From the Institution of Clinical Science, Department of Pediatrics,
Karolinska University Hospital Huddinge,
Karolinska Institutet, Stockholm, Sweden

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

Kajsa Bohlin, MD

Stockholm 2005

Cover: “Soap bubble image”
Macrophotography of thin soap film,
by Karl E. Deckart, 1999
(printed with permission)

Published and printed by Repro Print AB
Box 21085, SE-100 31 Stockholm, Sweden

ISBN 91-7140-229-2

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 (^{13}C) labeled precursors for surfactant phospholipid, the ^{13}C-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.

ORIGINAL PAPERS

This thesis is based on the following papers, listed in chronological order. Papers will be referred to by their Roman numerals:

I. Merchak A, Janssen DJ, Bohlin K, Patterson BW, Zimmermann LJ, Carnielli VP, Hamvas A. Endogenous pulmonary surfactant metabolism is not affected by mode of ventilation in premature infants with respiratory distress syndrome, Journal of Pediatrics, 140, 693-8, 2002.

II. Bohlin K, Merchak A, Spence K, Patterson BW, Hamvas A. Endogenous surfactant metabolism in newborn infants with and without respiratory failure, Pediatric Research, 54,185-91, 2003.

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*.

IV. Bohlin K, Patterson BW, Spence KL, Merchak A, Zozobrado JCG, Zimmerman LJI, Carnielli VP, Hamvas A. Metabolic kinetics of pulmonary surfactant in newborn infants using endogenous stable isotope techniques, Journal of Lipid Research, *resubmitted*.

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*.

CONTENTS

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
$T_{1/2}$	half-life
TA	tracheal aspirate
Tapp	time of appearance
Tmax	time of maximum enrichment
TTR	tracer to tracee ratio

PREFACE

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.

Kajsa Bohlin, Stockholm 2005

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 [1]. 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 [2]. 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 [3,4]. Already in 1903, Hochheim reported on hyaline membranes in the lungs of infants with respiratory distress [5]. 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 [6] 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 [7]. In the 1960s, pulmonary surfactant underwent further biochemical and functional characterization. The high content and functional importance of disaturated phosphatidylcholine was described [8,9]. 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 [10,11]. During the 1970s, ground-breaking experimental work of surfactant replacement in animal models performed by Robertson and Enhörning [12-16] 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 [17]. During this time, proteins were also recognized as important constituents of surfactant [18] and prenatal steroid treatment was first reported to decrease the incidence and severity of HMD in premature infants [19]. 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 [20-24]. 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].

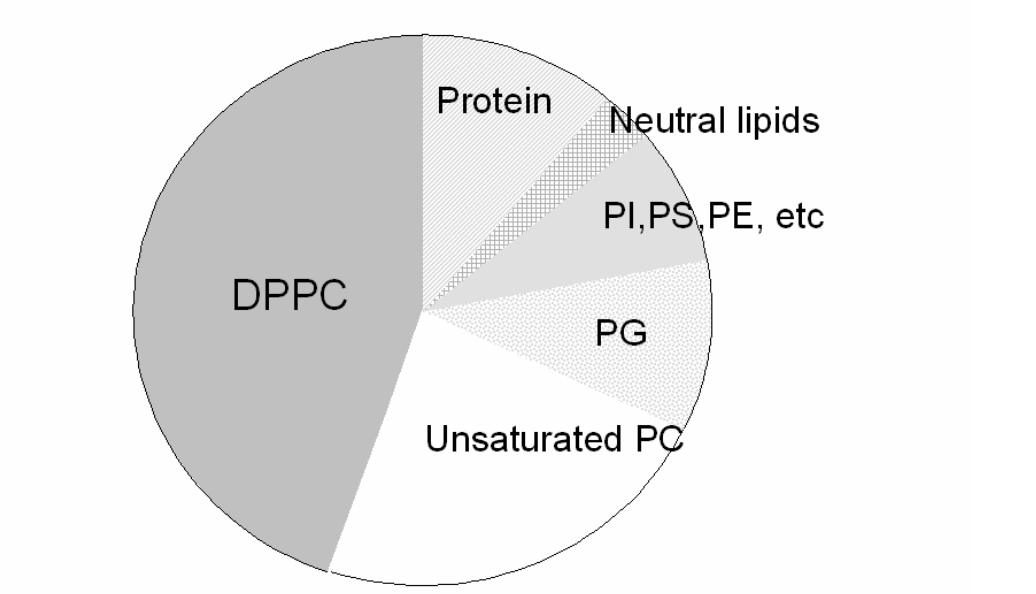

Figure 1. Composition of pulmonary surfactant

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 [27]. 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 [25]. SP-A also functions as an important non-immune host defence protein [28,29]. 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 [30]. 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 [27]. SP-D is not exclusively produced in the lung and less than 10% is associated with surfactant phospholipids. Like SP-A, SP-D s 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 [31].

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 [32]. 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 [33]. The phospholipids and proteins are subsequently degraded and recycled back into the type II cells or phagocytized by macrophages (Fig. 2).

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 [34]. 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 [35]. Experimental data suggest that *de novo* synthesis may be of greater importance for phospholipid formation in the perinatal period [36]. 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 [34,37,38]. 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 [36].

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

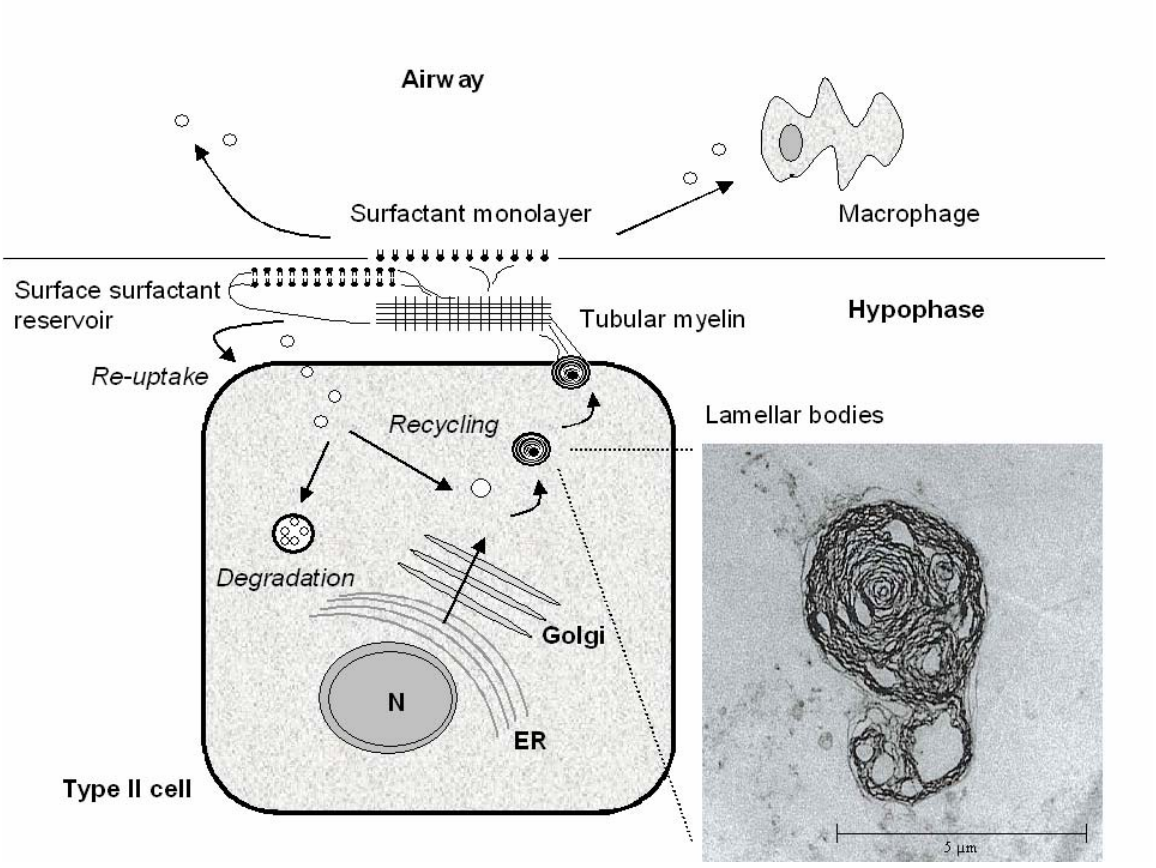

Figure 2. Schematic diagram of surfactant synthesis and clearance.
N=nucleus, ER=endoplasmatic reticulum. Electron micrograph of lamellar body (courtesy B-M Linderholm).

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 [33]. 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 [50].

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 [51,52]. In the newborn, only small amounts are degraded by macrophages or lost via the airway [36,53]. 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 [54]. In contrast, animal data suggest that, in the newborn lung the efficiency of recycling is greater than 90% and the catabolism is minimal [55,56]. 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 [57]. 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 [58]. 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 [59]. 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 [60]. 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 [61]. Preterm rabbits and lambs are also reported to have surfactant pool sizes of 3-10 mg/kg [62-64]; 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 [65]. 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 [66]. 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 [67]. Therefore, the PC concentration in airway samples are sometimes used as a reflection of pool size, at least for comparative purposes (paper II) [68]. 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 [69]. Additional markers are PG and SP-A levels in airway samples, as these surfactant components are decreased in RDS [26,70,71]. 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 [72] and thermally injured pigs exhibited a significantly reduced PC-pool size in alveolar washes [73]. Infants developing BPD showed higher apparent surfactant pool sizes compared to those not developing BPD [74] 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 [75]. 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 [76,77]. 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 [78]. 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 [56,79,80]. In term lambs, the $T_{1/2}$ was about 50 hours after i.v. administration of radiolabeled palmitate [81] 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 [82]. In the preterm and the term rabbits, the efficacy of recycling exceeded 90% and surfactant turnover times were longer compared to adult animals [55]. 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 [35]. The results showed that plasma free fatty acid was the primary source of palmitate for

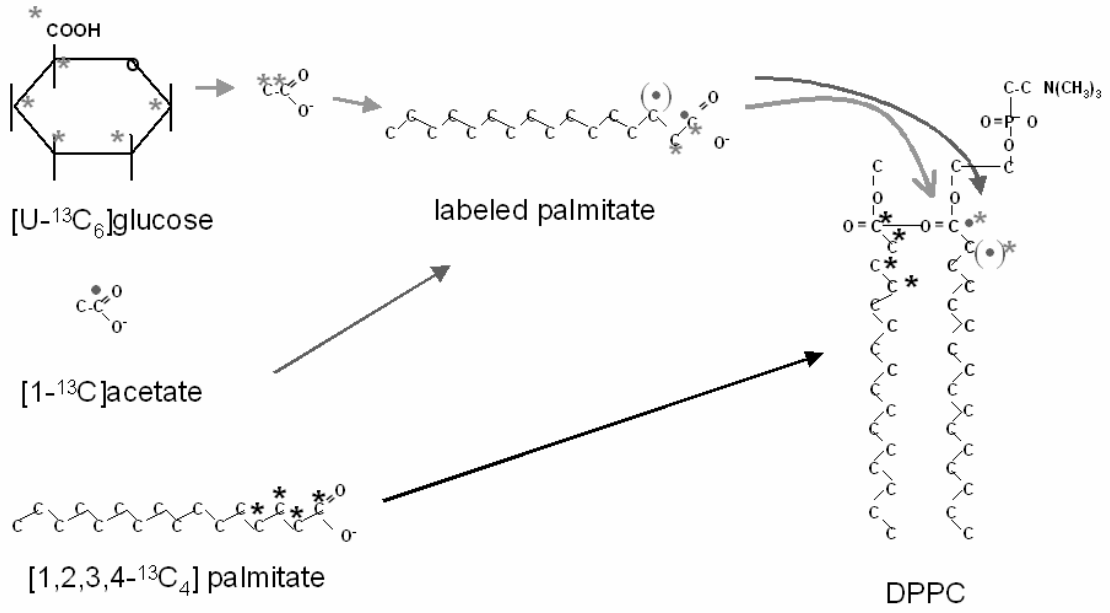

Figure 3. Tracer incorporation into surfactant phospholipid.
Stable isotope labeled precursors of DPPC (disaturated phosphatidylcholine) are administered as 24-h intravenous infusions. [U-$^{13}C_6$] glucose and [1-$^{13}C_1$] acetate are incorporated into palmitate by *de novo* fatty acid synthesis in the alveolar type II cell. [1,2,3,4-$^{13}C_4$] 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 [83]. 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 ^{13}C-glucose. The maximum ^{13}C-enrichment in palmitate from serial tracheal aspirate samples was found to be 48-96 hours after the start of infusion and the $T_{1/2}$ 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 ^{13}C-palmitate and linoleic acid to study a group of critically ill infants and found FSR values ranging from 10-82% per day and $T_{1/2}$ values of 17-178 hours [84]. The somewhat faster synthesis rate from plasma palmitate compared to glucose was in line with the findings of Martini et al. in pigs [35]. 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 theses 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 [103]. 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[104].

Respiratory Distress Syndrome (RDS)

Lung development

The normal development of the lung is divided into three periods: embryonic, fetal and postnatal [27]. 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 sub-divided 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 multi-vesicular 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 [105,106]. 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 [7]. In addition, RDS is associated with delayed absorption of fetal lung water due to defective sodium transport mechanisms [107]. 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 [108,109]. 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 [110], producing epithelial injury and pulmonary edema [111,112]. The leakage of plasma proteins into the alveolar space further aggravates surfactant deficiency by inactivation [113,114]. 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

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 $T_{1/2}$ 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

function [136]. 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 [137]. 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 [138]. In newborn rabbits with experimental GBS pneumonia, exogenous surfactant reduced bacterial proliferation and improved lung function [139]. Inactivation of SP-A, believed to be important in the pulmonary antimicrobial defense, did not influence the bacterial proliferation in the rabbit model [140]. Other studies have suggested that exogenous surfactant may promote bacterial growth by reducing the capacity of alveolar macrophages to eliminate GBS [141]. 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 [142]. 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 [143]. 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 [31]. 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 [144]. 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 [145]. 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 [146]. Ultrastructural changes include markedly abnormal lamellar bodies [147]. 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) [148]. However,

several other, often family-unique, SP-B mutations have been identified, some of which may result in only partial deficiency [149,150]. The carrier frequency of the common mutation has been estimated to 1 per 1,000, but the exact incidence is not known [151]. 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 [152,153]. SP-B knockout mice unable to produce SP-B die within minutes after birth [154]. 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 [155]. *In vitro* studies of lung explants from SP-B deficient infants showed normal synthesis rates for phospholipids [156]. 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 [129]. 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 [157]. 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 [158]. 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 [157,159,160]. 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 [158]. 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 [161]. 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 [162]. 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 reflects 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 is 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 favour 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 [168] 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 [169]. 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 arteriousus (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 post-natal 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 [190]. 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 [191-194]. The meta-analysis by Stevens et al. reported an increased number of surfactant doses per patients in the early treatment group [191]. However, in the experience of us and others [192-194] 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 [195,196]. After instillation into the airway there is a rapid tissue association of the exogenous surfactant that has been described in several experimental settings [54,197-200]. 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 [201,202]. 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 [203]. A poor response to surfactant treatment can also identify a group of infants at risk of dying [204]. 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

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 [205]. In addition, lung resistance appears to be increased in RDS, even during spontaneous breathing [206]. Mechanically ventilated infants with RDS have 3-6 times greater resistance than normal infants during spontaneous breathing [207], some of which can be attributed to the endotracheal tube [208].

