Surfactant Phosphatidylcholine Metabolism in Severe Neonatal Lung Disease

Studied with Stable Isotopes

Daphne Janssen

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CIP-data Koninklijke Bibliotheek, Den Haag
ISBN:

Printed by Optima Grafische Communicatie, Rotterdam.

The studies described in this thesis were supported by a grant from the Sophia Foundation for Medical Research.

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Het metabolisme van surfactant fosfatidylcholine in pasgeborenen met ernstige neonatale longziekten

bestudeerd met stabiele isotopen

PROEFSCHRIFT

Ter verkrijging van de graad van doctor aan de Erasmus Universiteit Rotterdam op gezag van de Rector Magnificus Prof. dr. ir. J.H. van Bemmel
en volgens besluit van Colleges van Promoties

De openbare verdediging zal plaatsvinden op donderdag 26 juni 2003 om 13.30 uur

door

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geboren te Geleen

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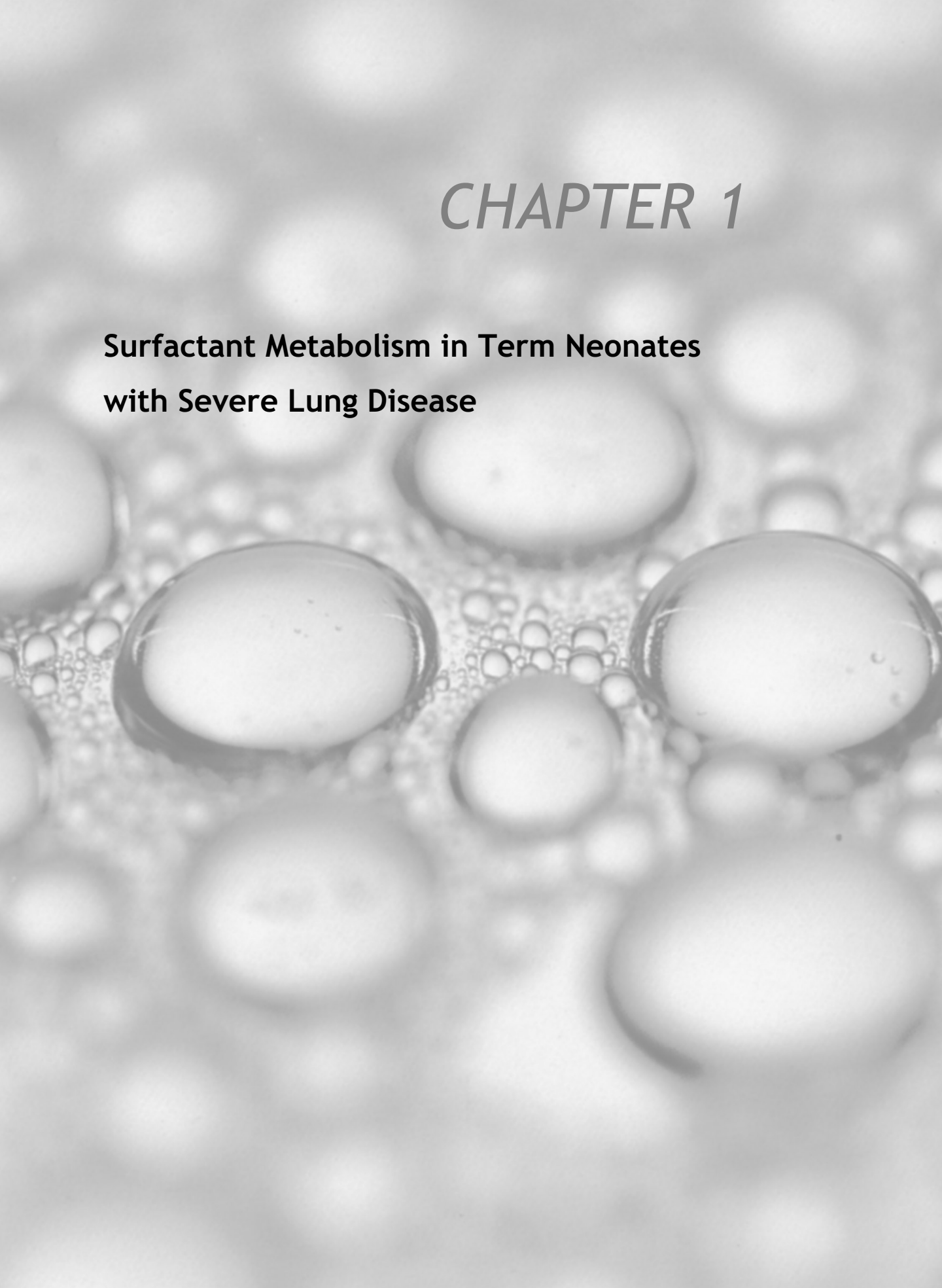


CHAPTER 1

Surfactant Metabolism in Term Neonates with Severe Lung Disease

Introduction

Avery and Mead showed in 1959 that pulmonary surfactant deficiency is a major factor in the pathophysiology of respiratory distress syndrome (RDS) (1). In 1980 Fujiwara et al. administered exogenous surfactant for the first time successfully to preterm infants with RDS (2). This was followed by numerous clinical trials that demonstrated a decrease in death rates and complications (3, 4). There are now accumulating data which suggest that a disturbed surfactant metabolism plays a role in several other neonatal lung diseases, such as congenital diaphragmatic hernia (CDH), meconium aspiration syndrome (MAS), surfactant protein-B (SP-B) deficiency, and neonatal pneumonia and/or sepsis. Also in adult respiratory distress syndrome, asthma, infectious lung diseases, and interstitial lung diseases (5-9). Insufficient surfactant function could be due to a disturbance in surfactant kinetics or secondary to an inactivation of surfactant by several components. Surfactant therapy possibly plays a therapeutical role in the management of these clinical conditions.

This review will focus on the surfactant metabolism in term neonatal lung diseases. We will discuss the role of surfactant in CDH, MAS, SP-B deficiency, and neonatal pneumonia and/or sepsis. First, we will briefly review the functions and composition of surfactant and the normal cellular metabolism of surfactant.

Surfactant function and composition

The primary function of surfactant is to decrease the surface tension at the air-liquid interface in the alveoli and distal bronchioli, to promote lung expansion during inspiration and to prevent alveolar collapse at expiration. Besides these biophysical properties, surfactant plays also an important role in pulmonary host defense (10, 11).

Surfactant is composed of a complex mixture of lipids (~90%) and proteins (~10%), which is strikingly comparable across species including human (12, 13). Of the surfactant lipids, 80 to 90% are phospholipids, from which phosphatidylcholine (PC) is the major phospholipid, accounting for 70-80% (13). Other lipids are phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin, cholesterol, triacylglycerols and free fatty acids (14). The PC molecule comprises a glycerol backbone, two fatty acids, phosphate, and a choline moiety. Approximately 60% of the PC contains two saturated fatty acids (sat PC, DSPC) of which most is dipalmitoyl (16:0/16:0) (DPPC). DPPC is the principal surface tension lowering component of surfactant. Most other PC molecules have a fatty acid with one double bound in the 2 position of the glycerol molecule.

