since the target population for INSURE treatment primarily is the moderately premature infants the inclusion criteria were chosen as (i) a gestational age of ≥27 weeks or <34 weeks and (ii) a diagnosis of RDS, defined as respiratory distress, increasing $FiO_2$ and
characteristic radiographic findings. Congenital malformations or chromosomal abnormalities were exclusion criteria. The time periods before and after the introduction of INSURE (1993-1997 versus 1998-2002) were compared. The groups were demographically similar during both time periods and at both centres. Mean gestational age was 29.5 (±2.0 SD) weeks, mean birth weight 1438 (±479 SD) g and 62% (261/420) of the population were males.

In order to further study the effects of spontaneous breathing in nCPAP compared to mechanical ventilation on surfactant treatment response, a sub-population of 109 infants in the later time period (1998-2002) was identified and reviewed separately. This group consisted of all infants receiving INSURE treatment at KH (n=42) and all infants receiving surfactant in conjunction with mechanical ventilation at KS (n=67). INSURE-treated infants requiring mechanical ventilation (according to criteria described above) during the first week after surfactant replacement were regarded as treatment failures. Outcome parameters included duration of ventilation support, air leaks and survival. BPD was defined as oxygen requirement at an age corresponding to 36 weeks of gestation. Repeated cranial ultrasounds were performed during the first 2 weeks of life and intraventricular haemorrhage (IVH) was defined and graded according to Papile et al. IVH more than grade II was recorded. Infants were screened for retinopathy of prematurity (ROP) at 4 weeks of age and thereafter once weekly as needed until vascularization was complete. ROP requiring treatment with laser or cryotherapy was reported.

**Stable isotopes**

- Naturally occurring
- Non-radioactive
- Differ in number of neutrons/atomic mass
  - $^{12}$C natural abundance 98.89%
  - $^{13}$C natural abundance 1.11%

**THE STABLE ISOTOPE TECHNIQUE (I, II, IV)**

**Tracer infusion and sample collection**

In humans, stable isotopes provide a method to study the kinetics of pulmonary surfactant *in vivo*. Previously, studies of surfactant metabolism required radioactive tracers and acquisition of lung tissue. These techniques were therefore necessarily limited to animal studies. Stable isotopes are naturally occurring, non-radioactive variants of an atom carrying a different number of neutrons resulting in a slightly different molecular weight (Box 1). In case of carbon, the $^{13}$C is heavier than $^{12}$C, thus permitting substances labeled with $^{13}$C to be distinguished by mass spectrometry. In studies of endogenous surfactant metabolism, the
method is based on the administration of labeled precursors of surfactant phospholipid synthesis (tracers) and the measurement of their appearance in airway samples (Fig. 5) 256,257. All infants received a 24-hour continuous intravenous infusion of tracer, included in their routine fluid intake. Three tracers were used:

\([1-^{13}C_1]\) acetate (Cambridge Isotope Laboratories, Inc. Andover, MA)
\([1,2,3,4-^{13}C_4]\) palmitate (Isotec, Miamisburg, OH)
\([U-^{13}C_6]\) glucose (Campro Scientific, Veenendaal, The Netherlands and Cambridge Isotope Laboratories, Inc., Andover, MA)

Tracheal aspirate samples were collected in a standardized fashion before the tracer infusion began (time 0) and every 6-12 h for 14 d or until infants were extubated 83. Samples containing visible blood were not included. In infants receiving glucose or palmitate tracers, plasma samples were obtained prior to tracer administration and at approximately 6-h intervals during the 24-h infusion period for measurements of plasma glucose or palmitate enrichment. Samples were stored at -70°C until processing.

Figure 5. Overview of the stable isotope methodology for studies of endogenous surfactant metabolism.

The labeled surfactant precursor (in the present investigation \([1-^{13}C_1]\) acetate, \([U-^{13}C_6]\) glucose or \([1,2,3,4-^{13}C_4]\) palmitate) is administered to mechanically ventilated infants by intravenous infusion. Tracheal aspirates are collected during routine suction of the endotracheal tube several times daily for as long as the infant is intubated, or a maximum of 14 d. Surfactant phospholipids are extracted from the tracheal aspirates and the incorporation of labeled precursor into surfactant phospholipid is measured with gas chromatography/mass spectrometry (GC/MS).
Analytical procedures

Tracheal aspirate samples were processed to yield disaturated phospholipids (DSPL) or total phosphatidylcholine (PC). For total PC (paper I), thin layer chromatography was used. For disaturated PL a modification of the method by Mason et al was used. Briefly, after chloroform-methanol extraction osmium tetroxide was added to the samples to form a complex with the unsaturated phospholipids, and phospholipids containing only saturated fatty acids were eluted over neutral alumina columns. In paper IV, a group of samples were split in half and osmium was omitted from one portion to recover the total phospholipid fraction. DSPL was compared against total phospholipid (PL) in paired samples. Fatty acid methyl esters were prepared for quantification by gas chromatography and measurements of the isotopic enrichment of methyl palmitate from surfactant and plasma by gas chromatography/mass spectrometry (GC/MS) or gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS). Enrichment is expressed as tracer to tracer ratio (TTR), representing the molar ratio of labeled to unlabeled palmitate in the sample (GC/MS) or atom percent excess (APE), representing the increase in the percentage of $^{13}$C atoms in total carbon dioxide from the combusted compound above baseline enrichment (GC/C/IRMS).

Figure 6. $^{13}$C-enrichment-over-time curve.