Animal studies have clearly established that surfactant treatment improves gas exchange and compliance [14,209]. Dynamic compliance reflects surfactant function and activity and has long been used to evaluate treatment response [111]. 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 en elevation of surface tension in lung lavage fluid suggesting surfactant inactivation [198]. Compliance measurements in surfactant treated, spontaneous breathing animals are sparse [210]. 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 [211]. 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 [212,213].

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 [214], modern ventilators can measure and even guarantee specific volume delivery. Barotrauma was previously considered to be the major cause of ventilator induced lung injury [115,215]. 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 [216]. 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 [115,217,218]. In rabbits,

mechanical ventilation increases surfactant aggregate conversion [52]. High tidal volumes result in higher ratios of small, inactive surfactant aggregates and correlate with severity of lung injury [219]. 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 radiolabeled palmitate in moderately preterm rabbits [220]. 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 [221]. 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 [222]. 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 [175]. The spreading of surfactant treatment doses was impaired resulting in an uneven and patchy distribution, however this could be alleviated by prophylactic surfactant treatment [223]. 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 [224]. Others have also shown that surfactant sufficient lungs tolerate mechanical ventilation well [111,225-227]. 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 [228].

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 [229]. PEEP decreased the recovery of intravascular labeled albumin in the lungs of surfactant treated preterm rabbits as an indicator of less lung injury [230] and improved compliance in surfactant treated preterm lambs [120]. 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 [231,232] and suggested a modest reduction in CLD and death in very-low-birth-weight infants [232]. 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 [233,234] and not verified in other [235]. Phospholipid composition and quantities have been reported to be unchanged after HFOV in non-human primates [236,237]. In contrast, a possibly decreased surfactant production is suggested by lower concentrations of SP-A in preterm infants receiving HFOV [238] and increasing PC concentrations after switching from HFOV to conventional ventilation [239]. Knowledge regarding the impact of ventilation strategy on surfactant synthesis, clearance and pool size in humans is still limited.

Continuous Positive Airway Pressure and INSURE

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

Table 1. Population characteristics of surfactant kinetic studies using stable isotopes

	Preterm infants (n=44)	Term infants (n=17)
Gestational age (weeks)	25.8±0.2	38.1±1.4
Weight at study start (g)	781±24	3667±227
Age at study start	34±3 hours	40±14 days
Sex (male/female)	25/19	11/6
Race (white/black/other)	12/30/2	14/1/2
Survival at discharge n (%)	35 (80)	14 (82)

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 F_iO_2. 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 F_iO_2 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 H_2O using the Infant Flow system (EME, UK) or a Benveniste jet system [252]. The fraction of inspired oxygen (F_iO_2) is adjusted to give an arterial or transcutaneous partial pressure of oxygen (PO_2) value of 8-10 kPa. The oxygenation is assessed by the arterial to alveolar ratio (a/A ratio), calculated as:

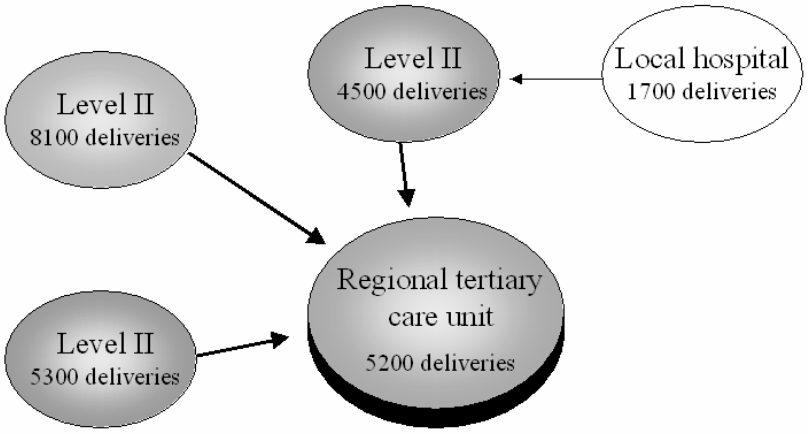

Figure 4. The organization of neonatal care in Stockholm County.
Approximately 24 800 deliveries/year (2004) are divided on five delivery centers, four of which are associated with neonatal units (grey). During the study period (1993-2002), all infants requiring mechanical ventilation were transferred to one regional level III neonatal unit.

a/A ratio = $P_aO_2/(F_iO_2 \times 95)-P_aCO_2$

P_aO_2 is the arterial partial pressure of oxygen (kPa); F_iO_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 tiopentalsodium (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 Zeeland), 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 F_iO_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 F_iO_2 and

characteristic radiographic findings [117]. 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 [253]. 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. [254]. 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 [255]. 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.

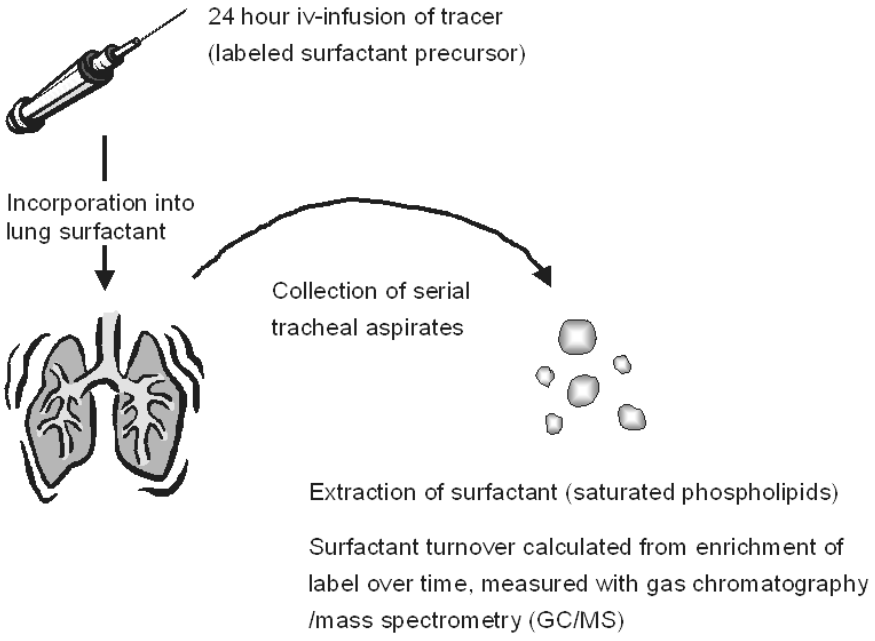

Figure 5. Overview of the stable isotope methodology for studies of endogenous surfactant metabolism.

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 [258]. For disaturated PL a modification of the method by Mason et al was used [259]. 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 [89] 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 tracee 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).

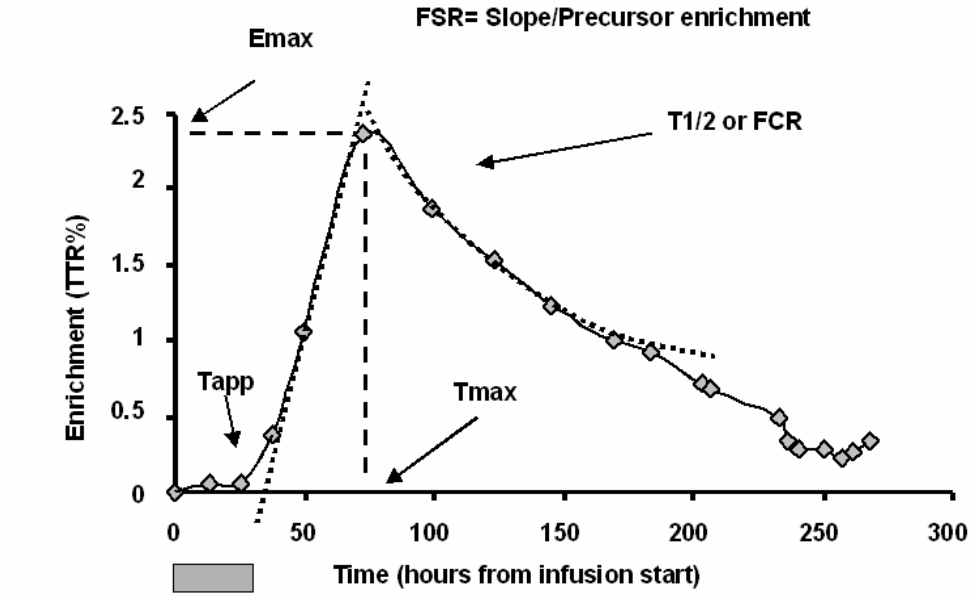

Figure 6. ^{13}C-enrichment-over-time curve.
After intravenous infusion of a ^{13}C-labeled precursor of surfactant lipid, the ^{13}C-enrichment of palmitate in serial tracheal aspirate is measured. From the enrichment-over-time curve indices of surfactant metabolic kinetics are derived; the time of 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) [35,89,257]. 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 37ºC and flushed with 100% oxygen. The duration of the procedure was 1-2 min. The next fetus was not delivered

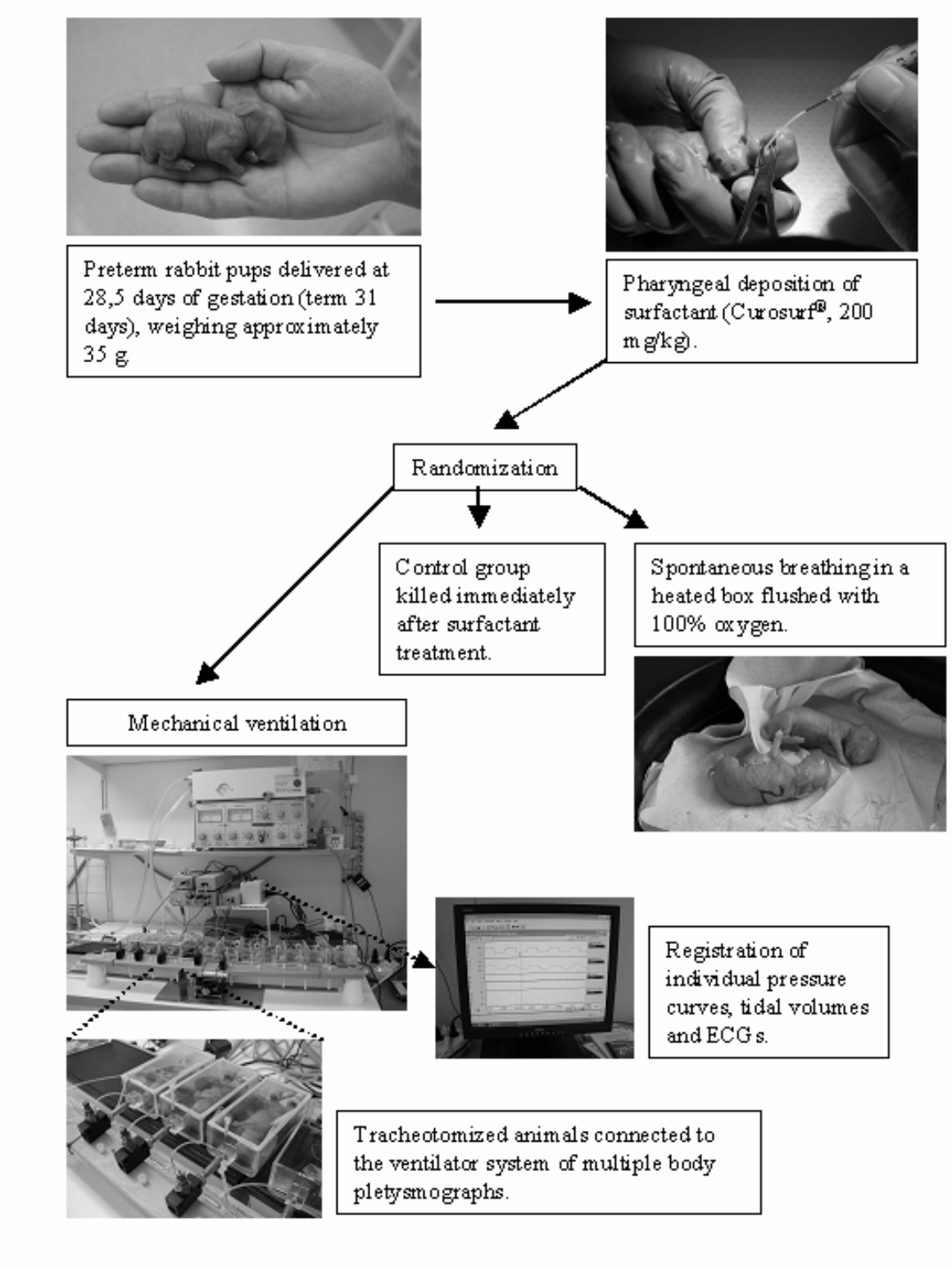

Figure 7. The rabbit model.

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 37ºC [111]. 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 (cmH_2O) 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 ^{14}C-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 ^{14}C-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 [261]. 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 [183] was performed in a sub-group of animals (3 litters, n= 15). Microbubbles were defined as bubbles with diameter <20 μm [184].

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-Withney 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 Stat graphics (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 $T_{1/2}$ (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 ^{13}C-enrichment in surfactant palmitate over time for the different study groups is shown in Fig. 8.

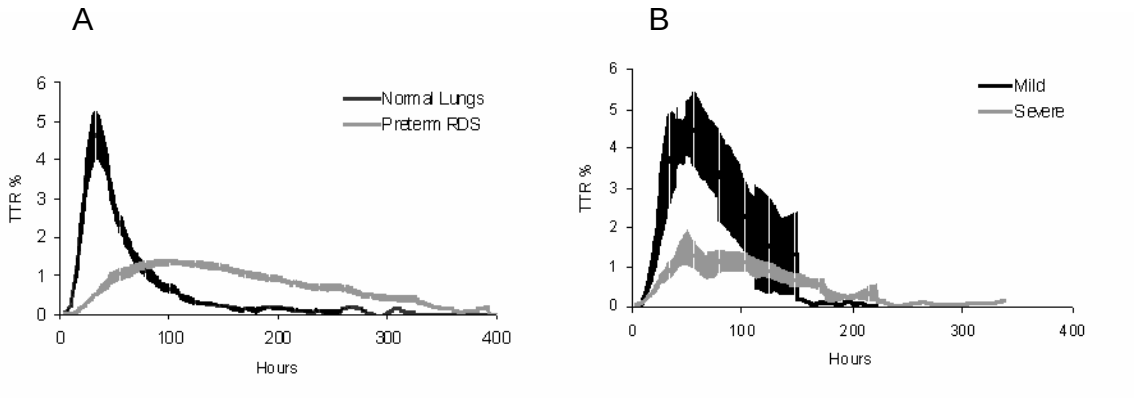

Figure 8. Surfactant turnover in term and preterm infants.