Four surfactant proteins have been identified (15, 16). SP-A and SP-D are hydrophilic, and SP-B and SP-C are hydrophobic. They are either exclusively lung-associated or predominately found in the lung. SP-A is the most abundant surfactant protein and is essential for the formation of tubular myelin, it functions as a regulator of phospholipid insertion into the monolayer, and modulates the uptake and secretion of phospholipids by type II cells (17). However, mice that lack SP-A have indeed no tubular myelin but have normal lung function and surfactant metabolism even when exercised (18). Furthermore, together

with SP-D, SP-A has an important role in the lung defense. SP-A can both depress and activate macrophage function, it binds to multiple pathogens and facilitates phagocytosis of pathogens by macrophages and clearance from the airspace (10). SP-D also binds pathogens and facilitates their clearance (10). The absence of SP-D results in increased surfactant lipid pools in the airspaces and emphysema in lungs of mice (19). SP-B also plays a role in the formation of tubular myelin, it promotes together with SP-C the rapid phospholipid insertion into the air-liquid interface (20, 21), and SP-B influences the molecular ordering of the phospholipid layer (22, 23). Infants with a genetic absence of SP-B develop lethal respiratory distress after birth, which can only be overcome by a lung transplantation (24). The lack of SP-B cause a loss of lamellar bodies, tubular myelin, and an incompletely processed SP-C (25). SP-C regulates the phospholipid ordering in the monolayer, enhances the reuptake of surfactant lipids in vitro, and may have a role in surfactant catabolism (20, 26). Mice that lack SP-C have a normal surfactant and lung function, and have no abnormalities in SP-B processing (27). However, the stability of surfactant at low lung volumes is decreased. On the other hand, overexpression of SP-C mature peptide in type II cells of transgenic mice results in lethal, neonatal RDS (28).

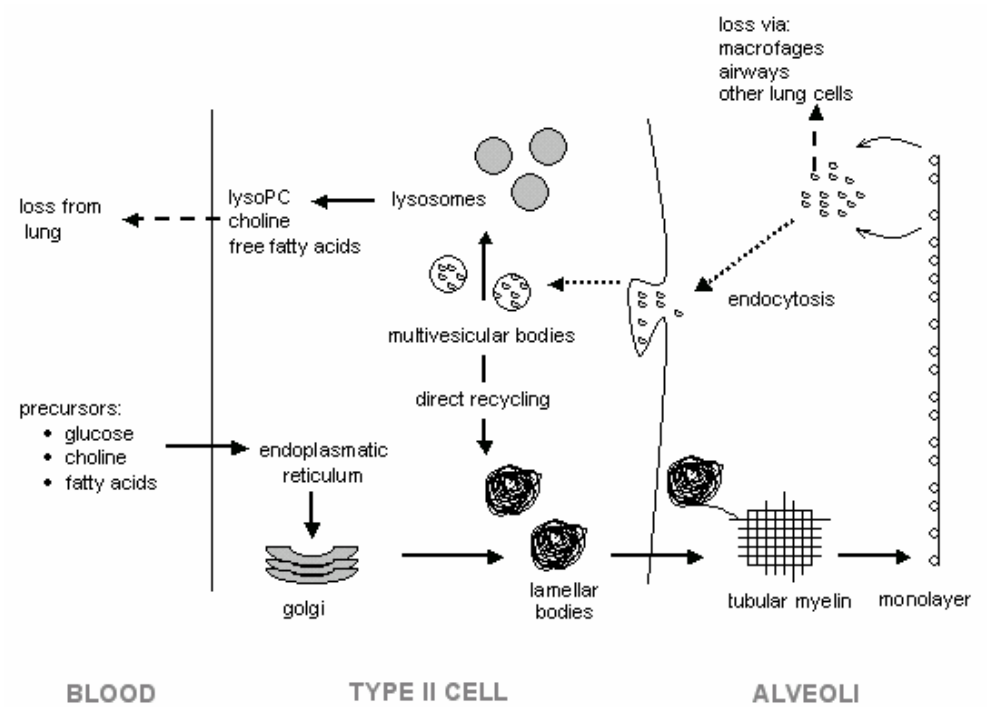


Figure 1. *Schematic illustration of surfactant phosphatidylcholine metabolism*

Surfactant synthesis, secretion and clearance

The synthesis, secretion and clearance of surfactant is a complex sequence of biochemical synthetic events, subcellular organelle growth, and maturational events which take place in the alveolar type II cell of the lung (Figure 1). Surfactant PC is synthesized from phospholipid precursors (eg, fatty acids, glycerol, choline, glucose) in the Golgi apparatus (29, 30). The CDP-choline pathway is the principle pathway involved in the *de novo* synthesis of surfactant PC (31). Shortly, choline is brought into the cell and is phosphorylated by choline kinase. The synthesis of the activated intermediate, CDP-choline is catalyzed by phosphocholine cytidyltransferase (CT). Finally, the phosphocholine is transferred via diacylglycerol to phosphocholine by the CDP-choline: 1,2-diacylglycerol phosphocholine-transferase. The enzyme CT plays an important regulatory role in *de novo* synthesis of PC (32, 33). In the fetal type II cell intracellular glycogen stores appear to be a major source of the glycerol backbone of PC (34), whereas in the adult lung glucose from the circulation is a major substrate for the glycerol backbone of PC. Choline is mainly derived from the diet (35). The fatty acids of surfactant phospholipids are synthesized *de novo* in the type II cell, or taken up from the blood, or are derived from recycling of alveolar surfactant phospholipids (29). The exact relative contribution of the fatty acids supplied by the circulation, those synthesized de novo, and those supplied by recycling of alveolar phospholipids to the formation of surfactant lipids by the type II cell is still unknown. Lipogenesis from glucose and lactate among other substrates, is probably of particular importance to supply fatty acids for surfactant phospholipid synthesis in the prenatal lung (36, 37). In the more mature lung, fatty acids of surfactant PC are derived mainly from uptake of fatty acids from plasma (38). The composition of the newly formed PC is modified to achieve high levels of DPPC, by acyl remodeling, mainly involving sequential actions of phospholipase and acyltransferase enzymes (39).

Lamellar bodies are condensed, highly structured lipoprotein packages, that serve as the intracellular storage form of surfactant. Lamellar bodies are secreted into the alveolar space by fusing of their limiting membrane with the plasma membrane (40). This can be stimulated by stretch of the type II cell, by beta agonists, by purinergic agonists, such as ATP, and various other agents (41, 42). After secretion the lamellar bodies unravel to form loose membranous arrays and lattice-like structures, the tubular myelin. At alveolar surface expansion during inspiration, surfactant components insert from the hypophase (epithelial lining fluid (ELF)) into the monolayer. At expiration the alveolar surface reduces and the monolayer is compressed, thereby squeezing out some surfactant proteins, unsaturated PC, and other lipids. By this mechanism, the monolayer comprises mainly the most surface tension lowering component DPPC during compression (43).