After intravenous infusion of a $^{13}$C-labeled precursor of surfactant lipid, the $^{13}$C-enrichment of palmitate in serial tracheal aspirate is measured. From the enrichment-over-time curve indices of surfactant metabolic kinetics are derived; the time of appearance ($T_{app}$); the maximum enrichment ($E_{max}$) and the time of maximum enrichment; ($T_{max}$). From the linear upslope of the curve the fractional synthesis rate (FSR) is calculated. The half-life of clearance ($T_{1/2}$) or fractional catabolic rate (FCR) is derived from the downslope of the curve.
Kinetic analysis

Indices of surfactant metabolism were determined from each individual curve of isotopic enrichment over time (Fig. 6). These included the time of appearance of tracer in surfactant palmitate ($T_{app}$, h), representing an estimate of the time from synthesis to secretion into the alveolar pool; the maximum enrichment ($E_{max}$, TTR or APE, percent) and the time of maximum enrichment ($T_{max}$, h). The half-life of clearance ($T_{1/2}$, h) was calculated from the monoexponential fitting of the downslope of the enrichment versus time curve (paper I, II), however this measure is suggested to be replaced by the fractional catabolic rate (FCR), i.e. the percentage of the surfactant pool removed and replaced by newly produced material per day, calculated from the natural log transformation of the monoexponential slope (paper IV). The fractional synthesis rate (FSR) from a given precursor per day was either calculated by dividing the slope of the linear increase in the $^{13}$C-enrichment curve by the steady state enrichment in the precursor pool or by using mass isotopomer distribution analysis (MIDA) \(^{35,80,257}\). The precursor pool was defined as plasma glucose or palmitate when labeled glucose or palmitate was used as tracers (paper I, IV). When labeled acetate was used as a tracer (paper II, IV), MIDA mathematically determined the acetyl-CoA-precursor enrichment by the ratio of doubly- to singly-labeled palmitate (corresponding to the incorporation of 2 and 1 labeled acetate subunits respectively). Lastly, the ratio of FSR/FCR, representing the contribution of newly synthesized surfactant from a given precursor to the total surfactant turnover, was described.

THE EXPERIMENTAL RABBIT MODEL (III)

Animal experiments

From a well-established model to test surfactant in mechanically ventilated preterm, newborn rabbits \(^{111}\) a new experimental set-up was designed to study the underlying mechanisms behind the different treatment response seen in infants after surfactant replacement followed by spontaneous breathing (INSURE) compared to mechanical ventilation. Radioactively labeled surfactant was prepared by adding 1-palmitoyl-2-[$1-^{14}$C] palmitoyl phosphatidylcholine to Curosurf® (Chiesi Farmaceutici, Parma, Italy), for details see paper III. The experiments were performed on 7 litters with a total of 54 rabbit fetuses delivered at a gestational age of 28.5 days (term 31 days). This represents a transitional stage of lung maturation at which the fetal rabbits have started to produce surfactant $^{260}$. Furthermore, pups delivered prematurely at this stage of gestation have sufficient respiratory drive to support spontaneous breathing for several hours $^{210}$. The experimental set-up is schematically described in Fig 7. The does were anaesthetized with intravenous propophol and local infiltration of the abdominal wall with lidocaine. The fetuses were sequentially obtained by hysterotomy and thorax compression was immediately applied to prevent breathing. A thin plastic catheter was introduced to the pharynx and 0.1 ml (corresponding to approximately 200 mg/kg) of $^{14}$C-Curosurf® was deposited with simultaneous release of the thorax compression and cutaneous stimulation to induce breathing. After aspiration, when there was no visible surfactant left in the pharynx, the animals were weighed and placed in a box heated to 37°C and flushed with 100% oxygen. The duration of the procedure was 1-2 min. The next fetus was not delivered
Figure 7. The rabbit model.
Schematic view of the experimental set-up. A total of 54 preterm rabbits received radiolabeled Curosurf® before their first breath. After randomisation, the animals were kept spontaneously breathing or mechanically ventilated for 4 h. At the end of the experiment, spontaneously breathing animals were anaesthetized and tracheotomized, as previously the mechanically ventilated, and dynamic compliance measurements were performed in both groups.
until the first had received surfactant. Animals in which less than 10% of the administered dose was delivered to the lungs were excluded (n=10). The animals were randomized to:

1. Mechanical ventilation (n=18).
2. Spontaneous breathing (n=18).
3. Control group (n=8).

Animals randomized to mechanical ventilation were anaesthetized with intraperitoneal pentobarbital sodium (0.1 ml, 6 mg/ml) and tracheotomized. They were then transferred to a system of multiple body pletysmographs heated to 37°C. After muscle relaxation with intraperitoneal pancuronium bromide (0.1 ml, 0.2 mg/ml), the animals were connected in parallel to a pressure-constant ventilator system (Servo Ventilator 900 B, Siemens-Elema, Solna, Sweden), delivering 100% oxygen at a frequency of 40 breaths/min and 1:1 inspiration:expiration ratio. The inspiratory pressure was set to generate a tidal volume of about 10 ml/kg and was monitored and adjusted regularly during the observation period. Individual pressures, flow rates/tidal volumes and ECGs were recorded. ECG indicating severe arrhythmia or bradycardia below 50 beats/min was regarded as exclusion criterion (n=7).

Spontaneously breathing animals were kept in the heated box during the 4-h observation period. Subjects who failed to establish spontaneous regular breathing or developed irregular breathing were excluded if the respiratory rate was below 6 breaths/min (n=3). At the end of the observation period the spontaneously breathing animals were anaesthetized, tracheotomized and connected to the ventilator-multipletysmograph system. Dynamic lung-thorax compliance was measured at 4 h of life in both mechanically ventilated and spontaneously breathing animals by dividing tidal volume (ml/kg) by peak inspiratory pressure (cmH2O) for individual animals in each treatment group. After compliance measurements animals were euthanized by intracerebral injection of lidocaine (2%, 0.5 ml). Control animals were similarly killed immediately after instillation of 14C-Curosurf®.

Lung preparations and analytical procedures

Bronchoalveolar lavage (BAL) was performed in all animals, using 40 ml/kg of normal saline that was instilled and withdrawn three times. The procedure was repeated five times and the lavage fluid was pooled. The total lavage volume was recorded and an average of 93±1% (mean±SEM) of the instilled volume was recovered. BAL fluid was used for measurements of:

1. Exogenous surfactant content (radioactivity)
2. Total phospholipid amount
3. Surfactant inactivation (microbubble stability test and lipid peroxidation)

The lavaged lungs were removed, weighed and mechanically homogenized in chloroform/methanol, filtered and mixed with distilled water to final proportions of chloroform/methanol/water, 2:2:1. The organic phases of BAL fluid and lung tissue samples were used in duplicate for radioactivity measurements. The total recovery from the lungs (i.e. the dose of 14C-Curosurf® deposited to the lungs) was calculated by dividing the sum of the
radioactivity (counts per minutes) in BAL fluid + lung tissue from each animal with the administered dose of radioactivity. The degree of tissue association of exogenous surfactant was determined by the distribution of $^{14}$C-Curosurf® in BAL fluid and lung tissue, calculated as the ratio of radioactivity in lung tissue relative to the dose deposited (radioactivity in BAL fluid + lung tissue), expressed in percent.