13C-enrichment in surfactant palmitate from serial tracheal aspirates after i.v. infusion of [1-$^{13}C_1$] 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 [76,77]. 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 [56,79,80]. 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 [68,75]. 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 $T_{1/2}$ 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 $T_{1/2}$ 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 [265]. Physical stretch stimulates surfactant synthesis and secretion [49,266] and repetitive alveolar distension during conventional mechanical ventilation was thought to have similar effects. Data regarding the possible the 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 [267]. Surfactant turnover measured with intraperitoneal administration of radiolabeled palmitate was also similar in adult rats after 90 minutes of CV or HFOV [235]. Regarding the effects on pulmonary function, inflammation, surfactant secretion, aggregate conversion, phospholipid quantity and composition some studies favoured HFOV [233,234,268-270] whereas others showed equal effectiveness of HFOV compared to CV [227,235-237]. 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 [215,271-273]. Previous human studies have suggested decreased surfactant production measured as lower concentrations of phospholipids and SP-A in tracheal aspirates [238,239]. 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 F_iO_2 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) [73,234]. 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 [75] 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 [92]. 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 $T_{1/2}$=ln2/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 ($FSR_{palmitate}$/FCR 29±6% and $FSR_{acetate}$/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 $FSR_{palmitate}$ reported by Cavicchioli et al [90] compared to the present data. The higher $FSR_{acetate}$ 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 [35,73,83-86,90,91], DSPC [59,68,74,75,87] or DSPL [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 $T_{1/2}$ 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±5% of the detectable enrichment at the peak of m+2 enrichment, but contributed 52±15% 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 [263]. In studies using [^{13}C] acetate and [$^{13}C_4$] 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 [U-^{13}C] glucose as tracer. The results from different mass spectrometry instruments are otherwise likely to be comparable.

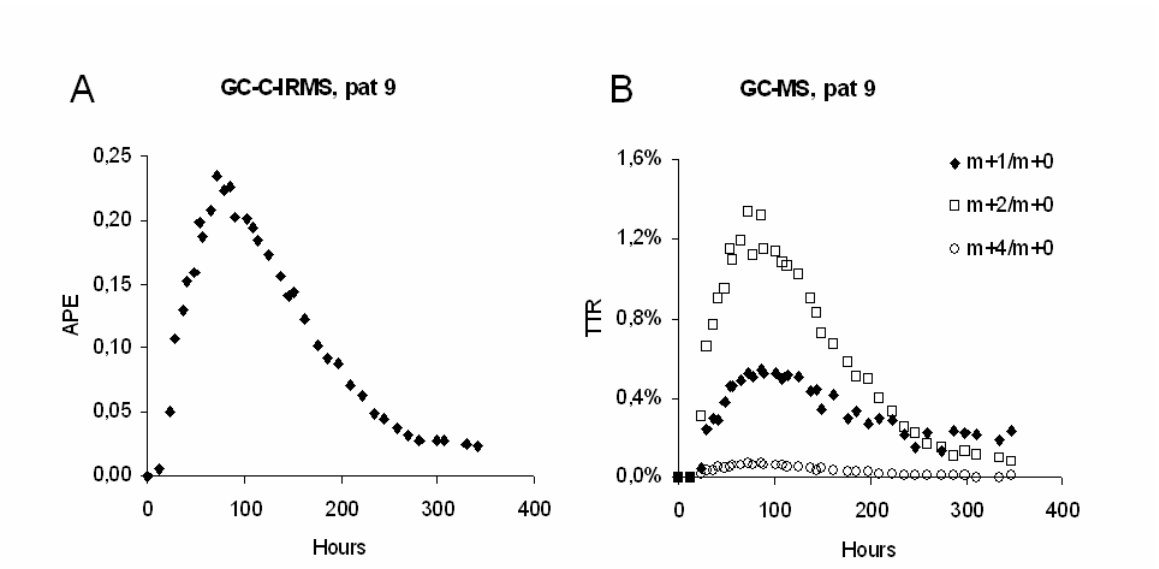

Figure 9. Tracer recycling.

Representative enrichment curves from a preterm infant with RDS after i.v. infusion of [U-$^{13}C_6$] 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).

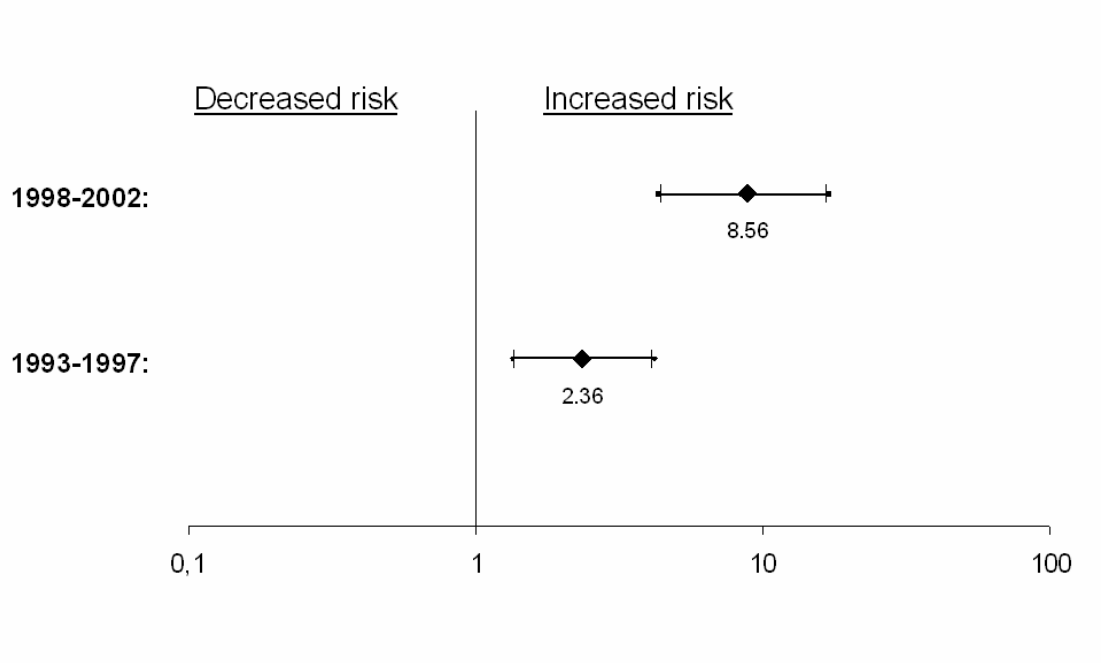

Figure 10. The risk of mechanical ventilation.
The Odds ratio for mechanical ventilation at KS compared to KH before (1993-1997) and after (1998-2002) the introduction of surfactant administration during a brief intubation (INSURE) at KH. After the introduction of INSURE the risk for mechanical ventilation 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 [192-194]. The number of infants developing BPD ranged from 8-17%, which is similar to what has been previously reported from Stockholm [247], 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 [274-276]. In addition, the clinical pattern of BPD is changing and factors such as infections [277], inflammatory reactions [278,279] and surfactant abnormalities [280] 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 [113,114,281]. 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 [181]. 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 [192]. 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

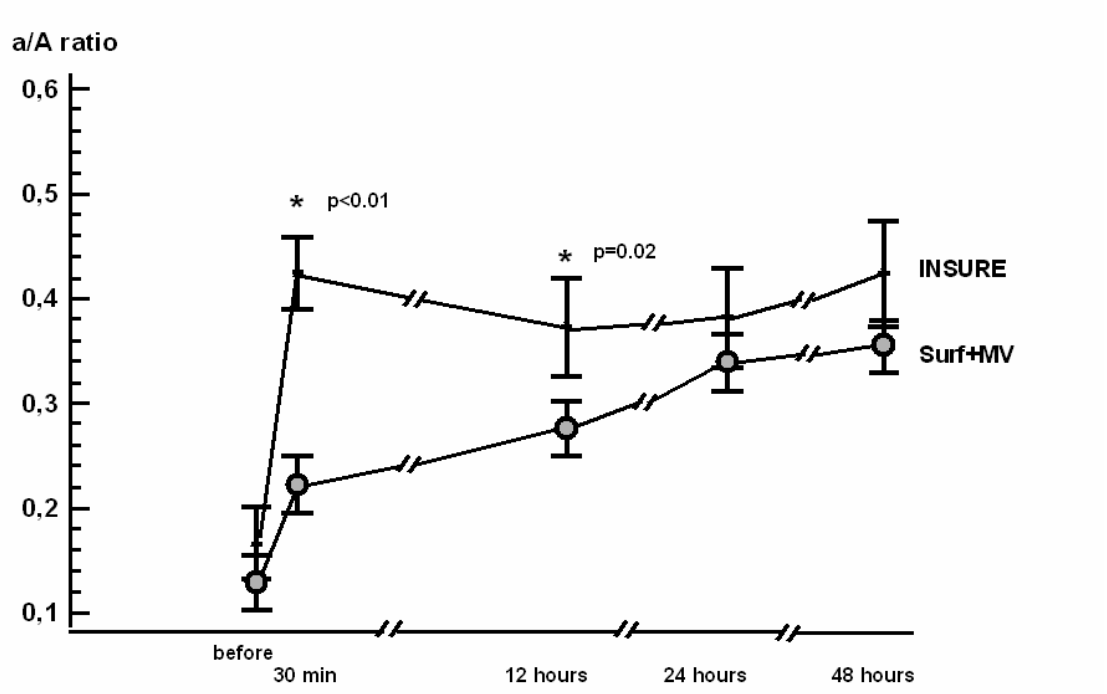

Figure 11. Oxygenation after surfactant treatment.

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.

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).

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 [54,197-200]. 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 [197]. 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 [282].

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) $ml \cdot kg^{-1} \cdot cmH_2O^{-1}$ 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 [115,217]. Activated phagocytes or bacteria produce free oxygen radicals that, at least under in vitro conditions, cause peroxidation of surfactant lipids [283-286]. 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 [287] that was associated with a poorer outcome [288].

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 [120,230]. 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.

SWEDISH SUMMARY - SVENSK SAMMANFATTNING

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 ytspänningsnedsättande 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 genes. 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 alltmer 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 inaktiverar surfactant och dels till att typ II cellerna skadas och surfaktantproduktionen därmed påverkas.

I vilken utsträckning lungsjukdomar och ventilationsstrategier påverkar produktion och omsättning av surfaktant hos nyfödda barn är ofullständigt känt eftersom det inte tidigare funnits någon bra metod att studera detta *in vivo*. Vår kunskap kommer huvudsakligen från djurmodeller där man använt radioaktivt märkta byggstenar (prekursorer) för att studera surfaktantmetabolism. Denna typ av studier går av etiska och medicinska skäl inte att genomföra på nyfödda barn. En nyligen utvecklad metod med stabila isotoper innebär dock att surfaktantomsättning nu också kan studeras hos barn. Tekniken innebär att kolatomer i glukos-, acetat- eller palmitatmolekyler märks med den stabila isotopen kol^{13}, en naturligt förekommande, icke-radioaktiv variant av kol som innehåller en extra neutron. Kol^{13} är därmed något tyngre än den vanligast förekommande kolisotopen, kol^{12}, och kan därför detekteras med hjälp av masspektrometri. Kol^{13}-märkt surfaktantprekursor ges som en intravenös infusion och hur mycket som inkorporerats i surfaktant mäts i luftvägssekret. Från anrikningen av kol^{13} i surfaktant över tid kan mått på surfaktantomsättningen, såsom fraktionell synteshastighet och halveringstid, räknas fram. Eftersom surfaktantomsättning hos nyfödda, och f.f.a. för tidigt födda barn, är långsam krävs upprepade prover av luftvägssekret under flera dagar. Detta kräver att barnet är intuberat och innebär att studier huvudsakligen kan göras på respiratorvårdade barn. Proverna tillvaratas i form av trakealaspirat i samband med rutinmässig rensugning av luftvägstuben.

Surfaktantbehandling av prematurfödda barn med RDS är mycket effektivt. Den minskar dödlighet och komplikationsrisk. Mycket grundläggande forskningsarbete och utveckling av surfaktantbehandling har utförts vid Karolinska Institutet i Stockholm av professorerna Bengt Robertson och Tore Curstedt. Surfaktant ges som en instillation i barnets luftrör och har därför traditionellt administrerats i samband med intubering för respiratorvård. Sedan 1998 praktiseras på neonatalavdelningen vid Karolinska Universitetssjukhuset Huddinge en metod där surfaktantbehandlingen ges under en mycket kortvarig intubation. Tuben

avlägsnas sedan direkt så att barnet kan andas själv hjälp av ett mottryck i näsluften, s.k. CPAP (Continuos 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 förlossningsenhet. Efter införandet av INSURE metoden har vi observerat att barn som INSURE behandlas svarar med förbättrad syresättning och att effekten oftast 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 oftast upprepade doser krävs.

Målsättningen 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 oscillerande ventilation (HFOV) minskar surfaktant produktionen hos preterma barn med RDS, (*3*) systematiskt utvärdera aktuella metoder att studera surfaktantmetabolism med hjälp av stabila 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 behandlingssvar jämfört med surfaktantbehandling som efterföljs av mekanisk ventilation.