Due to the cyclic changes during respiration the surfactant monolayer is degraded to small aggregates, representing ‘inactive surfactant’ or ‘used surfactant’. By differential centrifugation these small aggregates can be separated from the lamellar bodies and tubular myelin, the ‘large aggregates’ (active surfactant) (44-46).

The alveolar surfactant can be cleared by different pathways. Surfactant components can be reutilized through uptake by the type II cell, incorporated into the lamellar bodies and then directly resecreted (47, 48). Another way is by degradation and utilization of

surfactant components to synthesize new surfactant lipids or proteins. Finally, surfactant can be removed from the lung, either as intact molecules or as degraded products (41). The efficiency of recycling is age-dependent, and was calculated to be 90% in young pigs (38), >90% in the newborn rabbit and 50% in adult rabbits (47, 49).

Surfactant kinetics

The time required for *de novo* PC synthesis, secretion, and significant alveolar accumulation has been studied in animals using radioactive labeled substrates. Following the intravascular injection of radiolabeled palmitic acid into newborn rabbits or sheep, maximal incorporation of the labeled precursors into lung PC occurs within ~10 minutes (50, 51). However, recovery of labeled alveolar surfactant PC does not reach its peak until about 35 to 45 hours. Compared to adult animals the time to reach peak specific activity is longer in term newborns (52). Preterm ventilated lambs show similar to the term ventilated lambs a slow movement of surfactant PC from the synthetic sites to the alveolus (53).

In preterm infants with RDS that received a 24 hour intravenous infusion of the stable isotope [U-^{13}C]glucose, the palmitic acid became labeled after ~19 hours and was maximally labeled after ~70 hours (Table 1) (54). By using [U-^{13}C]palmitic acid or [U-^{13}C]linoleic acid as precursors in preterm infants with RDS, surfactant PC became labeled after ~20 hours and ~16 hours, respectively, after start of infusion, and was maximally labeled after ~71 hours and ~85 hours, respectively (55). Recently, Bohlin *et al*. measured [1-^{13}C]acetate incorporation in surfactant DPPC of preterm infants with RDS and term infants without lung disease (56). The preterm infants showed first incorporation of label after ~13 hours, and reached maximal enrichment after ~82 hours, whereas term infants reached first enrichment of label at ~7 hours and maximal enrichment at ~35 hours. Term infants without significant lung disease who received [U-^{13}C]palmitic acid had a first appearance of label in surfactant DPPC after ~9 hours, and a maximal incorporation of label at ~51 hours (57). Kinetic parameters in human infants studied with different precursors are depicted in Table 1. As in animal studies, these results of human stable isotopes studies show slower surfactant kinetics in preterm infants compared to term infants. The kind of precursor used is of no difference to the results.

The fractional synthesis rate (FSR) of surfactant after infusion of labeled glucose renders the percentage of surfactant that is synthesized from glucose per day. In premature infants who received a 24 hour ^{13}C-glucose infusion, the FSR was calculated to be ~3%/day (54). Preterm infants who received labeled palmitic acid and linoleic acid had a FSR of ~12%/day and ~25%/day, respectively (55). FSR in preterm and term infants after infusion of labeled acetate was ~2%/day and ~15%/day, respectively (56). When term infants received labeled palmitic acid, the FSR was found to be ~17%/day (57) (Table 1). These data show a lower FSR in preterm infants compared to term infants. When [U-^{13}C]palmitic acid was used as precursor, a higher FSR was found.

The slow secretion and alveolar accumulation of surfactant are balanced in the term and preterm lungs by slow catabolism and clearance. For example, the half-life of radiolabeled surfactant phospholipid given endotracheally to term lambs is about 6 days (58).

However, in premature baboons using the same method the half-life is about 30 hours (59), which may be due to more recycling in premature animals, and to species difference. Half-lives calculated in animal studies differ depending on gestational age, postnatal age, the labeled substrate used and the surfactant pool studied.

Half-life measurements in human infants have been performed mainly in preterm infants with RDS. Using different markers such as PG, ^{2}H-DPPC, [U-^{13}C]palmitic acid labeled DPPC which were given endotracheally, the half-life of PG, respectively surfactant PC was about 30 to 50 hours (60-62). The half-life of ^{13}C-labeled PC-palmitate measured in tracheal aspirates of preterm infants with RDS after labeled glucose infusion was ~113 hours (54, 63). In preterm infants the surfactant half-life after infusion of [U-^{13}C]palmitic acid and [U-^{13}C]linoleic acid was ~98 hours and ~47 hours, respectively (64). The half-life of preterm infants with RDS who received intravenous [1-^{13}C]acetate was ~106 hours, whereas term infants with normal lungs had a half-life of ~28 hours (Table 1) (56). Thus, surfactant half-live is different between preterm and term infants, with a slower disappearance of label in the preterm infants.

Table 1. *Surfactant kinetic parameters in infants studied with different precursors*

	Premature Infants				**Term Infants without lung disease**		**Term infants with severe lung disease**		
precursors	*^{13}C-GLU*	*^{13}C-PA*	*^{13}C-LA*	*^{13}C-AC*	*^{13}C-PA*	*^{13}C-AC*	*^{13}C-PA*	*^{13}C-LA*	*^{13}C-AC*
T_{app} (h)	19	20	16	13	9	7	9	10	11
T_{max} (h)	70	71	85	82	51	35	51	46	53
T½ (h)	113	98	47	106	43	28	64	59	68
FSR (%/d)	3	12	25	2	17	15	28	50	3

^{13}C-GLU, [U-^{13}C]glucose; ^{13}C-PA, [U-^{13}C]palmitic acid; ^{13}C-LA, [U-^{13}C]linoleid acid; ^{13}C-AC, [1-$^{13}C_1$]-acetate. All labels were administered via intravenous infusion during 24 hours.

In two reports, term critically ill patients were studied using different precursors for surfactant metabolism (56, 64). After infusing labeled fatty acids in a group of critically ill patients with various diagnoses, marked differences in PC kinetics among infants were noticed (64). Term infants with primary respiratory failure had a surfactant metabolism similar to preterm infants with RDS, suggesting either delayed maturity of the surfactant system or disruption from the underlying disease (56).

With stable isotopes, apparent differences in surfactant metabolism could result from differences in tracer metabolism, from true differences in surfactant synthesis and clearance, or from differences in the size of the unlabeled surfactant pool. That is, a small unlabeled pool would result in an apparent increase of FSR by an unchanged absolute synthesis of surfactant.

In summary, animal studies show a slower surfactant turnover in newborns compared to adults. In human studies using stable isotopes the surfactant PC metabolism is even slower in preterm infants with RDS compared to term infants without lung disease. Moreover, term infants with respiratory failure have abnormal surfactant metabolism that is similar to that of preterm infants with RDS.