The total phosphorus content in the BAL fluid was measured according to Bartlett. The alveolar pool of endogenous surfactant was estimated by subtracting the amount of exogenous surfactant (total recovery of $^{14}$C-Curosurf®) from the total phosphorus content in the BAL fluid, adjusted to the weight of the individual animal.

The degree of lipid peroxidation in the BAL fluid was determined by a colorimetric assay measuring the amounts of secondary products (i.e. malondialdehyde and 4-hydroxyalkenals). To adjust for differences between litters the value of each sample was divided by the mean value of the litter giving a lipid peroxidation score.

MST was performed in a sub-group of animals (3 litters, n= 15). Microbubbles were defined as bubbles with diameter <20 µm.

**STATISTICAL ANALYSES**

The data are presented as mean and standard deviation (SD) or standard error of the mean (SEM). Non-normally distributed data are shown as median and true range or interquartile range (IQR). Two-tailed $t$ tests were used for normally distributed data, paired-samples $t$ test for paired data, Wilcoxon Rank Sum, Mann-Whitney U or Kruskal Wallis tests for non-normally distributed data, Chi-Square and Fisher’s exact test for categorical data and Pearson coefficients for correlation analysis. A linear regression model was used to determine the influences of covariates (paper IV). The Odds ratio and relative risk were used as risk estimates. All statistical analyses were performed using Statistical Analysis System (SAS, version 8.1; SAS Institute, Cary, NC) or SPSS (version 11.5, SPSS Inc. Chicago, IL) and Statgraphics (Manugistics Inc, Rockville, ML).

**ETHICAL CONSIDERATIONS**

For the human studies (paper I, II, IV) written parental consent was obtained. Parents were approached only after assent of the attending physician. There were no direct benefits to the participating child from the study and the risks were judged to be minimal. One study (paper I) involved randomization between 2 different modes of ventilation (CV and HFOV). Although the scientific evidence has not proven one modality to be superior to the other, in clinical practise HFOV is most often used as the second line of treatment. The randomisation process was therefore modified so that infants already receiving HFOV as part their routine care continued to receive it.

The Washington University Human Studies Committee (paper I, II, IV) and the Human Ethical Committee at the Karolinska Institutet, Huddinge approved the studies (paper I, II, IV, V). The experimental study (paper III) was approved by the Karolinska Institutet Animal Ethics Committee, Stockholm.
RESULTS AND DISCUSSION

ENDOGENOUS SURFACTANT METABOLISM

Relation to lung disease and prematurity (II)

Normal surfactant turnover was faster in term infants (controls) than in preterm infants with RDS. This was demonstrated by higher synthesis rate (FSR 15.2 (6.9-24.4) versus 1.7 (0.9-5.1)% of pools/d, p<0.001) and a shorter T1/2 (28±3.2 versus 106±11.6 h, p<0.001). Term infants with respiratory failure and mild disease exhibited a surfactant turnover similar to that of the control infants. In contrast, the surfactant metabolic indices of infants with severe disease resembled those of preterm infants with RDS. The 13C-enrichment in surfactant palmitate over time for the different study groups is shown in Fig. 8.

Figure 8. Surfactant turnover in term and preterm infants.

13C-enrichment in surfactant palmitate from serial tracheal aspirates after i.v. infusion of [1-13C1] acetate. Tracer incorporation and clearance from the airways is significantly faster in term control infants (Normal Lungs) compared to preterm infants with RDS (panel A). Term infants with mild respiratory disease exhibited normal enrichment curves whereas infants with severe respiratory failure showed surfactant turnover similar to that of preterm infants with RDS (panel B). The curves are obtained by linear interpolation at hourly intervals for each individual subject, then obtaining the mean ± SEM for each group.

Data on normal surfactant turnover in term infants are limited. In vitro studies in fetal and neonatal lung sections have shown increased fatty acid synthesis toward the end of the gestation. Animal experiments, using radioactively labeled precursors of surfactant synthesis, have demonstrated that surfactant turnover is slower in the premature compared to the term 3-day old rabbit. In humans, the availability of stable isotopically labeled surfactant precursors has provided a mean to study surfactant turnover in newborns. To date, normal values for surfactant metabolic indices have only been reported in two studies. Cogo et
al. found FSR values of 15 and 17 %/d using labeled palmitate 68,72. In addition, FSR of 8.0 %/d in term control infants after labeled glucose infusion is described in the thesis by Janssen 129. These values are higher than previously reported values for preterm infants with RDS; FSR 12±8%/day using palmitate 90 and FSR 1.06-5.9 using glucose 83,85,86,89. Cogo et al. 84 also studied older, critically ill infants with varying mechanisms of respiratory failure with labeled palmitate and found a wide range of values for FSR (9.6-81.6%/day). These results are consistent with ours, although caution has to be made in comparing studies tracking direct incorporation of preformed fatty acids (palmitate) into surfactant phospholipids rather than the de novo fatty acid synthesis (glucose and acetate). The present study was the first to use labeled acetate as a tracer of surfactant metabolism in humans. Acetate offers several potential advantages; (1) it is the direct precursor for de novo synthesis of surfactant fatty acids, (2) it may be preferred to glucose in the neonatal lung and is exclusively incorporated into fatty acids in vitro whereas 20-40% of glucose is used for glyceride-glycerol 34,37,38, (3) it is less expensive and (4) MIDA can be used to measure the direct intracellular precursor pool 262,263. The enrichment of the precursor pool was similar for all groups of infants studied indicating that the observed differences in endogenous surfactant turnover were not caused by differences in tracer metabolism or availability, but rather were true differences in the synthesis and clearance of surfactant.