Surfaktantmetabolism *in vivo* studerades hos totalt 70 nyfödda barn vid neonatalenheten, St Louis Children's Hospital, USA. Barnen fick en 24-timmars intravenös infusion av stabil isotopmärkt surfaktantprekursor ([1-$^{13}C_1$] acetat, [U-$^{13}C_6$] glukos eller [1,2,3,4-$^{13}C_4$] palmitat). Från en serie trakealaspirat extraherades surfaktant fosfolipid och anrikningen av kol^{13} analyserades med gaskromatografi/masspektrometri. Resultaten visade att fullgångna barn utan lungsjukdom hade signifikant snabbare surfaktantomsättning jämfört med preterma barn med RDS och lungmognadsbetingad surfaktantbrist. Hos fullgångna barn med andningssvikt visade sig barn med svår lungsjukdom ha en störd och långsam surfaktantomsättning, liknande den hos preterma barn med RDS. Dessa barn hade också låga nivåer av surfaktant fosfolipider i trakealaspiraten vilket indikerar en minskad lungpool av surfaktant. De låga surfaktant fosfolipidnivåerna korrelerade med svårighetsgraden av lungsjukdomen. Fullgångna barn med lätt andningssjukdom hade normal surfaktantomsättning. Nitton mycket för tidigt födda barn med RDS randomiserades till antingen mekanisk ventilation i konventionell respirator eller HFOV vid 24 timmars ålder. Efter infusion av [U-$^{13}C_6$] glukos kunde ingen skillnad i surfaktantmetabolismen påvisas. Metodutvärderingen visade att stabil isotopteknik ger reproducerbara värden på surfaktantmetbolism. Hittills använda metoder var jämförbara avseende analysteknik och masspektrometri instrumentering. En ny parameter, fractional catabolic rate (FCR), introducerades. FCR beskriver den total surfaktantproduktionen, oberoende av vilken märkt surfaktantprekursor som används, och föreslås bli det primära måttet för surfaktantomsättning.

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 respiratorbehovet sjunkit 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 (^{14}C-Curosurf®) och randomiserades till spontanandning, mekanisk ventilation eller kontrolldjur. De mekaniskt ventilerade djuren uppvisade 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.

Slutsatser

Vår studie är en av de först hittills att beskriva surfaktantomsättning *in vivo* hos fullgångna, lungfriska, nyfödda barn. Resultaten visar också att svår lungsjukdom hos fullgångna barn resulterar i störd surfaktantmetabolism liknande den hos för tidigt födda barn. Detta talar för antingen en underliggande mognadsförsening eller en påverkad lungfunktion utlöst av lungsjukdomen i sig. Metoden med stabila isotoper för att studera surfaktantmetabolism hos nyfödda möjliggör adekvat jämförelse av olika sjukdomstillstånd och behandlingsstrategier. Det är en kraftfull teknik som kan bidra till att optimera omhändertagandet av denna känsliga grupp av patienter. En ventilationsstrategi med HFOV påverkar inte den kroppsegna surfaktantomsättningen hos mycket för tidigt födda barn med RDS. Vi har däremot visat att ventilationsstratgi har stor betydelse för svaret efter surfaktantbehandling. Experimentellt leder mekanisk ventilation till ett hämmat upptaget av tillfört surfaktant i lungvävnad och ökad surfaktantinaktivering, vilket kan förklara en kortvarig klinisk behandlingseffekt och behov av flera surfaktantdoser. Införandet av INSURE, en ny strategy där surfaktant ges under en kort intubation och efterföljs av spontanandning i CPAP, har visat sig halvera behovet av respiratorvård. Resultaten visar att surfaktantbehandling enligt INSURE är säkert, utan ökad risk för komplikationer. Tillsammans med uppenbara fördelar som minskade transporter, ökad närhet mor-barn och potentiellt reducerade sjukvårdskostnader etablerar studien INSURE som en viktig behandlingsstrategi hos måttligt för tidigt födda barn med RDS.

ACKNOWLEDGEMENTS

These studies were performed at Children's Hospital, Karolinska University Hospital Huddinge; the Laboratory of Surfactant Research, Department of Clinical Chemistry, Stockholm, Sweden; and the Division of Newborn Medicine, Washington University School of Medicine, St Louis Children's Hospital, St Louis, MO, USA.

I would like to thank all the babies and their parents for participating in these studies.
This work would not have been possible without the help and support of many great people. In particular, I wish to express my sincere gratitude to:

Mats Blennow, my supervisor, for enthusiastically inviting me into the world of neonatal research. From the very beginning you have always promoted me and looked out for me, even when I was overseas. Your generous support and trust in my ability have pushed me forward and made me grow independent. As a bonus, your great social skills have created a lot of fun.

Bengt Robertson, my co-supervisor, for not giving up on me when we couldn't get the rabbit pups to survive in our pilot experiments. Working with you is an honour and it has been a privilege for me to benefit from your vast knowledge and amazing experience in surfactant research.

Aaron Hamvas, my supervisor in St Louis, for letting me be part of the exciting development of stable isotope studies of surfactant metabolism. I am truly grateful for all the things I have learned from you, everything from the science of crunching a manuscript to perfection to the basic rules of baseball.

Tore Curstedt, for providing expert advice at all moments and for enthusiastically taking time off your busy schedule to do practical lab work and help me prepare labeled Curosurf®. You have a brilliant ability to pinpoint and solve a problem that has helped me many times when I have been stuck.

Bruce Pattersson, for providing an excellent mass spectrometry facility and for constantly pushing my boundaries in understanding lipid kinetics.

FS Cole, head of the Division of Newborn Medicine in St Louis, for inviting me in as a research fellow and for being such a sharp scientist.

Agne Larsson and *Claude Marcus*, professors of pediatrics, the former and current head of the Department of Pediatrics at the Institution of Clinical Science, for creating a scientific environment.

Ann-Britt Bohlin, my boss at Children's Hospital, Huddinge, for keeping track of my pediatric residency in spite of the many interruptions and for being a joining force at our department.

All the past and present members of the surfactant lab in Stockholm: *Bim Linderholm*, for your invaluable presence in the lab and skilful assistance in the animal experiments, I couldn't have made it without you; *Eva, Margareta, Guido, Andreas* and the people at the neighbouring alcohol lab for creating a great atmosphere.

My fellow research fellows: *Assaad Merchak*, for teaching me the ways around the NICU and the lab, for all your crazy jokes, many good laughs and for making great hummus; *Kim Spence* and *James Zozobrado*, for questioning old habits, improving the project and helping me out a lot when I was pregnant.

Attendings, fellows and *staff* at Division of Newborn Medicine, St Louis Children's Hospital, for always being friendly, helpful and interested in my work; and all the *NICU nurses* for your dedicated care of the babies and assistance with sample collection.

Freida Custodio and *Junyoung Kwon*, for excellent technical assistance in the mass spectrometry lab; *Kristina Bryowski*, for tracer preparation; *Michelle Trusgnich* in the Cole lab, for always providing a little gossip and helping me out with practical issues many times.

Virgilio Carnielli, Luc Zimmermann and *Daphne Janssen*, for fruitful collaborations.

Connie Jarstrand and *Linda Bouhafs*, for lipid peroxidation analyses, especially getting it all together for the additional experiments three years later.

Baldvin Jonsson, for encouragement and Icelandic wisdom; *Miriam Katz-Salamon*, for getting the statistics right; and *Thorgerdur Gudmundsdottir*, for working through all those charts with a smile.

The *staff* at Neo, Huddinge, for taking such good care of the babies - you are crucial for INSURE to work.
The fabulous Neo-boys, old and new; *Ihsan Sarman, Mikael Norman, Boubou Hallberg, Lasse Navér, Martino Corrias* and *Kari Arhimaa.*
All the other great Neo-people: *Sandra Horsch*, for becoming a good friend always willing to discuss the impossible task of getting research, work and family to work smoothly together; *Kristin* and *Marco* for seeking out the fun at meetings.

All my friends and colleagues and at Children's Hospital, Huddinge: *Ewa H*, my dedicated, hard-working room-mate and neo-doctor to be, who always manage to care a little for me too, *Kristina N*, also future neo-doctor, for putting up with my writing stress and sharing the soap-opera of real life, *Åsa G-J*, for trying to get me to work-out (maybe now we can finally get to it), *Märta E*, my first mentor, *Torun T*, for joint efforts in the intricate administration of preparing our dissertations, *Katarina L*, for great company at the "allmänvetenskaplig grundkurs", *Kerstin D*, my clinical mentor, *Björn F*, for the cholestasis project, *Nina P, Maria P, Ola E, Mona-Lisa E, Britt G, Annika J, Mari J, Ulrika B, Isabelle dM, Svante N, Birgit B, Anna N, Antal N, Ulla B* and all the rest – you all mean a lot to me!

The pediatric cardiology team at Huddinge; *Maria L*, for all your excellent advice during the thesis process, about cardiology and life; *Ulf E*, for good teaching and fun; *Birgitta* and *Helene*, for your great job and incredible flexibility to adjust to my schedule and for always knowing exactly when I desperately need a cup of coffee and some common sense.

All the friends that have meant so much for me over the years when working on the thesis: *Gunilla*, for always caring for me and being ready to help with the kids in spite of all the things going on in your own life; *Anna-Carin Z*, for long talks and moving support; the Swedish girls in St Louis, *Maria, Britta B, Anna, Malin, Sofia and Britta S*; *Judit*, *Mohammed* and *Celine* for happy Halloweens and good dinners.

My always-supportive family: *Gunnar*, my incredible brother, for contributing with the book of books, my brother *Anders* and *Ewa, Jesper* and *Viktor;* my bonus family *Monika, Linda, Fredrik* and *Wille*.
My parents-in law *Isidro* and *Dolores*, for staying with us in St Louis when Elisa was a baby so I could go back to work; and *Miguel* and *Ulrika.*

My mother, *Kerstin*, for always, always believing in me, helping me as much as you possibly can, caring for me, encouraging me and making sure I take care of myself.
My father, *Lars,* for your enthusiasm, interest and pride in my work, but also for never letting me forget that it is not what I accomplish that really matters, you love me anyway.

Antonio, for being the true scientist in the family and for scrutinizing the manuscript of the thesis (I treasure your advice even if it doesn't always seem that way), but most of all for loving me through the ups and downs of it all.

Oscar and *Elisa*, my very own babies, growing up so fast - nothing compares to you!

This work was supported by grants from Karolinska Institutet, European Society for Pediatric Research, The Swedish Society of Medicine, the Freemasons in Stockholm Foundation for Children's Welfare, Majblommans Research Fund, Stiftelsen Samariten and Stiftelsen Allmänna BB:s Minnesfond.

REFERENCES

1. Neergaard v. Neue Auffassungen uber einenGrund-begriff der Atemmechanik. *Z Gesamte Exp Med* 1929;66:373-394.

2. Pattle RE. Properties, function and origin of the alveolar lining layer. *Nature* 1955;175:1125-6.

3. Clements JA. Surface tension of lung extracts. *Proc Soc Exp Biol Med* 1957;95:170-2.

4. Clements JA, Brown ES, Johnson RP. Pulmonary surface tension and the mucus lining of the lungs: some theoretical considerations. *J Appl Physiol* 1958;12:262-8.

5. Hochheim K. Uber einige Befunde den Lungen von Neugeborenen und die Bziehung derselben zur Aspiration von Fruchtwasser. *Centralbl Pathol* 1903;14:537-538.

6. Gruenwald P. Surface tension as a factor in resistance of neonatal lung to aeration. *Am j Obstet Gynecol* 1947;53:996-1007.

7. Avery ME, Mead J. Surface properties in relation to atelectasis and hyaline membrane disease. *AMA J Dis Child* 1959;97:517-23.

8. Brown E. Chemical identification of a pulmonary surface active agent. *Fed Proc* 1962;21:438.

9. Brown E. Isolation and assay of dipalmitoyl lecithin in lung extracts. *Am J of Physiol* 1964;207:402-406.

10. Gluck L, Motoyama EK, Smits HL, Kulovich MV. The biochemical development of surface activity in mammalian lung. I. The surface-active phospholipids; the separation and distribution of surface-active lecithin in the lung of the developing rabbit fetus. *Pediatr Res* 1967;1:237-46.

11. Gluck L, Kulovich MV, Borer RC, Jr., Brenner PH, Anderson GG, Spellacy WN. Diagnosis of the respiratory distress syndrome by amniocentesis. *Am J Obstet Gynecol* 1971;109:440-5.

12. Enhorning G, Grossman G, Robertson B. Tracheal deposition of surfactant before the first breath. *Am Rev Respir Dis* 1973;107:921-7.

13. Enhorning G, Grossmann G, Robertson B. Pharyngeal deposition of surfactant in the premature rabbit fetus. *Biol Neonate* 1973;22:126-32.

14. Enhorning G, Robertson B. Lung expansion in the premature rabbit fetus after tracheal deposition of surfactant. *Pediatrics* 1972;50:58-66.

15. Enhorning G, Robertson B, Milne E, Wagner R. Radiologic evaluation of the premature newborn rabbit after pharyngeal deposition of surfactant. *Am J Obstet Gynecol* 1975;121:475-80.

16. Robertson B, Enhorning G. The alveolar lining of the premature newborn rabbit after pharyngeal deposition of surfactant. *Lab Invest* 1974;31:54-9.

17. Fujiwara T, Maeta H, Chida S, Morita T, Watabe Y, Abe T. Artificial surfactant therapy in hyaline-membrane disease. *Lancet* 1980;1:55-9.

18. King RJ, Klass DJ, Gikas EG, Clements JA. Isolation of apoproteins from canine surface active material. *Am J Physiol* 1973;224:788-95.

19. Liggins GC, Howie RN. A controlled trial of antepartum glucocorticoid treatment for prevention of the respiratory distress syndrome in premature infants. *Pediatrics* 1972;50:515-25.

20. Surfactant replacement therapy for severe neonatal respiratory distress syndrome: an international randomized clinical trial. Collaborative European Multicenter Study Group. *Pediatrics* 1988;82:683-91.

21. Merritt TA, Hallman M, Bloom BT, Berry C, Benirschke K, Sahn D, Key T, Edwards D, Jarvenpaa AL, Pohjavuori M, et al. Prophylactic treatment of very premature infants with human surfactant. *N Engl J Med* 1986;315:785-90.

22. Soll RF, Hoekstra RE, Fangman JJ, Corbet AJ, Adams JM, James LS, Schulze K, Oh W, Roberts JD, Jr., Dorst JP, et al. Multicenter trial of single-dose modified bovine surfactant extract (Survanta) for prevention of respiratory distress syndrome. Ross Collaborative Surfactant Prevention Study Group. *Pediatrics* 1990;85:1092-102.

23. Hallman M, Merritt TA, Jarvenpaa AL, Boynton B, Mannino F, Gluck L, Moore T, Edwards D. Exogenous human surfactant for treatment of severe respiratory distress syndrome: a randomized prospective clinical trial. *J Pediatr* 1985;106:963-9.

24. Fujiwara T, Konishi M, Chida S, Okuyama K, Ogawa Y, Takeuchi Y, Nishida H, Kito H, Fujimura M, Nakamura H, et al. Surfactant replacement therapy with a single postventilatory dose of a reconstituted bovine surfactant in preterm neonates with respiratory distress syndrome: final analysis of a multicenter, double-blind, randomized trial and comparison with similar trials. The Surfactant-TA Study Group. *Pediatrics* 1990;86:753-64.

25. Creuwels LA, van Golde LM, Haagsman HP. The pulmonary surfactant system: biochemical and clinical aspects. *Lung* 1997;175:1-39.

26. Hallman M, Gluck L. Phosphatidylglycerol in lung surfactant. II. Subcellular distribution and mechanism of biosynthesis in vitro. *Biochim Biophys Acta* 1975;409:172-91.