Surfactant pool sizes

In most species studied, phosphatidylcholine, DPPC in particular, increases in the last trimester of the pregnancy (65-67). Lamellar bodies containing PC are first identified in human lung at 24 weeks of gestation and at 19 days of a 22-day gestation in the rat (68). In amniotic fluid, surfactant concentrations increase, reflecting accumulation of alveolar surfactant. This increase is also reflected in an increasing lecithin/sphingomyelin (L/S) ratio (69). During and shortly after birth large amounts of surfactant are released into the alveolar space (70, 71). Since there is no depletion of the intracellular surfactant pool, this increase in alveolar pool shortly after birth is accompagnied by an increase in the *de novo* surfactant synthesis (72, 73). However, incorporation studies as mentioned above, showed a slow surfactant metabolism, which means that it takes a long time before *de novo* synthesized surfactant is detectable in the alveolar space. Increased lung tissue and alveolar surfactant pools can only be explained by the rapid mobilization of surfactant from other pools (lamellar bodies, small vesicles) within type II cells.

The amount of lung tissue and alveolar surfactant changes with age. Surfactant pools are very high in the newborn period in all species studied to date, and decrease subsequently with lung maturity. In the rat, alveolar and lung tissue pools of both saturated PC and SP-A decrease with age (74, 75). Surfactant pool size/kg body weight decreases to adult value by 12 days of age (76). In humans total amounts of saturated PC in alveolar washes at autopsy also decreases with age (77, 78). Saturated PC in lung tissue was similar in all age groups (78).

Animal studies showed a 10-fold higher surfactant pool size in term neonates compared to adults (79). Newborns from all animal species have from 30 to 100 µmol/kg (23-76 mg/kg) surfactant in alveolar washes (80). In preterm infants with RDS, surfactant pool sizes in the alveolus are low (2-10 mg/kg) (52, 80), due to immaturity of the lungs, especially of the type II cells. In adults pool sizes are smaller because the lung structure, alveolar lining fluid, and surfactant film have adapted maximally to optimal function. The mean amount of total lung saturated PC/kg for the adult human was ~22 mg/kg, and ~2 mg/kg in alveolar wash at autopsy (78).

Hallman *et al*. showed an apparent pool size of ~15 mg/kg in human preterm neonates with RDS (61). They used PG as label to measure surfactant pool size. With the same

method Griese *et al*. calculated an apparent pool size of ~17 mg/kg sat PC in preterm neonates with RDS (81). Pool size measurements in preterm infants with RDS using endotracheal administered stable isotope (^{13}C-DPPC) in combination with a treatment dose of surfactant (100 mg/kg) showed an endogenous surfactant PC pool size (before treatment) of ~10 mg/kg (62).

In conclusion, surfactant pool size increases in late pregnancy, followed by a gradually decrease after birth to adult values. Among species, humans have the smallest alveolar pool sizes, whereas the amount of saturated PC in lung tissue is similar across species. The lower alveolar surfactant pool sizes make the human lung particularly vulnerable to surfactant dysfunction in case of lung injury.

Congenital diaphragmatic hernia

Although CDH lungs are immature (82) and show morphologically some resemblance to lungs of preterm infants with RDS, it is still unclear whether a primary surfactant deficiency is present in human CDH. Several animal models have been developed to study the pathogenesis of CDH (83).

The classical model of CDH consists of creating CDH by surgical intervention at the 80th day of gestation in lambs. By thoracotomy of the fetus the left diaphragm is opened and the stomach, omentum and small intestines are gently pulled into the chest. Then the gestation is allowed to progress to term. In bronchoalveolar lavage fluid of these surgically created CDH lambs the amount of phospholipids, PC, SP-A and SP-B is decreased compared to controls (84-87). However, the amniotic L/S ratio is not different in CDH lambs compared to control lambs (84). In vitro studies in isolated type II cells of CDH lamb show a decreased incorporation of choline into PC (85, 87), suggesting decreased surfactant synthesis in CDH (Table 2).

In rodents (rats and mice) CDH can be created using a toxicological approach by giving nitrofen (2,4-dichlorophenyl-*p*-nitrophenyl ether) to pregnant rats on days 9 or 10 of gestation (88, 89). In the nitrofen-induced CDH rat model, total phospholipids, sat PC, and SP-A in lung tissue are lower compared to control rats (Table 2) (90-94). By using antibodies towards sphingomyelin and disaturated PC, the lungs of the nitrofen-exposed rats demonstrated an impaired secretion of sat PC into the alveoli by accumulation of antibodies in the type II cells (93). In controls antibodies to sat PC were distributed on the surface of the alveoli, showing secretion of surfactant.

Human studies show contradictionary results about the presence of a surfactant deficiency in CDH. L/S ratios in amniotic fluid have been reported to be both normal and decreased (95, 96). SP-A and sat PC concentrations in amniotic fluid were lower in fetuses with CDH who died or required extracorporeal membrane oxygenation (ECMO) (97). Autopsy studies in CDH patients who died at birth or within the first days of life showed decreased SP-A in lung tissue (98, 99). However, we reported earlier that the L/S ratio, PC and, phosphatidylglycerol concentrations in bronchoalveolar lavage fluid from CDH infants were not different from age-matched control patients (100). In this study we found a PC concentration of ~3 mg/ml and ~6 mg/ml ELF in ventilated and ECMO treated CDH pa-

tients, respectively. Cogo *et al*. measured lower amounts of DSPC and SP-A in tracheal aspirates of CDH patients who did not required ECMO compared to control subjects (~2 *versus* ~5 mg DSPC/ml ELF) (57). After a 24 hour infusion of [U-^{13}C]palmitic acid, the label was incorporated into surfactant PC after ~8 hours, and reached a maximum enrichment after ~50 hours. Fractional synthesis was ~20% per day, and half-life of DSPC was ~59 hours in CDH patients. Moreover, no significant difference was observed in these kinetics parameters of surfactant between the CDH and control subjects (Table 2) (57).

The use of surfactant therapy in the CDH animal models is still contorversial. In the experimental lamb model prophylactic exogenous surfactant therapy caused an improvement in gas exchange, lung mechanics, and hemodynamics (101-103). In the nitrofen-induced rat model exogenous surfactant administration only had a transient effect on lung volume (104). However, surfactant treatment was not given prophylactically in these rats. There are only a few reports about surfactant treatment in human CDH patients (105-108). In all these reports the groups of infants studied were small. Already in the early nineties, a study performed by Bos *et al*. showed an improvement of oxygenation in three of the five infants with CDH after surfactant administration (106). In another study in which surfactant was given prophylactically to high-risk neonates with CDH, all three infants survived (105). Surfactant treatment in nine infants with CDH who required ECMO had no beneficial effect on the lungs, morbidity, and survival (108).