We used the amount of disaturated phospholipids in the tracheal aspirate samples 58 as an indirect reflection of pool size and found similar absolute amounts in term controls and preterm infants with RDS. All preterm infants had received 1 or more doses of exogenous surfactant prior to the start of the study in order to normalize their developmentally deficient surfactant pool. This might explain that the obtained values were similar to those of term infants without lung disease. In fact, when normalized for body weight, the preterm infants had a significantly higher value than controls, likely reflecting that treatment doses often exceeds normal pools size. Augmenting the unlabeled pool with exogenous surfactant could theoretically result in an apparent decrease in turnover of labeled surfactant. Bunt et al. also reported decreasing FSR with increasing number of doses of exogenous surfactant 85. However, in the aforementioned study, the infants receiving multiple doses of surfactant also had the most severe RDS and had not been treated with antenatal steroids, either of which might have independently resulted in a lower FSR 86,92. Others have not been able to demonstrate any effect on either FSR or surfactant T1/2 from exogenous surfactant treatment 90. In the present study, the term infants with severe respiratory failure had significantly lower amounts of phospholipids in their tracheal aspirates than any other group, suggesting a smaller pool size. They also had low FSR, which directly contradicts what would have been expected had isotopic dilution significantly contributed to the differences in surfactant metabolic indices. We also found an inverse correlation between the amount of phospholipids and the disease severity score (r=-0.7, p=0.01). This is in line with the experimental findings in pigs and lambs showing that impaired lung function is associated with decreased surfactant pool size 62,73. Low surfactant synthesis rate can be secondary to lung injury and type II cell dysfunction, as seen in older patients with acute respiratory distress syndrome 264. Whether the low amount of surfactant phospholipid seen in infants with severe respiratory failure results from a developmental lag in surfactant production or from reduced synthesis rate remains to be further investigated.
Relation to ventilation strategy (I)

The metabolic kinetic indices of endogenous surfactant metabolism were similar for infants receiving HFOV and CV. Neither FSR nor T1/2 was found to be different suggesting no decrease in surfactant production or turnover in infants receiving HFOV. Thus, our hypothesis that HFOV would decrease surfactant production could not be confirmed. The hypothesis was based on the observation that surfactant protein B deficient infants had better gas exchange and less surfactant accumulation in the air spaces during HFOV. Physical stretch stimulates surfactant synthesis and secretion and repetitive alveolar distension during conventional mechanical ventilation was thought to have similar effects. Data regarding the possible impact on surfactant metabolism by HFOV are sparse and both experimental and human studies have yielded conflicting results. In surfactant-treated preterm lambs no differences between HFOV and CV were seen in surfactant metabolism after tracer doses of labeled PC. Surfactant turnover measured with intraperitoneal administration of radiolabeled palmitate was also similar in adult rats after 90 minutes of CV or HFOV. Regarding the effects on pulmonary function, inflammation, surfactant secretion, aggregate conversion, phospholipid quantity and composition some studies favoured HFOV whereas others showed equal effectiveness of HFOV compared to CV. Other factors, such as achieving and maintaining alveolar expansion, i.e. the open lung concept, are suggested to be as important as ventilation style. If an appropriate CV strategy with low tidal-volume and sufficient PEEP is applied, the impact on the surfactant system from HFOV and CV are likely to be comparable. Previous human studies have suggested decreased surfactant production measured as lower concentrations of phospholipids and SP-A in tracheal aspirates. The results are not directly comparable to ours since the study populations were more mature and SP-A is under different metabolic control than surfactant phospholipid. Other reasons for the absence of differences between HFOV and CV in the present study may be that we studied a population of extremely immature and critically ill infants in which surfactant metabolism might already have been sufficiently disrupted, below the threshold of detection for this technique. It is also possible that alveolar stretch is more important as a stimulus for surfactant secretion and synthesis in the immediate transition period following birth and that the effect is less obvious at 24 hours of age when the present study was started.

Infants in the HFOV group had a higher respiratory severity score (5.5±2.1 versus 3.4±1.5 for the CV group, p=0.02). Although statistically significant, it is doubtful that this had any clinical relevance. MAP, one of the parameters of the score value, is usually higher in the ventilatory settings for HFOV and was also shown to be significantly higher in the HFOV-group. However, the FIO2 was also higher (0.54±0.18 versus 0.32±0.13 for HFOV and CV respectively, p=0.008), which could indicate more severe lung disease in infants with HFOV and thereby a decreased surfactant synthesis as previously discussed (paper II). If this was the case, it would accentuate rather than mask any expected differences compared to the CV-group. In addition, no correlations could be found between any of the ventilatory parameters and the respiratory severity score or the surfactant metabolic indices.

Since tracheal aspirates are the only realistic samples in infants, our measurements are actually the net results of surfactant synthesis, secretion, catabolism, recycling, tracer metabolism and the rate at which surfactant ascends the tracheobronchial tree, all of which may be influenced by the mode of ventilation. The ongoing development of the methodology with addition of isotopically labeled exogenous surfactant and simultaneous
administration of tracers labeling different metabolic pathways (paper IV) will provide means to further interpret the dynamics of the surfactant system.

**Aspects on stable isotope methodology (IV)**

**Reproducibility and validity**

One term infant with normal lungs underwent two infusion of $^{13}$C-acetate 3 weeks apart, at age 24 and 46 days. Between infusions, the infant was tracheostomized due to poor respiratory drive, but her chest x-ray remained clear, her oxygen requirement low and the ventilatory settings were unchanged. The enrichment curves and surfactant metabolic indices were nearly identical for the two studies with FSR of 25.4 and 25.2%/d and $T_{1/2}$ 25.0 and 25.4 h respectively, suggesting that the measurements using this technique are reproducible.

The technique assumes that palmitate in phospholipid extracts derived from tracheal aspirate is representative of surfactant palmitate. Studies in adult pigs and premature baboons have suggested that tracheal aspirate samples reflect surfactant at the alveolar level.

We have also studied infants undergoing lung transplantation and found, as expected, higher amounts of DSPL in partial bronchoalveolar lavage from the explanted lung compared to a tracheal aspirate sample at the time of transplant. However, the $^{13}$C-enrichment was similar in tracheal aspirate and BAL-fluid samples suggesting that stable isotope technique yields representative data on the kinetics of surfactant (unpublished data).