27. Jobe A. Lung development and maturation. In: Fanaroff A, Martin R, eds. Neonatal-Perinatal Medicine: diseases of the fetus and infant. St Louis: Mosby, Inc, 2002:973-991.

28. Crouch E, Wright JR. Surfactant proteins a and d and pulmonary host defense. *Annu Rev Physiol* 2001;63:521-54.

29. Hawgood S, Poulain FR. The pulmonary collectins and surfactant metabolism. *Annu Rev Physiol* 2001;63:495-519.

30. Whitsett JA, Nogee LM, Weaver TE, Horowitz AD. Human surfactant protein B: structure, function, regulation, and genetic disease. *Physiol Rev* 1995;75:749-57.

31. Hallman M, Haataja R. Genetic influences and neonatal lung disease. *Semin Neonatol* 2003;8:19-27.

32. Jobe AH, Ikegami M. Biology of surfactant. *Clin Perinatol* 2001;28:655-69, vii-viii.

33. Pérez-Gil J. Molecular interactions in pulmonary surfactant films. *Biol Neonate* 2002;81(Suppl 1):6-15.

34. Patterson CE, Rhoades RA. Substrate utilization in the perinatal lung. *Am J Physiol* 1989;257:L318-30.

35. Martini WZ, Chinkes DL, Barrow RE, Murphey ED, Wolfe RR. Lung surfactant kinetics in conscious pigs. *Am J Physiol* 1999;277:E187-95.

36. Batenburg JJ. Surfactant phospholipids: synthesis and storage. *Am J Physiol* 1992;262:L367-85.

37. Yeh YY. Substrate utilization for phosphatidylcholine synthesis by type II pneumocytes of neonatal rats. *Pediatr Res* 1991;30:55-61.

38. Sheehan PM, Yeh YY. Pulmonary surfactant lipid synthesis from ketone bodies, lactate and glucose in newborn rats. *Lipids* 1985;20:835-41.

39. Rooney SA. Fatty acid biosynthesis in developing fetal lung. *Am J Physiol* 1989;257:L195-201.

40. Hallman M, Miyai K, Wagner RM. Isolated lamellar bodies from rat lung: correlated ultrastructural and biochemical studies. *Lab Invest* 1976;35:79-86.

41. Stratton CJ. The high resolution ultrastructure of the periodicity and architecture of lipid-retained and extracted lung multilamellar body laminations. *Tissue Cell* 1976;8:713-28.

42. Stratton CJ. The three-dimensional aspect of mammalian lung multilamellar bodies. *Tissue Cell* 1976;8:693-712.

43. Chander A, Fisher AB. Regulation of lung surfactant secretion. *Am J Physiol* 1990;258:L241-53.

44. Wright JR. Clearance and recycling of pulmonary surfactant. *Am J Physiol* 1990;259:L1-12.

45. Wirtz HR, Dobbs LG. Calcium mobilization and exocytosis after one mechanical stretch of lung epithelial cells. *Science* 1990;250:1266-9.

46. Rooney S, Gobran L, Greise M. Signal transduction mechanisms mediating surfactant phospholipid secretion in isolated type II cells. *Prog Resp Res* 1994;27:84-91.

47. Faridy EE. Effect of distension on release of surfactant in excised dogs' lungs. *Respir Physiol* 1976;27:99-114.

48. Lawson EE, Birdwell RL, Huang PS, Taeusch HW, Jr. Augmentation of pulmonary surfactant secretion by lung expansion at birth. *Pediatr Res* 1979;13:611-4.

49. Oyarzun MJ, Clements JA. Ventilatory and cholinergic control of pulmonary surfactant in the rabbit. *J Appl Physiol* 1977;43:39-45.

50. Wright J, JA C. Metabolism and turnover of lung surfactant. *Am Rev Respir Dis* 1987;136:426-444.

51. Veldhuizen RA, Ito Y, Marcou J, Yao LJ, McCaig L, Lewis JF. Effects of lung injury on pulmonary surfactant aggregate conversion in vivo and in vitro. *Am J Physiol* 1997;272:L872-8.

52. Ito Y, Veldhuizen RA, Yao LJ, McCaig LA, Bartlett AJ, Lewis JF. Ventilation strategies affect surfactant aggregate conversion in acute lung injury. *Am J Respir Crit Care Med* 1997;155:493-9.

53. Pettenazzo A, Jobe A, Humme J, Seidner S, Ikegami M. Clearance of surfactant phosphatidylcholine via the upper airways in rabbits. *J Appl Physiol* 1988;65:2151-5.

54. Jobe AH, Ikegami M. Surfactant metabolism. *Clin Perinatol* 1993;20:683-96.

55. Jacobs H, Jobe A, Ikegami M, Conaway D. The significance of reutilization of surfactant phosphatidylcholine. *J Biol Chem* 1983;258:4159-65.

56. Jacobs H, Jobe A, Ikegami M, Jones S. Surfactant phosphatidylcholine source, fluxes, and turnover times in 3-day-old, 10-day-old, and adult rabbits. *J Biol Chem* 1982;257:1805-10.

57. Adams FH, Fujiwara T, Emmanouilides GC, Raiha N. Lung phospholipids of human fetuses and infants with and without hyaline membrane disease. *J Pediatr* 1970;77:833-41.

58. Hallman M, Merritt TA, Pohjavuori M, Gluck L. Effect of surfactant substitution on lung effluent phospholipids in respiratory distress syndrome: evaluation of surfactant phospholipid turnover, pool size, and the relationship to severity of respiratory failure. *Pediatr Res* 1986;20:1228-35.

59. Torresin M, Zimmermann LJ, Cogo PE, Cavicchioli P, Badon T, Giordano G, Zacchello F, Sauer PJ, Carnielli VP. Exogenous surfactant kinetics in infant respiratory distress syndrome: A novel method with stable isotopes. *Am J Respir Crit Care Med* 2000;161:1584-9.

60. Janssen DJ, Carnielli VP, Cogo PE, Seidner SR, Luijendijk IH, Wattimena JL, Jobe AH, Zimmermann LJ. Surfactant phosphatidylcholine half-life and pool size measurements in premature baboons developing bronchopulmonary dysplasia. *Pediatr Res* 2002;52:724-9.

61. Jackson J, Palmer S, Standaert T. Developmental changes of surface active material in newborn non-human primates. *Am Rev Respir Dis* 1984;129:A204.

62. Jobe AH, Ikegami M, Jacobs HC, Jones SJ. Surfactant pool sizes and severity of respiratory distress syndrome in prematurely delivered lambs. *Am Rev Respir Dis* 1983;127:751-5.

63. Ikegami M, Jobe A, Yamada T, Priestly A, Ruffini L, Rider E, Seidner S. Surfactant metabolism in surfactant-treated preterm ventilated lambs. *J Appl Physiol* 1989;67:429-37.

64. Ikegami M, Jobe AH, Yamada T, Seidner S. Relationship between alveolar saturated phosphatidylcholine pool sizes and compliance of preterm rabbit lungs. The effect of maternal corticosteroid treatment. *Am Rev Respir Dis* 1989;139:367-9.

65. Rebello CM, Jobe AH, Eisele JW, Ikegami M. Alveolar and tissue surfactant pool sizes in humans. *Am J Respir Crit Care Med* 1996;154:625-8.

66. Jackson JC, Palmer S, Truog WE, Standaert TA, Murphy JH, Hodson WA. Surfactant quantity and composition during recovery from hyaline membrane disease. *Pediatr Res* 1986;20:1243-7.

67. Hallman M, Merritt TA, Akino T, Bry K. Surfactant protein A, phosphatidylcholine, and surfactant inhibitors in epithelial lining fluid. Correlation with surface activity, severity of respiratory distress syndrome, and outcome in small premature infants. *Am Rev Respir Dis* 1991;144:1376-84.

68. Cogo PE, Zimmermann LJ, Rosso F, Tormena F, Gamba P, Verlato G, Baritussio A, Carnielli VP. Surfactant synthesis and kinetics in infants with congenital diaphragmatic hernia. *Am J Respir Crit Care Med* 2002;166:154-8.

69. Gluck L, Kulowitch M, Eidelman A, Cordero L, Khazin A. Biochemical development of of surface activity in mammalian lung: IV. Pulmonary lecithinsynthesis in the human fetus and the newborn and the ethiology of respiratory distress syndrome. *Pediatr Res* 1972;6:81-99.

70. Chida S, Phelps D, Cordle C, Soll R, Floros J. Surfctant -associated proteins in thracheal aspirats of infants with respiratory distress syndrome after surfactant therapy. *Am Rev of Respir Dis* 1988;137:943-947.

71. Hallman M, Merritt T, Bry K. The fate of exogenous surfactant in neonates with respiratory distress syndrome. *Clin Pharmacokinet* 1994;26:215-232.

72. Seidner SR, Jobe AH, Coalson JJ, Ikegami M. Abnormal surfactant metabolism and function in preterm ventilated baboons. *Am J Respir Crit Care Med* 1998;158:1982-9.

73. Martini WZ, Irtun O, Chinkes DL, Barrow RE, Wolfe RR. Surfactant phosphatidylcholine in thermally injured pigs. *Crit Care Med* 2001;29:1417-22.

74. Cogo PE, Zimmermann LJ, Pesavento R, Sacchetto E, Burighel A, Rosso F, Badon T, Verlato G, Carnielli VP. Surfactant kinetics in preterm infants on mechanical ventilation who did and did not develop bronchopulmonary dysplasia. *Crit Care Med* 2003;31:1532-8.

75. Cogo PE, Zimmermann LJ, Verlato G, Midrio P, Gucciardi A, Ori C, Carnielli VP. A dual stable isotope tracer method for the measurement of surfactant disaturated-phosphatidylcholine net synthesis in infants with congenital diaphragmatic hernia. *Pediatr Res* 2004;56:184-90.

76. Chida N, Adams FH. Incorporation of palmitate, glucose and choline into lecithin by fetal and newborn lamb lung. *Pediatr Res* 1967;1:364-71.

77. Cockshutt A, Possmayer F. Metabolism of surfactant lipids and proteins in the developing lung. In: Robertson B, Van Golde L, Batenburg J, eds. Pulmonary Surfactant: From molecular Biology to Clinical Practice. Amsterdam: Elsevier Science Publishers, 1992:339-377.

78. Ikegami M, Jobe A, Nathanielsz PW. The labeling of pulmonary surfactant phosphatidylcholine in newborn and adult sheep. *Exp Lung Res* 1981;2:197-206.

79. Jobe A. The labeling and biological half-life of phosphatidylcholine in subcellular fractions of rabbit lung. *Biochim Biophys Acta* 1977;489:440-53.

80. Jobe A, Gluck L. The labeling of lung phosphatidylcholine in premature rabbits. *Pediatr Res* 1979;13:635-40.

81. Jobe A, Ikegami M, Sarton-Miller I, Barajas L. Surfactant metabolism of newborn lamb lungs studied in vivo. *J Appl Physiol* 1980;49:1091-8.

82. Jobe AH, Ikegami M, Seidner SR, Pettenazzo A, Ruffini L. Surfactant phosphatidylcholine metabolism and surfactant function in preterm, ventilated lambs. *Am Rev Respir Dis* 1989;139:352-9.

83. Bunt JE, Zimmermann LJ, Wattimena JL, van Beek RH, Sauer PJ, Carnielli VP. Endogenous surfactant turnover in preterm infants measured with stable isotopes. *Am J Respir Crit Care Med* 1998;157:810-4.

84. Cogo PE, Carnielli VP, Bunt JE, Badon T, Giordano G, Zacchello F, Sauer PJ, Zimmermann LJ. Endogenous surfactant metabolism in critically ill infants measured with stable isotope labeled fatty acids. *Pediatr Res* 1999;45:242-6.

85. Bunt JE, Carnielli VP, Janssen DJ, Wattimena JL, Hop WC, Sauer PJ, Zimmermann LJ. Treatment with exogenous surfactant stimulates endogenous surfactant synthesis in premature infants with respiratory distress syndrome. *Crit Care Med* 2000;28:3383-8.

86. Bunt JE, Carnielli VP, Darcos Wattimena JL, Hop WC, Sauer PJ, Zimmermann LJ. The effect in premature infants of prenatal corticosteroids on endogenous surfactant synthesis as measured with stable isotopes. *Am J Respir Crit Care Med* 2000;162:844-9.

87. Cogo PE, Zimmermann LJ, Meneghini L, Mainini N, Bordignon L, Suma V, Buffo M, Carnielli VP. Pulmonary surfactant disaturated-phosphatidylcholine (DSPC) turnover and pool size in newborn infants with congenital diaphragmatic hernia (CDH). *Pediatr Res* 2003;54:653-8.

88. Janssen DJ, Tibboel D, Carnielli VP, van Emmen E, Luijendijk IH, Darcos Wattimena JL, Zimmermann LJ. Surfactant phosphatidylcholine pool size in human neonates with congenital diaphragmatic hernia requiring ECMO. *J Pediatr* 2003;142:247-52.

89. Merchak A, Patterson BW, Yarasheski KE, Hamvas A. Use of stable isotope labeling technique and mass isotopomer distribution analysis of. *J Mass Spectrom* 2000;35:734-8.

90. Cavicchioli P, Zimmermann LJ, Cogo PE, Badon T, Giordano G, Torresin M, Zacchello F, Carnielli VP. Endogenous surfactant turnover in preterm infants with respiratory distress syndrome studied with stable isotope lipids. *Am J Respir Crit Care Med* 2001;163:55-60.

91. Martini WZ, Irtun O, Chinkes DL, Barrow RE, Wolfe RR. Glucose effects on lung surfactant kinetics in conscious pigs. *Am J Physiol Endocrinol Metab* 2000;279:E920-6.

92. Bunt JE, Carnielli VP, Seidner SR, Ikegami M, Darcos Wattimena JL, Sauer PJ, Jobe AH, Zimmermann LJ. Metabolism of endogenous surfactant in premature baboons and effect of prenatal corticosteroids. *Am J Respir Crit Care Med* 1999;160:1481-5.

93. Hjalmarson O. Epidemiology and classification of acute, neonatal respiratory disorders. A prospective study. *Acta Paediatr Scand* 1981;70:773-83.

94. Bonafe L, Rubaltelli FF. The incidence of acute neonatal respiratory disorders in Padova county: an epidemiological survey. *Acta Paediatr* 1996;85:1236-40.

95. Field DJ, Milner AD, Hopkin IE, Madeley RJ. Changing patterns in neonatal respiratory diseases. *Pediatr Pulmonol* 1987;3:231-5.

96. Rubaltelli FF, Dani C, Reali MF, Bertini G, Wiechmann L, Tangucci M, Spagnolo A. Acute neonatal respiratory distress in Italy: a one-year prospective study. Italian Group of Neonatal Pneumology. *Acta Paediatr* 1998;87:1261-8.