Other treatments that could influence the surfactant status in CDH are the administration of corticosteroids or thyroid hormones. Corticosteroids give an increase in synthesis of sat PC and surfactant proteins in the lung. This is predominately induced by stimulation of various key enzymes involved in the synthesis of surfactant (109). The administration of antenatal glucocorticoid treatment is nowadays an established therapy for all fetuses between 24 and 34 weeks of gestation at risk for preterm delivery (110). The effect of prenatal hormonal therapy in CDH patients is not clear yet. Animal studies showed an increase of DSPC, surfactant proteins, oxygenation and survival after antenatal corticosteroids (111-115). Combined use of antenatal corticosteroids with antenatal thyrotropin-releasing hormone (TRH) acts synergistically in lung maturation (116, 117). However, clinical trials showed no beneficial effects or even adverse maternal and neonatal effects when TRH was added to antenatal corticosteroid treatment in premature labor (118, 119). Only one report is available about the use of antenatal corticosteroids in human CDH (120). This study describes three high-risk CDH patients who survived, and were extubated within 10 days after up to 7 or 8 doses of antenatal corticosteroids (120). But with the increasing evidence of adverse perinatal and long-term effects of prenatal corticosteroids (121), this treatment option should be used with caution.

Tracheal occlusion is another antenatal therapeutic strategy which enhances pulmonary arterial and alveolar developmental growth (122-124). It has been hypothesized that the elevated intrapulmonary distending force from the increased volume of fetal lung fluid accounts for the increase in lung mass. However, some animal studies have demonstrated a profound decrease in type II cells and a deficiency of surfactant due to the tracheal occlusion (125-128). Especially timing and duration of tracheal occlusion are key factors determining outcome.

Until today, the presence of a primary surfactant deficiency in CDH is still not clear. We suggest an inactivation of surfactant function due to the intensive ventilation with negative side effects (129), which is often necessary in CDH patients. Although, a decreased surfactant synthesis due to severe hypoplasia of the lungs (especially in the more severe cases of CDH) cannot be excluded.

Table 2. *Review of literature about surfactant parameters in animal and human neonates with severe lung disease*

	(DS)PC in BAL or TAS	(DS)PC in lung tissue	surfactant proteins	L/S ratio	precursor incorporation	references
Congenital diaphragmatic hernia						
lambs	↓		↓ SP-A/B BAL	=	↓	*83-86*
rats		↓	↓ SP-A tissue	=		*89-93*
human			↓ SP-A tissue, TAS, amnion fluid	↓/=	=	*57, 94-99*
Meconium aspiration syndrome						
rats	=	=	↓ SP-A/B BAL	=		*141*
piglets	=					*142*
human	=		= SP-A BAL			*143*
Pneumonia						
rats					↑	*227*
human	↓		↓ SP-A = SP-B BAL			*209, 211*
SP-B deficiency						
mice		=	no SP-B pro SP-C		=	*189,190*
human	↑		no SP-B pro SP-C		↑/=	*194*

Meconium aspiration syndrome

Aspiration of meconium directly inhibits surfactant function and induces an inflammatory response in the lung with possible detrimental effects to the type II cell function and thereby surfactant metabolism. Surfactant function is inhibited by meconium in a concentration dependent way (130-132). Meconium increases the minimum and maximum surface tensions, and lowers the surface spreading rate of surfactant (130, 131, 133, 134). Separation of the lipid fraction from the water fraction of meconium by organic extraction exhibited a stronger inhibitory activity of meconium in the chloroform fraction (lipid fraction) which contains free fatty acids, triglycerides, and cholesterol, compared to the water-methanol fraction which contains proteins and bilirubin (130). Besides an effect on the

physical surface properties, meconium also changes the morphology of surfactant (133, 134).

Several mechanism are involved in the inactivation by meconium. One of these mechanisms is the infiltration of phospholipids other than DPPC, or other lipids (e.g. free fatty acids) in the surface film (135). This forms an unstable surface monolayer that easily collapses at high surface tension. Secondly, the formation of aggregates between surfactant and meconium constituents (usually proteins) may delay the formation of the surface film by competitive inhibition of phospholipid entry into the surface monolayer (136, 137). A third mechanism is degradation of surfactant lipids and proteins by enzymes (lipases, proteases) (138-140).

Studies about the surfactant concentration and composition in MAS are scarce. Cleary *et al*. found decreased SP-A and SP-B levels in the large aggregates of surfactant in a rat model of MAS (141). However, phospholipid and DPPC levels did not change significantly after meconium installation in either the lung tissue or BAL. In a newborn piglet model with MAS also no differences in phospholipids levels in BAL were found compared to controls (142). Analyses of BAL fluid from 8 infants with MAS receiving mechanical ventilation revealed no difference in phospholipid and SP-A content compared to control subjects (143). However, concentrations of non-surfactant protein and albumin were >3 times those found in normal lung. In MAS patients who required ECMO surfactant phospholipids, PC, and SP-A in tracheal aspirates increased during the ECMO treatment (144-146). To our knowledge, only one study investigated the surfactant kinetics in the presence of meconium (147). This *in vitro* study in type II cells of adult rats showed that meconium in low concentrations (1%) increases the PC secretion by type II cells, but had no effect on surfactant PC synthesis. Higher meconium concentrations were toxic to the cultured type II cells, though the effect of these higher concentrations on surfactant synthesis is not known (Table 2).

Exogenous administration of surfactant in animal models of MAS improves lung function and morphology, especially when surfactant is given at a high dose (200 mg/kg) (148-151). The clinical efficacy of surfactant therapy in MAS has been suggested by several nonrandomized studies (6, 8, 152, 153). These studies showed an improvement in oxygenation, although most infants required two or more doses of exogenous surfactant. Two randomized controlled trials have been reported (154, 155). Findlay *et al*. administered three doses of bovine surfactant in a higher than standard dose (150 mg/kg per dose) to 20 infants with MAS. Surfactant treated infants had improved oxygenation, a reduction in severity of pulmonary morbidity, a decrease in ECMO requirement, and a decrease in hospitalization time compared to the control group (n=20) (154). In the latter trial surfactant was not given as a bolus, but was continuously infused over a period of 20 minutes, started within 6 hours after birth. In a larger trial performed by Lotze *et al*. term infants with severe respiratory distress received four doses of bovine surfactant (100 mg/kg per dose) at ~30 hours after birth. Half of these patients had MAS as the cause of their respiratory failure. These authors were unable to demonstrate a difference in oxygenation, pulmonary morbidity, or hospitalization, although the need for ECMO in the surfactant treated group was decreased (155). From these studies it can be suggested that surfactant

therapy in MAS is most effective when given in the early phase of the respiratory failure, and at a high dose.

Moreover, the type of surfactant may play a role in the degree of benefit (156). Natural lavage surfactant containing SP-A is most resistant to meconium. Followed by recombinant surfactant protein-C and leucine/lysine polypeptide surfactant (KL_4-surfactant), and the modified natural surfactants (Curosurf, Alveofact, Survanta). These data suggest that the surfactant used for treatment of MAS requires another composition than the surfactant used for treatment of RDS.