**New approaches to assess surfactant kinetics**

In addition to previously used descriptive parameters of surfactant kinetics, such as $T_{app}$, $E_{max}$, $T_{max}$ and $T_{1/2}$, we apply new parameters with a clearer physiological significance. The fractional catabolic rate (FCR), representing the percentage of the surfactant pool being removed and replaced by newly produced material per day, and the ratio of FSR/FCR, i.e. the contribution of newly synthesized surfactant from a given precursor to the total surfactant turnover, are proposed as primary measures of surfactant kinetics in the future. The FCR is determined from the negative of the monoexponential down-slope of the enrichment curve. If desired, $T_{1/2}$ can be calculated from the FCR as $T_{1/2}=\ln 2/FCR$ (%/h). Taking the natural log values and selecting points that fall on a straight line provide a more reliable means of curve fitting than non-selectively fitting all post-peak points to an exponential function. We found the FCR measurements to be similar for the three different tracers used in our studies so far, [U-$^{13}$C] glucose, [$^{13}$C] acetate and [$^{13}$C$_4$] palmitate. This was expected as the FCR value represents the total fractional turnover rate from all sources of surfactant production, regardless of which pathway was initially labeled with tracer. The differences between the tracers instead lie in the relative contribution of each pathway to total surfactant production, described by the FSR/FCR ratio. By simultaneous infusion of labeled palmitate and acetate both synthesis from preformed fatty acids and de novo synthesis can be traced and the contribution of each pathway partitioned. We demonstrated that the fractional contribution of plasma palmitate to surfactant phospholipid production was higher than that of de novo synthesized palmitate (FSR$_{\text{palmitate}}$/FCR 29±6% and FSR$_{\text{acetate}}$/FCR 18±3%). The two sources together accounted for only approximately half of the total surfactant production in preterm infants studied during the first 72 hours of life. The remaining unlabeled production may come from turnover of tissue lipid components or
plasma triglycerides, although it is likely that a significant portion comes from recycling of lung surfactant itself. This is consistent with recent data showing that FCR increases with postnatal age. The relative contribution of newly synthesized surfactant (from acetate and plasma palmitate) also increases with postnatal age, suggesting less importance of recycling in preterm infants of 1 month of age compared to immediately after birth (Spence et al., unpublished data). The importance of age and maturity might explain the higher FSR\textsubscript{palmitate} reported by Cavicchioli et al.\textsuperscript{90} compared to the present data. The higher FSR\textsubscript{acetate} found in the group of preterm infants with RDS studied in paper IV compared to the infants in paper II is likely attributed to the latter group being more immature.

When MIDA was used to determine surfactant FSR, we were able to show similar values for acetate and glucose tracers. This was expected since glucose is converted to acetyl-CoA and therefore labels the same de novo pathway as acetate. In infants receiving glucose tracer the FSR values from previously measured plasma glucose were approximately 75% of the FSR values from MIDA, using intracellular acetate as the precursor pool (p=0.02 by paired t-test). It is reasonable to believe that most of the alveolar acetyl-CoA is derived from plasma glucose in a newborn infant and that the remaining 25% is derived form other sources, resulting in isotopic dilution. MIDA was hereby proven to be a reliable mean to estimate an otherwise inaccessible precursor compartment that may be more appropriate to use in the determination of FSR.

**Phospholipid pools and instrumentation**

Paired samples processed as DSPL and total PL were compared and no differences in the surfactant kinetic indices, using $^{13}$C-acetate as tracer, were found. The amount of phospholipids isolated from tracheal aspirates was 2-fold higher in total PL samples, but the proportion of palmitate was lower than in DSPL samples (p<0.001). When analysed on tandem mass spectrometry, total PL contained similar amounts of palmitate as total PC (55% and 62% respectively) and of the DSPL 89% was palmitate (Hamvas and Hsu, unpublished data). In previous stable isotope studies of surfactant metabolism in the newborn, either total PC\textsuperscript{35,73,83-86,90,91}, DSPC\textsuperscript{59,68,74,75,87} or DSPL\textsuperscript{89} have been extracted from tracheal aspirates with the assumption that they all are representative subfractions of surfactant phospholipid. However in the case of lung disease, with leakage of plasma into the alveoli, significant amounts of non-surfactant phospholipids may be present in the airway. If these phospholipids have a different turnover rate than surfactant phospholipid, measurements of surfactant metabolic indices may be influenced, depending on which lipid pool is being interrogated. Our results did not prove non-surfactant phospholipids to have any major impact on the surfactant kinetic indices, suggesting either similar turnover rate or that all the phospholipid subfractions used to date are primarily derived from surfactant phospholipid and therefore comparisons are valid.

Mass spectrometry instrumentation was compared in paired samples from preterm infants receiving [U-$^{13}$C] glucose. GC/MS and GC/C/IRMS yielded very similar indices of surfactant synthesis but revealed differences on the clearance side. GC/C/IRMS resulted in apparently longer T\textsubscript{1/2} and lower FCR values (23.4±1.8 and 21.1±1.5% pools/d respectively, p<0.01). Enrichment curves for GC/C/IRMS and GC/MS from a representative subject are shown in Fig. 9. With GC/MS the number of $^{13}$C-atoms incorporated into palmitate can be distinguished and is represented in the figure as mass+1 (m+1), mass+2 (m+2) etc. Since uniformly labeled $^{13}$C-glucose was used, most of the enrichment was found in the m+2 fraction as glucose is metabolised into acetyl units containing 2 carbons (Fig 3). However, at the end of
the time-enrichment curve there was an increase in the enrichment of the $m+1$ fraction, eventually even exceeding the $M+2$ enrichment. Upon further analysis, the $m+1$ fraction of the palmitate enrichment represented $28\pm5\%$ of the detectable enrichment at the peak of $m+2$ enrichment, but contributed $52\pm15\%$ at the tail ($p=0.001$). The $m+1$ fraction represents recycling of the $^{13}$C-label in intermediary metabolism and will attenuate the down slope and create a bi-exponential curve when samples are analysed with GC/C/IRMS. As GC/C/IRMS depicts the sum of all mass fractions, the result is a consistent overestimation of the $T_{1/2}$ and an apparently lower FCR. The possibility of tracer recycling is important to be aware of since it would underestimate the true FCR of surfactant. In studies using $[^{13}\text{C}]$ acetate and $[^{13}\text{C}_4]$ palmitate as tracers the down slope of the enrichment curve is usually monoexponential, consistent with the assumption that there is no significant tracer recycling. Hence, the inability of GC/C/IRMS to distinguish tracer recycling appears to be a problem confined to the use of $[^{13}\text{C}]$ glucose as tracer. The results from different mass spectrometry instruments are otherwise likely to be comparable.