97. Chard T, Soe A, Costeloe K. The risk of neonatal death and respiratory distress syndrome in relation to birth weight of preterm infants. *Am J Pernatol* 1997;14:523-526.

98. Hack M, Fanaroff AA. Outcomes of extremely-low-birth-weight infants between 1982 and 1988. *N Engl J Med* 1989;321:1642-7.

99. Hack M, Horbar JD, Malloy MH, Tyson JE, Wright E, Wright L. Very low birth weight outcomes of the National Institute of Child Health and Human Development Neonatal Network. *Pediatrics* 1991;87:587-97.

100. Crowley P, Chalmers I, Keirse MJ. The effects of corticosteroid administration before preterm delivery: an overview of the evidence from controlled trials. *Br J Obstet Gynaecol* 1990;97:11-25.

101. Kari MA, Hallman M, Eronen M, Teramo K, Virtanen M, Koivisto M, Ikonen RS. Prenatal dexamethasone treatment in conjunction with rescue therapy of human surfactant: a randomized placebo-controlled multicenter study. *Pediatrics* 1994;93:730-6.

102. Koivisto M, Marttila R, Kurkinen-Raty M, Saarela T, Pokela ML, Jouppila P, Hallman M. Changing incidence and outcome of infants with respiratory distress syndrome in the 1990s: a population-based survey. *Acta Paediatr* 2004;93:177-84.

103. Soll R. Clinical Trials of Surfactant Therapy in the Newborn. In: Robertson B, Taeusch H, eds. Surfactant therapy for lung disease. New York: Marcel Dekker, Inc, 1995:407-441.

104. Lee K, Khoshnood B, Wall SN, Chang Y, Hsieh HL, Singh JK. Trend in mortality from respiratory distress syndrome in the United States, 1970-1995. *J Pediatr* 1999;134:434-40.

105. Jobe AJ. The new BPD: an arrest of lung development. *Pediatr Res* 1999;46:641-3.

106. Coalson JJ, Winter VT, Siler-Khodr T, Yoder BA. Neonatal chronic lung disease in extremely immature baboons. *Am J Respir Crit Care Med* 1999;160:1333-46.

107. O'Brodovich H. Immature epithelial Na^+ channel expression is one of the pathogenic mechanisms leading to human neonatal respiratory distress syndrome. *Proc Assoc Am Phys* 1996;108:345-355.

108. Robertson PA, Sniderman SH, Laros RK, Jr., Cowan R, Heilbron D, Goldenberg RL, Iams JD, Creasy RK. Neonatal morbidity according to gestational age and birth weight from five tertiary care centers in the United States, 1983 through 1986. *Am J Obstet Gynecol* 1992;166:1629-41; discussion 1641-5.

109. Robert MF, Neff RK, Hubbell JP, Taeusch HW, Avery ME. Association between maternal diabetes and the respiratory-distress syndrome in the newborn. *N Engl J Med* 1976;294:357-60.

110. Nilsson R, Robertson B. Bronchiolar epithelial lesions in spontaneously breathing premature newborn rabbits. *Biol Neonate* 1985;48:357-61.

111. Sun B, Kobayashi T, Cursted T, Grossmann G, Robertson B. Application of a new ventilator-multi-plethysmograph system for testing efficacy of surfactant replacement in newborn rabbits. *Eur Respir J* 1991;4:364-70.

112. Robertson B, Berry D, Curstedt T, Grossmann G, Ikegami M, Jacobs H, Jobe A, Jones S. Leakage of protein in the immature rabbit lung; effect of surfactant replacement. *Respir Physiol* 1985;61:265-76.

113. Kobayashi T, Nitta K, Ganzuka M, Inui S, Grossmann G, Robertson B. Inactivation of exogenous surfactant by pulmonary edema fluid. *Pediatr Res* 1991;29:353-6.

114. Seeger W, Stohr G, Wolf HR, Neuhof H. Alteration of surfactant function due to protein leakage: special interaction with fibrin monomer. *J Appl Physiol* 1985;58:326-38.

115. Dreyfuss D, Saumon G. Ventilator-induced lung injury: lessons from experimental studies. *Am J Respir Crit Care Med* 1998;157:294-323.

116. Rudolph AJ, Desmond MM, Pineda RG. Clinical diagnosis of respiratory difficulty in the newborn. *Pediatr Clin North Am* 1966;13:669-92.

117. Donald I, Steiner RE. Radiography in the diagnosis of hyaline membrane. *Lancet* 1953;265:846-9.

118. Rodriguez R, Martin R, Fanaroff A. Respiratory Distress Syndrome and its management. In: Fanaroff A, Martin R, eds. Neonatal-perinatal medicine: diseases of the fetus and infant. St Louis: Mosby, Inc, 2002:1001-1011.

119. Kamper J. Early nasal continuous positive airway pressure and minimal handling in the treatment of very-low-birth-weight infants. *Biol Neonate* 1999;76 Suppl 1:22-8.

120. Michna J, Jobe AH, Ikegami M. Positive end-expiratory pressure preserves surfactant function in preterm lambs. *Am J Respir Crit Care Med* 1999;160:634-9.

121. Matthews TG, Warshaw JB. Relevance of the gestational age distribution of meconium passage in utero. *Pediatrics* 1979;64:30-1.

122. Sun B, Curstedt T, Robertson B. Surfactant inhibition in experimental meconium aspiration. *Acta Paediatr* 1993;82:182-189.

123. Moses D, Holm BA, Spitale P, Liu MY, Enhorning G. Inhibition of pulmonary surfactant function by meconium. *Am J Obstet Gynecol* 1991;164:477-81.

124. Cleary GM, Antunes MJ, Ciesielka DA, Higgins ST, Spitzer AR, Chander A. Exudative lung injury is associated with decreased levels of surfactant proteins in a rat model of meconium aspiration. *Pediatrics* 1997;100:998-1003.

125. Dargaville PA, South M, McDougall PN. Surfactant and surfactant inhibitors in meconium aspiration syndrome. *J Pediatr* 2001;138:113-5.

126. Bui KC, Walther FJ, David-Cu R, Garg M, Warburton D. Phospholipid and surfactant protein A concentrations in tracheal aspirates from infants requiring extracorporeal membrane oxygenation. *J Pediatr* 1992;121:271-4.

127. Higgins ST, Wu AM, Sen N, Spitzer AR, Chander A. Meconium increases surfactant secretion in isolated rat alveolar type II cells. *Pediatr Res* 1996;39:443-7.

128. Carroll JL, Jr., McCoy DM, McGowan SE, Salome RG, Ryan AJ, Mallampalli RK. Pulmonary-specific expression of tumor necrosis factor-alpha alters surfactant lipid metabolism. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L735-42.

129. Janssen D. Surfactant phosphatidylcholine metabolism in severe neonatal lung disease Department of Pediatrics. Thesis: Erasmus University Rotterdam, 2003.

130. Sun B, Herting E, Curstedt T, Robertson B. Exogenous surfactant improves lung compliance and oxygenation in adult rats with meconium aspiration. *J Appl Physiol* 1994;77:1961-71.

131. Herting E, Rauprich P, Stichtenoth G, Walter G, Johansson J, Robertson B. Resistance of different surfactant preparations to inactivation by meconium. *Pediatr Res* 2001;50:44-9.

132. Wiswell TE, Knight GR, Finer NN, Donn SM, Desai H, Walsh WF, Sekar KC, Bernstein G, Keszler M, Visser VE, Merritt TA, Mannino FL, Mastrioianni L, Marcy B, Revak SD, Tsai H, Cochrane CG. A multicenter, randomized, controlled trial comparing Surfaxin (Lucinactant) lavage with standard care for treatment of meconium aspiration syndrome. *Pediatrics* 2002;109:1081-7.

133. Nakamura Y, Yamamoto I, Fukuda S, Hashimoto T. Pulmonary acinar development in diaphragmatic hernia. *Arch Pathol Lab Med* 1991;115:372-6.

134. Thebaud B, Mercier JC, Dinh-Xuan AT. Congenital diaphragmatic hernia. A cause of persistent pulmonary hypertension of the newborn which lacks an effective therapy. *Biol Neonate* 1998;74:323-36.

135. Colby CE, Lally KP, Hintz SR, Lally PA, Tibboel D, Moya FR, VanMeurs KP. Surfactant replacement therapy on ECMO does not improve outcome in neonates with congenital diaphragmatic hernia. *J Pediatr Surg* 2004;39:1632-7.

136. Bouhafs RK, Jarstrand C, Robertson B. Lipid peroxidation of lung surfactant in experimental neonatal group B streptococcal pneumonia. *Lung* 2004;182:61-72.

137. Rudiger M, Friedrich W, Rustow B, Schmalisch G, Wauer R. Disturbed surface properties in preterm infants with pneumonia. *Biol Neonate* 2001;79:73-8.

138. Song GW, Robertson B, Curstedt T, Gan XZ, Huang WX. Surfactant treatment in experimental Escherichia coli pneumonia. *Acta Anaesthesiol Scand* 1996;40:1154-60.

139. Herting E, Sun B, Jarstrand C, Curstedt T, Robertson B. Surfactant improves lung function and mitigates bacterial growth in immature ventilated rabbits with experimentally induced neonatal group B streptococcal pneumonia. *Arch Dis Child Fetal Neonatal Ed* 1997;76:F3-8.

140. Herting E, Strayer DS, Jarstrand C, Sun B, Robertson B. Lung function and bacterial proliferation in experimental neonatal pneumonia in ventilated rabbits exposed to monoclonal antibody to surfactant protein A. *Lung* 1998;176:123-31.

141. Sherman MP, D'Ambola JB, Aeberhard EE, Barrett CT. Surfactant therapy of newborn rabbits impairs lung macrophage bactericidal activity. *J Appl Physiol* 1988;65:137-45.

142. Herting E, Gefeller O, Land M, van Sonderen L, Harms K, Robertson B. Surfactant treatment of neonates with respiratory failure and group B streptococcal infection. Members of the Collaborative European Multicenter Study Group. *Pediatrics* 2000;106:957-64; discussion 1135.

143. Fehrenbach H, Brasch F, Uhlig S, Weisser M, Stamme C, Wendel A, Richter J. Early alterations in intracellular and alveolar surfactant of the rat lung in response to endotoxin. *Am J Respir Crit Care Med* 1998;157:1630-9.

144. Nogee LM, de Mello DE, Dehner LP, Colten HR. Brief report: deficiency of pulmonary surfactant protein B in congenital alveolar proteinosis. *N Engl J Med* 1993;328:406-10.

145. Hamvas A, Nogee LM, Mallory GB, Jr., Spray TL, Huddleston CB, August A, Dehner LP, deMello DE, Moxley M, Nelson R, Cole FS, Colten HR. Lung transplantation for treatment of infants with surfactant protein B deficiency. *J Pediatr* 1997;130:231-9.

146. Vorbroker DK, Profitt SA, Nogee LM, Whitsett JA. Aberrant processing of surfactant protein C in hereditary SP-B deficiency. *Am J Physiol* 1995;268:L647-56.

147. deMello DE, Heyman S, Phelps DS, Hamvas A, Nogee L, Cole S, Colten HR. Ultrastructure of lung in surfactant protein B deficiency. *Am J Respir Cell Mol Biol* 1994;11:230-9.

148. Nogee LM, Garnier G, Dietz HC, Singer L, Murphy AM, deMello DE, Colten HR. A mutation in the surfactant protein B gene responsible for fatal neonatal respiratory disease in multiple kindreds. *J Clin Invest* 1994;93:1860-3.

149. Ballard PL, Nogee LM, Beers MF, Ballard RA, Planer BC, Polk L, deMello DE, Moxley MA, Longmore WJ. Partial deficiency of surfactant protein B in an infant with chronic lung disease. *Pediatrics* 1995;96:1046-52.

150. Dunbar AE, 3rd, Wert SE, Ikegami M, Whitsett JA, Hamvas A, White FV, Piedboeuf B, Jobin C, Guttentag S, Nogee LM. Prolonged survival in hereditary surfactant protein B (SP-B) deficiency associated with a novel splicing mutation. *Pediatr Res* 2000;48:275-82.

151. Cole FS, Hamvas A, Rubinstein P, King E, Trusgnich M, Nogee LM, deMello DE, Colten HR. Population-based estimates of surfactant protein B deficiency. *Pediatrics* 2000;105:538-541.

152. Tokieda K, Iwamoto HS, Bachurski C, Wert SE, Hull WM, Ikeda K, Whitsett JA. Surfactant protein-B-deficient mice are susceptible to hyperoxic lung injury. *Am J Respir Cell Mol Biol* 1999;21:463-72.

153. Clark JC, Weaver TE, Iwamoto HS, Ikegami M, Jobe AH, Hull WM, Whitsett JA. Decreased lung compliance and air trapping in heterozygous SP-B-deficient mice. *Am J Respir Cell Mol Biol* 1997;16:46-52.

154. Clark JC, Wert SE, Bachurski CJ, Stahlman MT, Stripp BR, Weaver TE, Whitsett JA. Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. *Proc Natl Acad Sci U S A* 1995;92:7794-8.

155. Tokieda K, Whitsett JA, Clark JC, Weaver TE, Ikeda K, McConnell KB, Jobe AH, Ikegami M, Iwamoto HS. Pulmonary dysfunction in neonatal SP-B-deficient mice. *Am J Physiol* 1997;273:L875-82.

156. Beers MF, Hamvas A, Moxley MA, Gonzales LW, Guttentag SH, Solarin KO, Longmore WJ, Nogee LM, Ballard PL. Pulmonary surfactant metabolism in infants lacking surfactant protein B. *Am J Respir Cell Mol Biol* 2000;22:380-91.

157. Nogee LM, Dunbar AE, 3rd, Wert SE, Askin F, Hamvas A, Whitsett JA. A mutation in the surfactant protein C gene associated with familial interstitial lung disease. *N Engl J Med* 2001;344:573-9.

158. Nogee LM. Genetic mechanisms of surfactant deficiency. *Biol Neonate* 2004;85:314-8.

159. Thomas AQ, Lane K, Phillips J, 3rd, Prince M, Markin C, Speer M, Schwartz DA, Gaddipati R, Marney A, Johnson J, Roberts R, Haines J, Stahlman M, Loyd JE. Heterozygosity for a surfactant protein C gene mutation associated with usual interstitial pneumonitis and cellular nonspecific interstitial pneumonitis in one kindred. *Am J Respir Crit Care Med* 2002;165:1322-8.

160. Hamvas A, Nogee LM, White FV, Schuler P, Hackett BP, Huddleston CB, Mendeloff EN, Hsu FF, Wert SE, Gonzales LW, Beers MF, Ballard PL. Progressive lung disease and surfactant dysfunction with a deletion in surfactant protein C gene. *Am J Respir Cell Mol Biol* 2004;30:771-6.