The optimal method of administration of surfactant in MAS is still a point of discussion. Paranka *et al*. first described a surfactant lavage of MAS in piglets (157). A lavage with surfactant could remove meconium, inflammatory cells, edema fluid, proteins, and other debris from the lungs, leaving behind a layer of functional exogenous surfactant. Similar studies were performed in an adult rabbit model and in newborn rhesus monkeys (158-160). All these animal studies showed a beneficial effect from surfactant lavage on pulmonary function, radiographic and histological appearance. There are a few reports about lavage therapy in human neonates with MAS (161-164). Two studies performed first a lavage with saline followed by standard surfactant administration, to overcome the airway obstruction and prevent non-homogenous distribution of surfactant (161, 162). Improvement in saturation and oxygen index were noticed, however, the patients groups were small and no control group was included. Lam *et al*. treated 6 infants with MAS with a surfactant lavage (15 ml/kg surfactant solution with a concentration of 5 mg phospholipids/ml) (163). They found a more rapid improvement in oxygenation and a shorter duration of ventilation in the lavaged group compared to historic controls. Recently, a multicenter, randomized controlled trial comparing surfactant lavage with standard treatment of MAS has been reported (164). In lavaged infants, diluted Surfaxin (KL_4-surfactant, 2.5 mg/ml in a volume of 8 ml/kg) was instilled twice, followed by third, more concentrated (10 mg/ml) surfactant lavage. A shorter duration of ventilation and a persisting improvement in oxygenation were noted, but the differences were not significant.

Combinations of surfactant therapy with other approaches to treatment in MAS have been investigated (165, 166). These studies suggest that surfactant treatment in combination with high frequency ventilation or partial liquid ventilation had better effects on oxygenation and lung function than conventional ventilation alone. However, the relative efficacy of these combination therapies remains to be tested.

A variety of substances are found to play a preventive role in the inactivation of surfactant. (136, 167-169). Polymers (such as polyethylene glycol and dextran) added to surfactant and instilled into the trachea of rats with MAS improves gas exchange, pulmonary mechanics, and histological appearance of the lungs (170, 171). The effect of polymers is not limited to a single inhibitor, or even a single mode of inhibition. This makes it possible to use polymers in the treatment of all kinds of lung injury where inactivation of surfactant plays a role (172). However, also contradictionary results have been reported (173). Polyethylene glycol added to surfactant attenuated surfactant responses in adult rabbits with lung injury. The exact mechanism of overcoming the surfactant inhibition with polymers has yet to be elucidated. Also the safety and efficacy of the use of polymers in hu-

mans with lung disease have to be considered. Almaas *et al*. found that by the addition of ferric chloride, copper chloride, or acetic acid the inhibitory effect of meconium on surfactant could be abolished (136). They suggested that the contra-inhibition was caused by lowering the pH, and that meconium inhibition of surfactant is pH-dependent.

In conclusion, surfactant inactivation seems to play a more important role in the pathophysiology of MAS than does a surfactant deficiency. However, surfactant metabolism in MAS has not been studied yet. Surfactant therapy seem to be effective in MAS, and should probably be given at a high dose and at an early stage in the development of the disease. The usefulness of surfactant lavages, polymers and combination with other treatment options in MAS have to be further investigated.

Congenital SP-B deficiency

Congenital SP-B deficiency is a lethal inherited disease of the term neonate. It was first recognized in 1993 by Nogee *et al*. (24), and it is characterized by the absence of SP-B, an aberrant form of SP-C (174), altered phospholipid composition, and disorganized lamellar bodies and tubular myelin (175). Clinically, the neonate with SP-B deficiency presents with severe progressive respiratory distress which does not respond to surfactant therapy, corticosteroid administration, and ECMO (176, 177). The infants will die in the first few weeks of life, unless they undergo a lung transplantation (178).

SP-B is a hydrophobic peptide with several functions related to surface tension reduction and metabolism of pulmonary surfactant (21, 23). It enhances the rate of absorption and surface spreading of the phospholipids, it maintains the surface active layer of surfactant, and plays a role in the organization and turnover of pulmonary phospholipids. Furthermore, SP-B counteracts the inhibition of surfactant function by plasma proteins, and contributes to the recycling of surfactant from the alveolar space (22). In humans the SP-B is encoded by a 9.5 kb gene that contains 11 exons on chromosome 2, and is expressed exclusively in the epithelium of the lung (23). Mature SP-B is detectable in the human lung after 19 weeks of gestation, and is found in the amniotic fluid after 31 to 33 weeks of gestation (179).

The most common mutation described to date is a net 2 base-pair insertion at codon 121 in exon 4 of the SP-B gene (121ins2), causing a frameshift and premature stop signal for the termination of the translation (180). Affected patients are homozygous for this mutation, their parents heterozygous carriers. But also other mutations have been identified resulting in total (181-185), partial (186, 187), or transient SP-B deficiency (188).

Clark *et al*. described the creation of homozygous SP-B knockout mice (189). These mice die within minutes after birth, have poorly compliant lungs, and express the aberrant SP-C. Ultrastructural examination of the lungs revealed an absence of lamellar bodies and tubular myelin. However, phospholipid content in lungs from SP-B knockout mice was not altered compared to normal mice. Neither was the incorporation of labeled choline and labeled palmitic acid in lung saturated PC of SP-B deficient fetuses after injection of the labels in pregnant mice (190). Mice heterozygous for the disrupted gene express 50% of normal SP-B. These heterozygous mice show evidence of air trapping, abnormalities of

lung compliance, and are susceptible to hyperoxic lung injury (191, 192). No changes in the amount of lung PC and incorporation of choline and palmitate into saturated PC were found (190). Lung function studies in human subjects heterozygous for SP-B deficiency revealed no abnormalities (193).

Beers *et al*. investigated the phospholipid content, composition and synthesis in lung tissue and lavage fluid acquired at transplantation or postmortem from SP-B deficient infants (194). They found elevated phosphatidylinositol and percent DSPC in lung tissue, and decreased levels of PG in both lavage and lung tissue compared to normal lungs. The incorporation rate of choline into PC, and acetate into phospholipids was increased in SP-B deficient tissue compared to lung tissue of infants with chronic lung disease (194). The incorporation of glycerol into PC was not different between groups. Infants with chronic lung disease who underwent lung transplantation were used as comparison, as they had undergone the same therapeutic interventions as the SP-B deficient patients.

In summary, although the tremendous intracellular and functional changes in SP-B deficient type II cells, surfactant PC synthesis and clearance seems not disturbed. The exact mechanisms which lead to these changes of pulmonary type II cells still have to be elucidated. By studying the surfactant metabolism in SP-B deficiency a better insight of the general surfactant metabolism in normal and other disease states can be obtained. Functions of certain surfactant structures can become more clear, and a possible way of treatment could be revealed.

Neonatal pneumonia

Pneumonia is an important source of morbidity and mortality in newborn infants. Group B streptococci (GBS) and Escheria Coli (E. Coli) are the most common pathogens causing neonatal pneumonia (195). There is evidence of an impaired surfactant system due to viral and bacterial pneumonia. In viral pneumonia surfactant function is reduced due to injury to the type II cells of the lungs (196, 197). The interaction of bacteria or endotoxin with surfactant results in functional changes of surfactant, which results in an abnormal concentration and composition of surfactant due to injury of type II cells (198). Additionally, surfactant function is further inactivated by plasma proteins due to the increased alveolar permeability.