**Figure 9. Tracer recycling.**
Representative enrichment curves from a preterm infant with RDS after i.v. infusion of $[^{13}\text{C}_6]$ glucose. With the GC/C/IRMS instrumentation (panel A), the analysed compound is combusted and the total enrichment of $^{13}$C over baseline is depicted. With GC/MS (panel B), palmitate molecules incorporating on, two or more $^{13}$C-atoms can be distinguished. Since uniformly labeled glucose is metabolised to doubly labeled acetate, most of the enrichment was found in the $m+2$ fraction of palmitate. At the tail end of the curve there is an increase in $m+1$ species representing recycled glucose. As this distinction cannot be made by GC/C/IRMS the $T_{1/2}$ is consistently overestimated.
EXOGENOUS SURFACTANT AND VENTILATION STRATEGY

Clinical data (V)

During the 5-year period following the implementation of surfactant replacement during nCPAP (INSURE) in 1998, the number of infants requiring mechanical ventilation at KH was reduced by 50% (p<0.01), compared to the preceding 5-year period. At KS, where the conventional treatment approach with surfactant replacement at initiation or during mechanical ventilation was practised throughout the 10-year study period, the rate of mechanical ventilation remained unchanged. The data confirms previously reported results \(^{192-194}\) and shows that the INSURE approach effectively reduces the need for mechanical ventilation. Already during the first 5-year period of the study the risk for a moderately preterm infant born at KS to be treated with mechanical ventilation was significantly higher compared to that of an infant born at KH, but increased greatly after the introduction of INSURE, as reflected by the Odds ratio (Fig. 10).

![Figure 10. The risk of mechanical ventilation.](image)

The Odds ratio for mechanical ventilation at KS compared to KH before (1993-1997) and after (1998-2002) the introduction of surfactant administration during a brief intubation (INSURE) at KH. After the introduction of INSURE the risk for mechanical ventilation decreased significantly at KH, as reflected in a significantly increased risk for an infant born at KS to receive mechanical ventilation, from Odds ratio 2.36 (CI 95%: 1.34-4.17) to 8.56 (CI 95%: 4.31-17.01).

This difference could not be explained by population differences such as maturity, birth weight or the use of antenatal steroids. Neither could it be shown that that population at KS had more severe lung disease. The a/A ratio, reflecting disease severity, was
similar immediately before the first dose of surfactant at both centers. The a/A ratio immediately before the initiation of mechanical ventilation was lower at KH, which might be reflective of the fact the infants at KH required transportation to KS for mechanical ventilation although no significant difference was found in the age at which mechanical ventilation was started. Mortality was higher at KS for the total time period, but not in the later time period (1998-2002) after the introduction of INSURE. Outcome parameters were otherwise similar between the two centers and time periods. In agreement with previous trials of surfactant and CPAP, no reduction on the incidence of BPD could be shown. The number of infants developing BPD ranged from 8-17%, which is similar to what has been previously reported from Stockholm, but slightly higher than the results from Denmark. It is generally difficult to compare the incidence of BPD since it varies greatly between studies, from 2.6-34.5% depending on the population, underlying disease, ventilation strategies and the definition of BPD. In addition, the clinical pattern of BPD is changing and factors such as infections, inflammatory reactions, and surfactant abnormalities are proposed to play a more important role in the development of chronic lung disease than ventilator induced lung injury. Thus, the impact of INSURE on BPD incidence may not be as evident. On the other hand, we found the development of BPD to be associated with a longer duration of mechanical ventilation (a median of 8 d compared to 2 d in infants without BPD, p<0.001), and an impaired treatment response to surfactant. The immediate improvement in oxygenation measured by the a/A ratio was lower compared to infants not developing BPD and the difference was even more pronounced after 48 hours (p<0.01). Infants who later developed BPD also tended to have a lower a/A ratio before surfactant administration suggestive of a more severe lung disease. Pulmonary oedema and infection are features in more severe disease that also predispose to BPD. Inactivation of surfactant might explain a poor treatment response. However, the exact mechanism behind our observation remains to be further investigated.

It has been previously shown that surfactant treatment early in the course of the disease can reduce the risk of complications and improve the outcome. Verder et al. also found that early INSURE treatment could further reduce the need for mechanical ventilation. However, early nCPAP alone prevents progression to respiratory failure and in combination with antenatal steroids many infants with RDS >27 weeks of gestation will develop only mild clinical symptoms. Hence, prophylactic or early rescue surfactant administration carries the risk of unnecessary treating a number of infants whose disease progress would not warrant surfactant, which has both ethical and economic consequences. Therefore, in Stockholm surfactant has been routinely administered as rescue treatment to infants with progressing respiratory failure and an a/A ratio of 0.22 or below. Of the INSURE treated infants only 19% were considered failures, i.e. progressed to needing mechanical ventilation, which is even lower than the 25% of the early-treated infants in the Danish study. The present follow-up shows that, in our hands, surfactant administration by the INSURE-approach as later rescue treatment is as efficient in reducing the need for mechanical ventilation as treatment earlier in the course of the disease.

Infants receiving surfactant by INSURE showed a greater improvement in oxygenation in compared to infants receiving surfactant treatment in conjunction with mechanical ventilation. The rapid and pronounced increase in a/A ratio after INSURE was sustained over the 48 hours following treatment (Fig. 11). In infants treated with surfactant together with mechanical ventilation the increase in a/A ratio was less dramatic and continued
Surfactant administration by INSURE resulted in a greater improvement in oxygenation compared to surfactant treatment followed by mechanical ventilation (Surf + MV). The arterial to alveolar ratio (a/A ratio) was significantly higher during the first 12 h after surfactant replacement. In the surf + MV group, the a/A ratio more often deteriorated after the initial surfactant dose and 58% received additional doses. To increase slowly over the following 2 days. During that time, many infants also exhibited deteriorating a/A ratios qualifying them for additional surfactant doses. Of infants treated with surfactant in conjunction with mechanical ventilation, 58% required more than one dose of surfactant compared to only 17% of the INSURE-treated infants (p<0.01). In the latter group, all infants receiving additional doses were INSURE-failures progressing to mechanical ventilation. This finding contradicts a recent meta-analysis of 4 studies suggesting that the number of surfactant doses per patient was significantly greater in patients receiving early surfactant administration with brief mechanical ventilation compared to later, selective treatment followed by continued mechanical ventilation [191]. However, the European Multicenter trial reported that mechanically ventilated infants often require multiple surfactant doses [190] and in previous studies of surfactant together with nCPAP, a single dose was sufficient to reverse the clinical course of RDS in most patients [192-194], supporting the present finding.