161. Amin RS, Wert SE, Baughman RP, Tomashefski JF, Jr., Nogee LM, Brody AS, Hull WM, Whitsett JA. Surfactant protein deficiency in familial interstitial lung disease. *J Pediatr* 2001;139:85-92.

162. Shulenin S, Nogee LM, Annilo T, Wert SE, Whitsett JA, Dean M. ABCA3 gene mutations in newborns with fatal surfactant deficiency. *N Engl J Med* 2004;350:1296-303.

163. Robertson B. Experimental Models for Evaluation of Exogenous Surfactants. In: Robertson B, Taeusch H, eds. Surfactant therapy for lung disease. New York: Marcel Dekker, Inc, 1995:239-267.

164. Anceschi MM, Robertson B, Broccucci L, Barbati A, Grossmann G, Hedenborg L, Lundberg E, Petrelli A, Zaccardo G, Cosmi EV. Immunochemical and immunohistochemical evaluation of lung permeability in ventilated newborn rabbits. *Exp Lung Res* 1990;16:593-605.

165. Jobe A, Ikegami M. The prematurely delivered lamb as a model for studies of neonatal adaption. In: Nathanielsz P, ed. Animal models in fetal medicine. Ithaca, NY: Perinatology Press, 1984:1-30.

166. Berggren P, Lachmann B, Curstedt T, Grossmann G, Robertson B. Gas exchange and lung morphology after surfactant replacement in experimental adult respiratory distress syndrome induced by repeated lung lavage. *Acta Anaesthesiol Scand* 1986;30:321-8.

167. Liechty EA, Donovan E, Purohit D, Gilhooly J, Feldman B, Noguchi A, Denson SE, Sehgal SS, Gross I, Stevens D, et al. Reduction of neonatal mortality after multiple doses of bovine surfactant in low birth weight neonates with respiratory distress syndrome. *Pediatrics* 1991;88:19-28.

168. Halliday H, CP S. Strategies for Surfactant Therapy in Established neonatal Respiratory Syndrome. In: Robertson B, HW T, eds. Surfactant therapy for lung disease. New York: Marcel dekker, Inc, 1995:443-454.

169. Soll RF, Morley CJ. Prophylactic versus selective use of surfactant in preventing morbidity and mortality in preterm infants. *Cochrane Database Syst Rev* 2001:CD000510.

170. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. *Am J Respir Crit Care Med* 2001;163:1723-9.

171. Long W, Corbet A, Cotton R, Courtney S, McGuiness G, Walter D, Watts J, Smyth J, Bard H, Chernick V. A controlled trial of synthetic surfactant in infants weighing 1250 g or more with respiratory distress syndrome. The American Exosurf Neonatal Study Group I, and the Canadian Exosurf Neonatal Study Group. *N Engl J Med* 1991;325:1696-703.

172. Horbar JD, Soll RF, Schachinger H, Kewitz G, Versmold HT, Lindner W, Duc G, Mieth D, Linderkamp O, Zilow EP, et al. A European multicenter randomized controlled trial of single dose surfactant therapy for idiopathic respiratory distress syndrome. *Eur J Pediatr* 1990;149:416-23.

173. Raju TN, Vidyasagar D, Bhat R, Sobel D, McCulloch KM, Anderson M, Maeta H, Levy PS, Furner S. Double-blind controlled trial of single-dose treatment with bovine surfactant in severe hyaline membrane disease. *Lancet* 1987;1:651-6.

174. Stahlman MT, Gray ME, Ross GF, Hull WM, Wikenheiser K, Dingle S, Zelenski-Low KR, Whitsett JA. Human surfactant protein-A contains blood group A antigenic determinants. *Pediatr Res* 1992;31:364-71.

175. Björklund LJ, Ingimarsson J, Curstedt T, John J, Robertson B, Werner O, Vilstrup CT. Manual ventilation with a few large breaths at birth compromises the therapeutic effect of subsequent surfactant replacement in immature lambs. *Pediatr Res* 1997;42:348-55.

176. Egberts J, de Winter JP, Sedin G, de Kleine MJ, Broberger U, van Bel F, Curstedt T, Robertson B. Comparison of prophylaxis and rescue treatment with Curosurf in neonates less than 30 weeks' gestation: a randomized trial. *Pediatrics* 1993;92:768-74.

177. Kattwinkel J, Bloom BT, Delmore P, Davis CL, Farrell E, Friss H, Jung AL, King K, Mueller D. Prophylactic administration of calf lung surfactant extract is more effective than early treatment of respiratory distress syndrome in neonates of 29 through 32 weeks' gestation. *Pediatrics* 1993;92:90-8.

178. Kendig JW, Notter RH, Cox C, Reubens LJ, Davis JM, Maniscalco WM, Sinkin RA, Bartoletti A, Dweck HS, Horgan MJ, et al. A comparison of surfactant as immediate prophylaxis and as rescue therapy in newborns of less than 30 weeks' gestation. *N Engl J Med* 1991;324:865-71.

179. Vaucher YE, Harker L, Merritt TA, Hallman M, Gist K, Bejar R, Heldt GP, Edwards D, Pohjavuori M. Outcome at twelve months of adjusted age in very low birth weight infants with lung immaturity: a randomized, placebo-controlled trial of human surfactant. *J Pediatr* 1993;122:126-32.

180. Early versus delayed neonatal administration of a synthetic surfactant--the judgment of OSIRIS. The OSIRIS Collaborative Group (open study of infants at high risk of or with respiratory insufficiency--the role of surfactant. *Lancet* 1992;340:1363-9.

181. Yost CC, Soll RF. Early versus delayed selective surfactant treatment for neonatal respiratory distress syndrome. *Cochrane Database Syst Rev* 2000:CD001456.

182. Eckert Seitz E, Fiori HH, Luz JH, Fiori RM. Stable microbubble test on tracheal aspirate for the diagnosis of respiratory distress syndrome. *Biol Neonate* 2005;87:140-4.

183. Chida S, Fujiwara T. Stable microbubble test for predicting the risk of respiratory distress syndrome. *Eur J Pediatr* 1993;152:147-150.

184. Fiori H, Linderholm B, Fiori R, Robertson B. Bubbles and computer-aided image analysis for evaluation of surfactant inhibition. *Acta Paediatr* 2002;90:1402-1404.

185. Verder H, Ebbesen F, Linderholm B, Robertson B, Eschen C, Arroe M, Lange A, Grytter C, Bohlin K, Bertelsen A. Prediction of respiratory distress syndrome by the microbubble stability test on gastric aspirates in newborns of less than 32 weeks' gestation. *Acta Paediatr* 2003;92:728-33.

186. Osborn DA, Jeffery HE, Bredemeyer SL, Polverino JM, Reid S. Targeted early rescue surfactant in ventilated preterm infants using the click test. *Pediatrics* 2000;106:E30.

187. Parkinson CE, Supramaniam G, Harvey D. Bubble clicking in pharyngeal aspirates of newborn babies. *Br Med J* 1978;1:758-9.

188. Stucin Gantar I, Babnik J. Surfactant inhibitors in amniotic fluid from gastric aspirates in premature infants with respiratory distress syndrome. *Biol neonate* 1999;76 Suppl 1:43-44.

189. Verder H, Staun-Olsen P, Brandt J, Qin G, Ebbesen F, B R. Prediction of Respiratory Distress Syndrome by Lamellar body Counts on gastric aspirates in newborns less than 32 weeks gestation. *Biol Neonate* 2003;84:32-41A.

190. Speer CP, Robertson B, Curstedt T, Halliday HL, Compagnone D, Gefeller O, Harms K, Herting E, McClure G, Reid M, et al. Randomized European multicenter trial of surfactant replacement therapy for severe neonatal respiratory distress syndrome: single versus multiple doses of Curosurf. *Pediatrics* 1992;89:13-20.

191. Stevens TP, Blennow M, Soll RF. Early surfactant administration with brief ventilation vs selective surfactant and continued mechanical ventilation for preterm infants with or at risk for RDS. *Cochrane Database Syst Rev* 2002:CD003063.

192. Verder H, Albertsen P, Ebbesen F, Greisen G, Robertson B, Bertelsen A, Agertoft L, Djernes B, Nathan E, Reinholdt J. Nasal continuous positive airway pressure and early surfactant therapy for respiratory distress syndrome in newborns of less than 30 weeks' gestation. *Pediatrics* 1999;103:E24.

193. Verder H, Robertson B, Greisen G, Ebbesen F, Albertsen P, Lundstrom K, Jacobsen T. Surfactant therapy and nasal continuous positive airway pressure for newborns with respiratory distress syndrome. Danish-Swedish Multicenter Study Group. *N Engl J Med* 1994;331:1051-5.

194. Dani C, Bertini G, Pezzati M, Cecchi A, Caviglioli C, Rubaltelli F. Early extubation and nasal continuous positive airway pressure after surfactant treatment for respiratory distress syndrome among preterm infants <30 weeks gestation. *Pediatrics* 2004;113:560-563.

195. Berggren E, Liljedahl M, Winbladh B, Andreasson B, Curstedt T, Robertson B, Schollin J. Pilot study of nebulized surfactant therapy for neonatal respiratory distress syndrome. *Acta Paediatr* 2000;89:460-4.

196. Arroe M, Pedersen-Bjergaard L, Albertsen P, Bode S, Greisen G, Jonsbo F. Inhalation of aerosolized surfactant (Exosurf) to neonates treated with continuous positive airway pressure. *Prenatal and Neonatal Medicine* 1998;3:346-52.

197. Alberti A, Pettenazzo A, Enzi GB, Palamidese A, Mapp C, Ventura P, Baritussio A. Uptake and degradation of Curosurf after tracheal administration to newborn and adult rabbits. *Eur Respir J* 1998;12:294-300.

198. Kobayashi T, Curstedt T, Grossmann G, Robertson B. Inhibition of exogenous surfactant in ventilated immature newborn rabbits. *Respir Physiol* 1989;76:1-12.

199. Seidner SR, Jobe AH, Ruffini L, Ikegami M, Pettenazzo A. Recovery of treatment doses of surfactants from the lungs and vascular compartments of mechanically ventilated premature rabbits. *Pediatr Res* 1989;25:423-8.

200. Stevens PA, Wright JR, Clements JA. Surfactant secretion and clearance in the newborn. *J Appl Physiol* 1989;67:1597-605.

201. Factors influencing the clinical response to surfactant replacement therapy in babies with severe respiratory distress syndrome. Collaborative European Multicentre Study Group. *Eur J Pediatr* 1991;150:433-9.

202. Hallman M. The severity of RDS during the first two neonatal days in relationship to fluid intake. *Acta Paediatr Scand Suppl* 1989;360:93-100.

203. Charon A, Taeusch W, Fitzgibbon C, Smith GB, Treves ST, Phelps DS. Factors associated with surfactant treatment response in infants with severe respiratory distress syndrome. *Pediatrics* 1989;83:348-54.

204. Hamvas A, Devine T, Cole FS. Surfactant therapy failure identifies infants at risk for pulmonary mortality. *Am J Dis Child* 1993;147:665-8.

205. Cotton R, Olsson T. Lung mechanics in Respiratory Distress Syndrome. In: Robertson B, Taeusch H, eds. Surfactant therapy for lung disease. New York: Marcel Dekker, Inc, 1995:121-143.

206. Hjalmarson O, Olsson T. III. Mechanical and ventilatory parameters in healthy and diseased newborn infants. *Acta Paediatr Scand Suppl* 1974:26-48.

207. Bhutani VK, Abbasi S, Long WA, Gerdes JS. Pulmonary mechanics and energetics in preterm infants who had respiratory distress syndrome treated with synthetic surfactant. *J Pediatr* 1992;120:S18-24.

208. Sly PD, Brown KA, Bates JH, Spier S, Milic-Emili J. Noninvasive determination of respiratory mechanics during mechanical ventilation of neonates: a review of current and future techniques. *Pediatr Pulmonol* 1988;4:39-47.

209. Sandberg K, Edberg KE, Benton W, Silberberg A, Sladek M, Sundell HW. Surfactant improves gas mixing and alveolar ventilation in preterm lambs. *Pediatr Res* 1991;30:181-9.

210. Lachmann B, Grossmann G, Nilsson R, Robertson B. Lung mechanics during spontaneous ventilation in premature and fullterm rabbit neonates. *Respir Physiol* 1979;38:283-302.

211. Davis JM, Veness-Meehan K, Notter RH, Bhutani VK, Kendig JW, Shapiro DL. Changes in pulmonary mechanics after the administration of surfactant to infants with respiratory distress syndrome. *N Engl J Med* 1988;319:476-9.

212. Bhat R, Dziedzic K, Bhutani VK, Vidyasagar D. Effect of single dose surfactant on pulmonary function. *Crit Care Med* 1990;18:590-5.

213. Cotton RB, Olsson T, Law AB, Parker RA, Lindstrom DP, Silberberg AR, Sundell HW, Sandberg K. The physiologic effects of surfactant treatment on gas exchange in newborn premature infants with hyaline membrane disease. *Pediatr Res* 1993;34:495-501.

214. Mariani G, Cifuentes J, Carlo WA. Randomized trial of permissive hypercapnia in preterm infants. *Pediatrics* 1999;104:1082-8.

215. Froese AB, McCulloch PR, Sugiura M, Vaclavik S, Possmayer F, Moller F. Optimizing alveolar expansion prolongs the effectiveness of exogenous surfactant therapy in the adult rabbit. *Am Rev Respir Dis* 1993;148:569-77.

216. Ricard JD, Dreyfuss D, Saumon G. Ventilator-induced lung injury. *Curr Opin Crit Care* 2002;8:12-20.

217. Parker JC, Hernandez LA, Peevy KJ. Mechanisms of ventilator-induced lung injury. *Crit Care Med* 1993;21:131-43.

218. Plavka R, Keszler M. Interaction between surfactant and ventilatory support in newborns with primary surfactant deficiency. *Biol Neonate* 2003;84:89-95.

219. Veldhuizen RA, Welk B, Harbottle R, Hearn S, Nag K, Petersen N, Possmayer F. Mechanical ventilation of isolated rat lungs changes the structure and biophysical properties of surfactant. *J Appl Physiol* 2002;92:1169-75.

220. Seidner S, Rider E, Jobe A, Yamada T, Ikegami M. Effects of antenatal thyrotropin-releasing hormone, antenatal corticosteroids, and postnatal ventilation on surfactant mobilization in premature rabbits. *Am J Obstet Gynecol* 1992;166:1551-9.

221. Ennema JJ, Reijngoud DJ, Wildevuur CR, Egberts J. Effects of artificial ventilation on surfactant phospholipid metabolism in rabbits. *Respir Physiol* 1984;58:15-28.