SP-A and SP-D are believed to be molecules of the innate immune system through their ability to recognize a broad spectrum of pathogens (10, 199). These surfactant proteins interact with a number of viruses, bacteria and fungi (200, 201). By binding to components of bacteria and viral surfaces the uptake of pathogens by phagocytes of the innate immune system (alveolar macrophages, neutrophils) is enhanced. Furthermore, SP-A and SP-D also regulate the production of cytokines and free radicals by innate immune cells (202, 203). SP-A and SP-D deficient mice show increased lung inflammation during bacterial infection (204). Recently, a study in transgenic mice also suggested a role of SP-B in protecting against endotoxin-induced lung inflammation by enhancing surfactant function (205).

Lavage fluid of adult patients with bacterial pneumonia showed a decrease in concentration of PC, PG, and SP-A, while PI and sphingomyelin were increased, and SP-B unaltered (206-208). The same results were found in tracheal aspirates of children with bacterial pneumonia or viral pneumonitis (209). The fatty acid composition of surfactant phospholipids had also changed in adult patients with pneumonia; palmitic acid being significantly reduced (210). During nosocomial infection in ventilated preterm neonates a decrease in total phospholipids, especially the amount of PC in the "small aggregate" fraction, was reported (211). There were no other changes in phospholipid composition. The surface activity of the surfactant recovered in the "large aggregate" fraction was reduced during the peak of the infection, and returned towards normal levels afterwards (212). In tracheal aspirates of 21 preterm neonates with pneumonia, minimal surface tension was increased, whereas L/S ratio was not decreased compared to healthy term infants (213). The authors suggested that surfactant inhibition has a more important role in causing the respiratory failure in congenital pneumonia than a surfactant deficiency. Unfortunately, surfactant composition and concentration were not measured. There are no data about the surfactant content and composition in term neonates with pneumonia.

Exogenous surfactant therapy in experimental neonatal GBS pneumonia in near-term rabbits resulted in a decreased bacterial proliferation, and less prominent inflammatory changes of the lungs (214). The same results were found in immature ventilated rabbits with experimentally induced GBS pneumonia (215). By adding a specific immunoglobulin against GBS in the same experimental model bacterial proliferation was even more effectively reduced than treatment with surfactant alone (216). Surfactant treatment in experimental E. Coli pneumonia in adult rats showed an improvement in oxygenation (217). However, the animals were only studied for 30 minutes, and total phospholipids and PC in lavage fluid were not different between the infectious group and the control group. Moreover, exogenous surfactant in E. Coli endotoxin-treated neonatal piglets had a detrimental effect on oxygenation and pulmonary function (218). Lung lavages with diluted surfactant had better effects (219).

Herting *et al*. reviewed the clinical course and outcome of 118 mostly preterm infants who were treated with surfactant due to respiratory failure, but were also found to be infected with GBS (220). Surfactant treatment in these neonates improved gas exchange. However, the response to surfactant in neonates with GBS was slower than in infants with RDS alone, and repeated surfactant doses were often needed. A study in seven full term neonates with pneumonia, who were treated with surfactant showed an improvement in oxygenation (8). In four newborns with respiratory insufficiency due to bacterial sepsis, surfactant treatment showed decreases in mean airway pressures and oxygen demands (221). In a randomized double-blind placebo-controlled trial, the use of surfactant significantly decreased the need for ECMO (155). However, only about 30% of the patients in both groups had a respiratory failure due to sepsis, other diagnoses were MAS and persistent pulmonary hypertension.

Although the above mentioned studies suggest some effect of surfactant therapy in pneumonia, others suggested, however, that exogenous surfactant may impair host defense (222-225). Furthermore, there are indications that exogenous surfactant compo-

nents may serve as nutrients for bacteria (226). Bacterial growth in different surfactant preparations is influenced by the microbial species and the composition and the dose of surfactant.

Incubation of isolated type II cells with lipopolysaccharide (LPS) increased the amount of choline incorporation into PC in vitro (227). LPS exposure in rats has been shown to decrease phospholipid levels in lung lavage as early as 2 hours after exposure in ex vivo experiments (228). At 72 hours, rats exposed to LPS showed an increased intracellular surfactant phospholipid pool as well as a decreased extracellular surfactant phospholipid pool (229). Neutrophils and activated macrophages degrade surfactant lipids in vitro and contribute to the alterations in lipid pool size (230). Clearance of large amounts of phospholipids could lead to decreased phopholipid levels, which may result in smaller alveolar pools of functional surfactant. SP-A seems to play a role in the regulation of extracellular surfactant phospholipid pools after LPS induced lung injury in vivo (231). Absence of SP-A resulted in decreased clearance of surfactant from the alveolar space in vivo. In the same study no change was found in surfactant synthesis and secretion in normal mice exposed to LPS as well as in SP-A knock-out mice exposed to LPS.

In conclusion, pneumonia seems to effect surfactant function by decreasing surfactant concentration. However, most studies have been performed in animals or adult human patients. Little is known about the effect of pneumonia on surfactant metabolism in human infants.

Conclusions

Surfactant metabolism has been studied thoroughly in preterm animals and neonates with RDS. In term infants with severe lung disease, such as CDH, MAS, SP-B deficiency and pneumonia, the role of a disturbed surfactant metabolism in the pathophysiology is not clear yet. There are a few studies about surfactant concentration and composition in human infants with severe lung disease. Also the effect of surfactant therapy has been evaluated in several groups of infants. However, the results are controversial and the studied groups are often small. Surfactant synthesis and catabolism have hardly been studied in these groups of human patients. An understanding of the surfactant metabolism in several lung diseases provides not only information about the pathophysiology of the concerning disease, but contributes also to a better understanding of the surfactant metabolism in general.

CHAPTER 2

Introduction to the Thesis & Aims of the Study

Introduction to the thesis

In the development of RDS in the preterm neonate a primary surfactant deficiency plays an important role, and exogenous surfactant has become a standard treatment of RDS. Since the introduction of surfactant therapy for the treatment of neonatal RDS, mortality and morbidity due to RDS have dramatically decreased. However, 20 years later (2) still discussion exists about the dosage, the timing, and the composition of exogenous surfactant. Bunt *et al*. have developed a novel method to study surfactant metabolism in preterm infants. With the use of this method the influence of prenatal corticosteroids and surfactant therapy on the endogenous surfactant synthesis in preterm infants was studied (232).

Another factor that could influence surfactant metabolism is the method of ventilation. Repetitive alveolar distension during conventional mechanical ventilation, stimulates surfactant synthesis and secretion. During high frequency oscillation (HFO), this alveolar excursion might be limited, which could decrease surfactant production. Studies evaluating the effect of HFO on surfactant metabolism have yielded conflicting results.