The improved treatment response after INSURE could not be explained by differences in maturity or disease severity. The a/A ratio before INSURE treatment was similar in comparison to infants receiving surfactant treatment followed by mechanical ventilation. Instead, the improvement was attributed the avoiding of mechanical ventilation. In lambs, mechanical ventilation produces more severe lung injury than CPAP, with a possible disruption of the surfactant system as a consequence [245]. In neonates with RDS, dynamic compliance was increased after surfactant treatment, an improvement that could not be detected during mechanical ventilation [211]. In order to further investigate the mechanisms behind the positive effects of INSURE an experimental set-up in an animal model was designed (paper III).

Figure 11. Oxygenation after surfactant treatment.

Surfactant administration by INSURE resulted in a greater improvement in oxygenation compared to surfactant treatment followed by mechanical ventilation (Surf + MV). The arterial to alveolar ratio (a/A ratio) was significantly higher during the first 12 h after surfactant replacement. In the surf + MV group, the a/A ratio more often deteriorated after the initial surfactant dose and 58% received additional doses. To increase slowly over the following 2 days. During that time, many infants also exhibited deteriorating a/A ratios qualifying them for additional surfactant doses. Of infants treated with surfactant in conjunction with mechanical ventilation, 58% required more than one dose of surfactant compared to only 17% of the INSURE-treated infants (p<0.01). In the latter group, all infants receiving additional doses were INSURE-failures progressing to mechanical ventilation. This finding contradicts a recent meta-analysis of 4 studies suggesting that the number of surfactant doses per patient was significantly greater in patients receiving early surfactant administration with brief mechanical ventilation compared to later, selective treatment followed by continued mechanical ventilation [191]. However, the European Multicenter trial reported that mechanically ventilated infants often require multiple surfactant doses [190] and in previous studies of surfactant together with nCPAP, a single dose was sufficient to reverse the clinical course of RDS in most patients [192-194], supporting the present finding.

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Experimental data (III)

When preterm, newborn rabbits were treated with labeled surfactant at birth and allowed to breathe spontaneously, a greater fraction of the surfactant became associated with the lung tissue after 4 h compared to mechanically ventilated rabbits (53±4% and 26±3% respectively, p<0.001). The immediate tissue association, i.e. label that could not be recovered by bronchoalveolar lavage, was 25±5% in the control group, which is in agreement with previous reports. The tissue association was similar in mechanically ventilated animals and controls indicating that after the immediate tissue association no further association occurred in mechanically ventilated animals during the 4-h observation period. This suggests that mechanical ventilation impairs or delays the process of continuous tissue association/uptake taking place in animals allowed to breathe spontaneously. The functional significance of the rapid tissue association of surfactant after intratracheal administration has not yet been fully elucidated. Presumably this phenomenon reflects the entering of exogenous surfactant into the metabolic pathways of endogenous surfactant and is therefore a desired process. The first immediate loss of surfactant recoverable by the lavage procedure appears to be due to tissue binding rather than uptake. It has been suggested that the lipids and proteins of surfactant are “sticky”, allowing them to adhere to the epithelial surface of the alveolar type II cells.

The lung function measurements showed that dynamic compliance was generally adequate in all animals. This was expected since the animals were only moderately immature and all had received surfactant replacement at birth. Still, the dynamic compliance of the spontaneously breathing rabbits was significantly higher compared to the mechanically ventilated animals with median (IQR) values of 0.90 (0.76-1.13) and 0.75 (0.70-0.80) ml · kg⁻¹ · cmH₂O⁻¹ respectively (p<0.05). We found a weak, but statistically significant, positive correlation between dynamic lung-thorax compliance and the degree of tissue association (r=0.41, p=0.04). Hence, the larger alveolar pool of exogenous surfactant did not contribute to any improvement in lung function. Instead, we found evidence for surfactant inactivation that could explain the lower dynamic compliance seen in the mechanically ventilated group. The lipid peroxidation score was higher in mechanically ventilated compared to spontaneously breathing animals (1.26 (1.10-1.66) versus 0.99 (0.80-1.12) median (IQR), p<0.05). There was a trend towards a lower percentage of microbubbles on MST (88 (63-93%) versus 95 (91-100%), p=0.11). Mechanical ventilation can induce varying degrees of lung injury, leading to fluid leakage into the alveoli and inflammatory responses. Activated phagocytes or bacteria produce free oxygen radicals that, at least under in vitro conditions, cause peroxidation of surfactant lipids. Our data suggests that mechanical ventilation results in inactivation of surfactant by lipid peroxidation, either by increased production or reduced clearance of free oxygen radicals. This may be aggravated in preterm human infants as prematurity has been linked to a higher rate of free radical mediated lipid peroxidation that was associated with a poorer outcome.

In our rabbit model, the application of CPAP to spontaneously breathing animals was not feasible. Consequently, we did not use PEEP in the ventilator settings for the mechanically ventilated animals. PEEP is known to be important for the efficacy of surfactant treatment by facilitating an even lung expansion pattern and preventing end-expiratory collapse. The absence of PEEP might therefore have impaired the treatment response and aggravated lung injury in the mechanically ventilated group. However, surfactant-deficient
spontaneously breathing preterm rabbits develop bronchiolar epithelial lesions similar to those observed after mechanical ventilation \(^{110}\) and, to our knowledge, there are no experimental studies of lung mechanics and injury in spontaneously breathing subjects with and without CPAP. Furthermore, the tidal volume of 10 ml/kg used in this animal study would be considered excessive in the clinical setting, carrying the risk of volutrauma and also predispose to lung injury \(^{216}\). Our ventilator system for preterm rabbits is well established and a tidal volume of 8-10 ml/kg has previously been evaluated in rabbits with the same degree of lung maturation, yielding similar values of dynamic lung-thorax compliance and no histological evidence of lung injury \(^{111}\). In addition, all animals received prophylactic surfactant, shown to protect against ventilator induced lung injury \(^{224,225,227}\). Thus, the ventilator settings used in our moderately immature, surfactant treated animals are not to be considered injurious. However, the results indicate that the negative effects of mechanical ventilation are still significant compared to spontaneous breathing.
CONCLUSIONS

- This investigation is one of the first to describe normal endogenous surfactant metabolism using stable isotope technique. It shows that term infants without significant lung disease have faster surfactant turnover than preterm infants with RDS. Obtaining normal reference values is crucial for correct interpretation of surfactant kinetics in newborn infants.

- Severe respiratory disease in term infants disrupts endogenous surfactant metabolism similar to that of preterm infants with RDS, whereas in mild respiratory disease a normal surfactant turnover is preserved. Severe disease is inversely correlated with low amounts of surfactant phospholipids in tracheal aspirate samples, suggesting reduced surfactant pool size. Delayed maturity of the surfactant system or impairment from underlying disease may cause the disruption of endogenous surfactant metabolism in term infants with severe respiratory disease.