222. Pinkerton KE, Ikegami M, Dillard LM, Jobe AH. Surfactant treatment effects on lung structure and type II cells of preterm ventilated lambs. *Biol Neonate* 2000;77:243-52.

223. Ingimarsson J, Bjorklund LJ, Curstedt T, Gudmundsson S, Larsson A, Robertson B, Werner O. Incomplete protection by prophylactic surfactant against the adverse effects of large lung inflations at birth in immature lambs. *Intensive Care Med* 2004;30:1446-53.

224. Wada K, Jobe AH, Ikegami M. Tidal volume effects on surfactant treatment responses with the initiation of ventilation in preterm lambs. *J Appl Physiol* 1997;83:1054-61.

225. Nilsson R, Grossmann G, Robertson B. Lung surfactant and the pathogenesis of neonatal bronchiolar lesions induced by artificial ventilation. *Pediatr Res* 1978;12:249-55.

226. Taskar V, John J, Evander E, Wollmer P, Robertson B, Jonson B. Healthy lungs tolerate repetitive collapse and reopening during short periods of mechanical ventilation. *Acta Anaesthesiol Scand* 1995;39:370-6.

227. Ikegami M, Wada K, Emerson GA, Rebello CM, Hernandez RE, Jobe AH. Effects of ventilation style on surfactant metabolism and treatment response in preterm lambs. *Am J Respir Crit Care Med* 1998;157:638-44.

228. Van Marter LJ, Allred EN, Pagano M, Sanocka U, Parad R, Moore M, Susser M, Paneth N, Leviton A. Do clinical markers of barotrauma and oxygen toxicity explain interhospital variation in rates of chronic lung disease? The Neonatology Committee for the Developmental Network. *Pediatrics* 2000;105:1194-201.

229. Robertson B, Halliday HL. Principles of surfactant replacement. *Biochim Biophys Acta* 1998;1408:346-61.

230. Rider ED, Jobe AH, Ikegami M, Sun B. Different ventilation strategies alter surfactant responses in preterm rabbits. *J Appl Physiol* 1992;73:2089-96.

231. Johnson AH, Peacock JL, Greenough A, Marlow N, Limb ES, Marston L, Calvert SA. High-frequency oscillatory ventilation for the prevention of chronic lung disease of prematurity. *N Engl J Med* 2002;347:633-42.

232. Courtney SE, Durand DJ, Asselin JM, Hudak ML, Aschner JL, Shoemaker CT. High-frequency oscillatory ventilation versus conventional mechanical ventilation for very-low-birth-weight infants. *N Engl J Med* 2002;347:643-52.

233. Ennema JJ, Reijngoud DJ, Egberts J, Mook PH, Wildevuur CR. High-frequency oscillation affects surfactant phospholipid metabolism in rabbits. *Respir Physiol* 1984;58:29-39.

234. Solimano A, Bryan C, Jobe A, Ikegami M, Jacobs H. Effects of high-frequency and conventional ventilation on the premature lamb lung. *J Appl Physiol* 1985;59:1571-7.

235. Ward E, Nicholas T. Effect of artificial ventilation and anaesthesia on surfactant turnover in rats. *Respir Physiol* 1992;87:115-129.

236. Truog WE, Standaert TA, Murphy JH, Woodrum DE, Hodson WA. Effects of prolonged high-frequency oscillatory ventilation in premature primates with experimental hyaline membrane disease. *Am Rev Respir Dis* 1984;130:76-80.

237. deLemos RA, Coalson JJ, deLemos JA, King RJ, Clark RH, Gerstmann DR. Rescue ventilation with high frequency oscillation in premature baboons with hyaline membrane disease. *Pediatr Pulmonol* 1992;12:29-36.

238. Gerdes JS, Abbasi S, Karp K, Hull W, Whitsett JA. Surfactant protein-A in bronchoalveolar lavage fluid from neonates with RDS on conventional and high-frequency oscillatory ventilation. *Pediatr Pulmonol* 1990;9:166-9.

239. Dargaville PA, South M, McDougall PN. Pulmonary surfactant concentration during transition from high frequency oscillation to conventional mechanical ventilation. *J Paediatr Child Health* 1997;33:517-21.

240. Gregory GA, Kitterman JA, Phibbs RH, Tooley WH, Hamilton WK. Treatment of the idiopathic respiratory-distress syndrome with continuous positive airway pressure. *N Engl J Med* 1971;284:1333-40.

241. De Paoli AG, Davis PG, Faber B, Morley CJ. Devices and pressure sources for administration of nasal continuous positive airway pressure (NCPAP) in preterm neonates. *Cochrane Database Syst Rev* 2002:CD002977.

242. Avery ME, Tooley WH, Keller JB, Hurd SS, Bryan MH, Cotton RB, Epstein MF, Fitzhardinge PM, Hansen CB, Hansen TN, et al. Is chronic lung disease in low birth weight infants preventable? A survey of eight centers. *Pediatrics* 1987;79:26-30.

243. Horbar JD, McAuliffe TL, Adler SM, Albersheim S, Cassady G, Edwards W, Jones R, Kattwinkel J, Kraybill EN, Krishnan V, et al. Variability in 28-day outcomes for very low birth weight infants: an analysis of 11 neonatal intensive care units. *Pediatrics* 1988;82:554-9.

244. Thomson MA, Yoder BA, Winter VT, Martin H, Catland D, Siler-Khodr TM, Coalson JJ. Treatment of immature baboons for 28 days with early nasal continuous positive airway pressure. *Am J Respir Crit Care Med* 2004;169:1054-62.

245. Jobe AH, Kramer BW, Moss TJ, Newnham JP, Ikegami M. Decreased indicators of lung injury with continuous positive expiratory pressure in preterm lambs. *Pediatr Res* 2002;52:387-92.

246. Ho JJ, Subramaniam P, Henderson-Smart DJ, Davis PG. Continuous distending pressure for respiratory distress syndrome in preterm infants. *Cochrane Database Syst Rev* 2002:CD002271.

247. Jonsson B. Neonatal care of very-low-birthweight infants in special-care units and neonatal intensive care units in Stockholm. Early nasal continuous positive airway pressure versus mechanical ventilation: gains and losses. *Acta Paediatr* 1997;419 Suppl:4-10.

248. Kamper J, Wulff K, Larsen C, Lindequist S. Early treatment with nasal continuous positive airway pressure in very low-birth-weight infants. *Acta Paediatr* 1993;82:193-7.

249. Tooley J, Dyke M. Randomized study of nasal continuous positive airway pressure in the preterm infant with respiratory distress syndrome. *Acta paediatr* 2003;92:1170-1174.

250. Victorin LH, Deverajan LV, Curstedt T, Robertson B. Surfactant replacement in spontaneously breathing babies with hyaline membrane disease--a pilot study. *Biol Neonate* 1990;58:121-6.

251. Thibeault DW, Mabry SM, Ekekezie, II, Truog WE. Lung elastic tissue maturation and perturbations during the evolution of chronic lung disease. *Pediatrics* 2000;106:1452-9.

252. Benveniste D, Berg O, Pedersen JE. A technique for delivery of continuous positive airway pressure to the neonate. *J Pediatr* 1976;88:1015-9.

253. Shennan A, Dunn M, Ohlsson A, Lennox K, Hoskins E. Abnormal pulmonary outcomes in premature infants: Prediction from oxygen requirement in the neonatal period. *Pediatrics* 1988;82:527-32.

254. Papile LA, Burstein J, Burstein R, Koffler H. Incidence and evolution of subependymal and intraventricular hemorrhage: a study of infants with birth weights less than 1,500 gm. *J Pediatr* 1978;92:529-34.

255. Gobel W, Richard G. Retinopathy of prematurity--current diagnosis and management. *Eur J Pediatr* 1993;152:286-90.

256. Bier D. Stable isotopes in biosciences, their measurements and models for amino acid metabolism. *Eur J Pediatr* 1997;156:S2-8.

257. Patterson BW. Use of stable isotopically labeled tracers for studies of metabolic kinetics: an overview. *Metabolism* 1997;46:322-9.

258. Touchstone J, Chen J, Beaver K. Improved separation of phospholipids in thin layer chromatography. *Lipids* 1979;15:61-62.

259. Mason RJ, Nellenbogen J, Clements JA. Isolation of disaturated phosphatidylcholine with osmium tetroxide. *J Lipid Res* 1976;17:281-4.

260. Nilsson R. The artificially ventilated preterm rabbit neonate as experimental model of hyaline membrane disease. *Acta Anaesthesiol Scand* 1982;26:89-103.

261. Bartlett GR. Phosphorus assay in column chromatography. *J Biol Chem* 1959;234:466-8.

262. Hellerstein MK, Kletke C, Kaempfer S, Wu K, Shackleton CH. Use of mass isotopomer distributions in secreted lipids to sample lipogenic acetyl-CoA pool in vivo in humans. *Am J Physiol* 1991;261:E479-86.

263. Chinkes DL, Aarsland A, Rosenblatt J, Wolfe RR. Comparison of mass isotopomer dilution methods used to compute VLDL production in vivo. *Am J Physiol* 1996;271:E373-83.

264. Schmidt R, Meier U, Yabut-Perez M, Walmrath D, Grimminger F, Seeger W, Gunther A. Alteration of fatty acid profiles in different pulmonary surfactant phospholipids in acute respiratory distress syndrome and severe pneumonia. *Am J Respir Crit Care Med* 2001;163:95-100.

265. King EL, Shackelford GD, Hamvas A. High-frequency oscillation and paralysis stabilize surfactant protein-B--deficient infants. *J Perinatol* 2001;21:421-5.

266. Nicholas TE, Barr HA. Control of release of surfactant phospholipids in the isolated perfused rat lung. *J Appl Physiol* 1981;51:90-8.

267. Ikegami M, Kallapur S, Michna J, Jobe AH. Lung injury and surfactant metabolism after hyperventilation of premature lambs. *Pediatr Res* 2000;47:398-404.

268. Imai Y, Nakagawa S, Ito Y, Kawano T, Slutsky AS, Miyasaka K. Comparison of lung protection strategies using conventional and high-frequency oscillatory ventilation. *J Appl Physiol* 2001;91:1836-44.

269. Kerr CL, Veldhuizen RA, Lewis JF. Effects of high-frequency oscillation on endogenous surfactant in an acute lung injury model. *Am J Respir Crit Care Med* 2001;164:237-42.

270. Yoder BA, Siler-Khodr T, Winter VT, Coalson JJ. High-frequency oscillatory ventilation: effects on lung function, mechanics, and airway cytokines in the immature baboon model for neonatal chronic lung disease. *Am J Respir Crit Care Med* 2000;162:1867-76.

271. Gommers D, Hartog A, Schnabel R, De Jaegere A, Lachmann B. High-frequency oscillatory ventilation is not superior to conventional mechanical ventilation in surfactant-treated rabbits with lung injury. *Eur Respir J* 1999;14:738-44.

272. Vazquez de Anda GF, Gommers D, Verbrugge SJ, De Jaegere A, Lachmann B. Mechanical ventilation with high positive end-expiratory pressure and small driving pressure amplitude is as effective as high-frequency oscillatory ventilation to preserve the function of exogenous surfactant in lung-lavaged rats. *Crit Care Med* 2000;28:2921-5.

273. Vazquez de Anda GF, Hartog A, Verbrugge SJ, Gommers D, Lachmann B. The open lung concept: pressure-controlled ventilation is as effective as high-frequency oscillatory ventilation in improving gas exchange and lung mechanics in surfactant-deficient animals. *Intensive Care Med* 1999;25:990-6.

274. Sahni R, Ammari A, Suri MS, Milisavljevic V, Ohira-Kist K, Wung JT, Polin RA. Is the New Definition of Bronchopulmonary Dysplasia More Useful? *J Perinatol* 2004.

275. Charafeddine L, D'Angio CT, Phelps DL. Atypical chronic lung disease patterns in neonates. *Pediatrics* 1999;103:759-65.

276. Aly H, Milner JD, Patel K, El-Mohandes AA. Does the experience with the use of nasal continuous positive airway pressure improve over time in extremely low birth weight infants? *Pediatrics* 2004;114:697-702.

277. Rojas MA, Gonzalez A, Bancalari E, Claure N, Poole C, Silva-Neto G. Changing trends in the epidemiology and pathogenesis of neonatal chronic lung disease. *J Pediatr* 1995;126:605-10.

278. Jonsson B, Tullus K, Brauner A, Lu Y, Noack G. Early increase of TNF-alfa and IL-6 levels intracheobronchial aspirate fluid indicator of subsequent chronic lung disease in preterm infants. *Arch of Dis Child* 1997;77:F198-201.

279. Groneck P, Gotze-Speer B, Oppermann M, Eiffert H, Speer CP. Association of pulmonary inflammation and increased microvascular permeability during the development of bronchopulmonary dysplasia: a sequential analysis of inflammatory mediators in respiratory fluids of high-risk preterm neonates. *Pediatrics* 1994;93:712-8.

280. Coalson JJ, King RJ, Yang F, Winter V, Whitsett JA, Delemos RA, Seidner SR. SP-A deficiency in primate model of bronchopulmonary dysplasia with infection. In situ mRNA and immunostains. *Am J Respir Crit Care Med* 1995;151:854-66.

281. Bouhafs RK, Jarstrand C. Lipid peroxidation of lung surfactant by bacteria. *Lung* 1999;177:101-10.

282. Jobe A, Rider E. Catabolism and recycling of surfactant. In: Robertson B, Van Golde L, Batenburg J, eds. Pulmonary surfactant; From molecular biology to clinical practice: Elsevier, 1992:313-338.

283. Moison RM, Palinckx JJ, Roest M, Houdkamp E, Berger HM. Induction of lipid peroxidation of pulmonary surfactant by plasma of preterm babies. *Lancet* 1993;341:79-82.

284. Bouhafs RK, Samuelson A, Jarstrand C. Lipid peroxidation of lung surfactant due to reactive oxygen species released from phagocytes stimulated by bacteria from children with cystic fibrosis. *Free Radic Res* 2003;37:909-17.

285. Bouhafs RK, Rauprich P, Herting E, Schroder A, Robertson B, Jarstrand C. Direct and phagocyte-mediated lipid peroxidation of lung surfactant by group B streptococci. *Lung* 2000;178:317-29.

286. Bouhafs RK, Jarstrand C. Effects of antioxidants on surfactant peroxidation by stimulated human polymorphonuclear leukocytes. *Free Radic Res* 2002;36:727-34.

287. Varsila E, Hallman M, Andersson S. Free-radical-induced lipid peroxidation during the early neonatal period. *Acta Paediatr* 1994;83:692-695.

288. Pitkanen O, Hallman M, Andersson S. Correlation of free-radiccal-induced lipid peroxidation with outcome in very low borth weight infants. *J Pediatr* 1990;116:760-764.