- The stable isotope technique for studies of endogenous surfactant metabolism *in vivo* yields reproducible data and similar indices of surfactant kinetics regardless of mass spectrometry instrumentation and surfactant phospholipid pool being analysed. Fractional catabolic rate is shown to be independent of the tracer for surfactant synthesis being used and is therefore suggested as the primary measure of surfactant turnover.

- Mode of mechanical ventilation (conventional ventilation or high frequency oscillatory ventilation) has no impact on endogenous surfactant metabolism in preterm infants with RDS.

- Mechanical ventilation impairs the treatment response to exogenous surfactant. In the clinical setting, surfactant administration during a brief intubation (INSURE) is associated with a more pronounced and sustained improvement in oxygenation and a reduced need for additional surfactant doses compared to surfactant administration in conjunction with mechanical ventilation. Experimentally, mechanically ventilated preterm rabbits have impaired tissue association of exogenous surfactant, lower dynamic compliance and evidence of surfactant inactivation, consistent with a poorer treatment response compared to spontaneously breathing animals. These mechanisms may explain the reduced surfactant requirements after INSURE treatment.

- Implementation of the INSURE strategy in a population of moderately preterm infants reduced the need for mechanical ventilation by 50%, without adverse effects on outcome. With associated potential advantages such as reductions in postnatal transfers to tertiary care centers for mechanical ventilation and costs for neonatal intensive care, the results establish INSURE as a powerful strategy to safely reduce the mechanical ventilation rate and improve the surfactant treatment response in preterm infants with RDS.
Surfaktantmetabolism hos nyfödda: inverkan av ventilationsstrategier och lungsjukdom

Surfaktant är en blandning av fett och protein som bildas i lungornas alveolära typ II celler. Det verkar ytspänningsnedersättande och hindrar lungblåsorna från att falla samman vid utandning. Barn som föds mer än 6-8 veckor för tidigt har omogna lungor och därför varierande grad av brist på surfaktant. Många utvecklar en för surfaktantbrist karaktäristisk andningsstörning, s.k. respiratory distress syndrome (RDS). Trots stora framsteg inom neonatalvården under senare år, med bl. a. möjlighet att behandla med tillfört surfaktant, kvarstår RDS som en av de främsta orsakerna till morbiditet och mortalitet hos underburna barn. Även fullgångna barn kan ha störningar i surfaktantsystemet sekundärt till lungsjukdom av annan gen. Lunginfektioner och aspiration av mekoniumtillblandat fostervatten är exempel på tillstånd som leder till inaktivering av surfactant och därmed försämrad lungfunktion. Respiratorvård är en viktig del i behandlingen av svåra lungsjukdomar hos nyfödda barn. Trots allt mer fysiologiskt anpassade respiratorer finns det en risk för att mekanisk ventilation med övertryck leder till mikroskopiska skador i lungblåsorna. Detta kan dels leda till att det läcker in plasma i alveolerna, vilket inaktiviterar surfactant och dels till att typ II cellerna skadas och surfaktantproduktionen därmed påverkas.


Surfactant metabolism in the newborn

avlägsnas sedan direkt så att barnet kan andas själv hjälp av ett mottryck i näsluften, s.k. CPAP (Continuous Positive Airway Pressure). Metoden kallas INSURE (INtubation-SURfaktant-Extubation) och förväntas minska antalet barn med RDS som kräver respiratorvård. Respiratorvård innebär inte bara en ökad risk för komplikationer och kroniska lungskador hos det nyfödda barnet utan har också i Stockholm inneburit att barnet behövt transporterats till Karolinska Universitetssjukhuset Solna om det fötts på annan förlössningsenhet. Efter införandet av INSURE metoden har vi observerat att barn som INSURE behandlas svarar med förbättrade syresättning och att effekten ofta bibehålls under den akuta sjukdomsfasen. Detta gör att mer än en dos surfaktant sällan är nödvändigt till skillnad från om behandlingen ges i samband med respiratorvård då ofta upprepade doser krävs.

Målsetningen med denna avhandling var att (1) med hjälp av stabil isotopteknik beskriva normal och störd surfaktantmetabolism hos fullgångna och för tidigt födda barn, (2) testa hypotesen att högfrekvens oscillering ventilation (HFOV) minskar surfaktantproduktionen hos presterma barn med RDS, (3) systematiskt utvärdera aktuella metoder att studera surfaktantmetabolism med hjälp av stabil isotoper, (4) göra en populationsbaserad uppföljning av effekterna efter införandet av INSURE, samt (5) i en experimentell djurmodell testa hypotesen att surfaktantbehandling som efterföljs av spontanandning ger ett bättre och mer bibehållet behandlingsvar jämfört med surfaktantbehandling som efterföljs av mekanisk ventilation.


Retrospektivt studerades alla barn med RDS (n=420) födda i graviditetsvecka 27+0 till 33+6 vid Karolinska Huddinge och Karolinska Solna i Stockholm under en 10-års period (1993-2002). Efter införandet INSURE metoden vid Karolinska Huddinge 1998 har respiratorbehandlet sjuknit med 50 % utan att några negativa effekter på utfallet har kunnat påvisas. INSURE behandling resulterade i signifikant bättre syresättningen (mätt som
arteriell/alveolär kvot) under de första 12 timmarna jämfört med hos barn som surfaktantbehandlats i samband med respiratorvård. Endast 17 % av de INSURE behandlade barnen krävde mer än en dos surfaktant jämför med 58 % av respiratorvårdade barn.

Mot bakgrund av detta utvecklade vi en djurexperimentell modell för att studera effekterna av surfaktantbehandling vid mekanisk ventilation. Femtifyra prematura kaniner behandlades med faryngeal deposition av radioaktivt märkt surfaktant (¹⁴C-Curosurf®) och randomiserades till spontanandning, mekanisk ventilation eller kontrolldjur. De mekaniskt ventilerade djuren upvisade utebliven vävnadsassociation av tillfört surfaktant och ökad lipidperoxidation i bronchoalveolär lavagevätska som tecken till surfaktantinaktivering. Detta var associerat till en lägre dynamisk lungcompliance jämfört med spontanandande djur och visade på ett sämre behandlingssvar.

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