Despite the progress in neonatal care and the introduction of surfactant therapy, many very preterm infants develop bronchopulmonary dysplasia (BPD), characterized by lung injury, inflammation, and an arrest in alveolar development. It is presently unclear in what way the surfactant system is involved in the 'natural history' of BPD. Studies of surfactant metabolism have been performed during the first days of life. However, almost no information about surfactant metabolism during the development of BPD is available.

There are (controversial) indications of a disturbed surfactant metabolism in several neonatal lung diseases, such as CDH, MAS, sepsis/pneumonia, and inherited SP-B deficiency. Surfactant therapy seems to be effective in some cases, but the effect is often of short duration and/or unpredictable. Occasionally, surfactant metabolism has been studied in animal models of the lung diseases in question. In human infants surfactant analyses of tracheal aspirates or broncheoalveolar lavages have been performed, and have provided data on the quality and the concentration of surfactant. However, these data give no information on the synthesis and catabolism of endogenous surfactant.

Aim of the studies

1. To determine if high frequency oscillation (HFO) decreases surfactant production in preterm infants with respiratory distress syndrome (chapter 3).
2. To study the surfactant metabolism and surfactant pool size during the early development of bronchopulmonary dysplasia (chapter 4)
3. To study the apparent surfactant pool size in neonates with severe lung disease (chapter 5).
4. To study the endogenous surfactant metabolism in neonates with congenital diaphragmatic hernia, neonates with meconium aspiration syndrome, and neonates with inherited surfactant protein-B deficiency (chapter 6,7 & 8).

Methods

Stable isotopes

The surfactant metabolism in this thesis was studied with the use of stable isotopes. Stable isotopes are non-radioactive and can therefore be safely used in neonates. In the studies presented in this thesis we used two stable isotopes: ^{2}H (deuterium) and ^{13}C. The stable isotope of the carbon atom (^{13}C) has a natural occurrence (enrichment) (1.11%). Baseline samples are taken prior to the isotope administration to account for the natural enrichment. In the samples, the ratio of $^{13}C/^{12}C$ is measured directly, so sampling techniques and sample amount are of little influence.

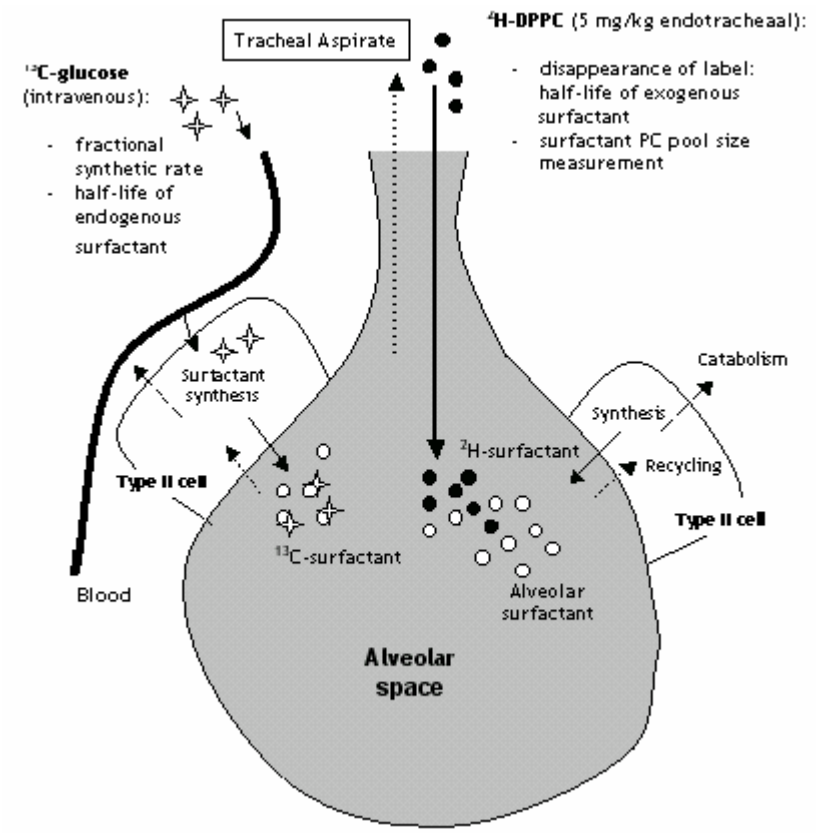


Figure 1. *Methods of stable isotope administration*

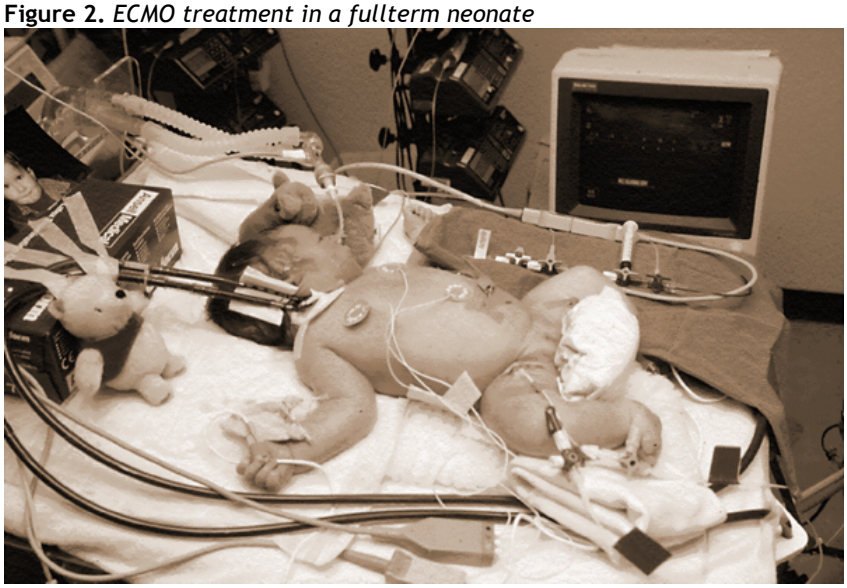
In this thesis two different methods of tracer administration were used. Dipalmitoylphosphatidylcholine (DPPC), which is the most surface active compound of surfactant was labeled with ^{2}H (^{2}H-DPPC). After endotracheal administration of the tracer, the ^{2}H-DPPC mixes with the endogenous alveolar surfactant, will be taken up by the type II cells and mix with the intracellular surfactant, and will gradually disappear from the lung (Figure 1). From the disappearance of the label we can calculate the half-life of the exogenous tracer and the surfactant PC pool size.

The other tracer used in this thesis was ^{13}C-glucose, which was administered by intravenous infusion during 24 hours. The stable isotopes are incorporated into the fatty acids in surfactant PC by endogenous synthesis of PC, which will be secreted into the alveolar space (Figure 1). The rate of incorporation is a measure for synthesis of surfactant PC. The disappearance of the stable isotope from surfactant PC is a reflection of the turnover (i.e. synthesis and clearance) of endogenous surfactant PC.

Extracorporeal membrane oxygenation (ECMO)

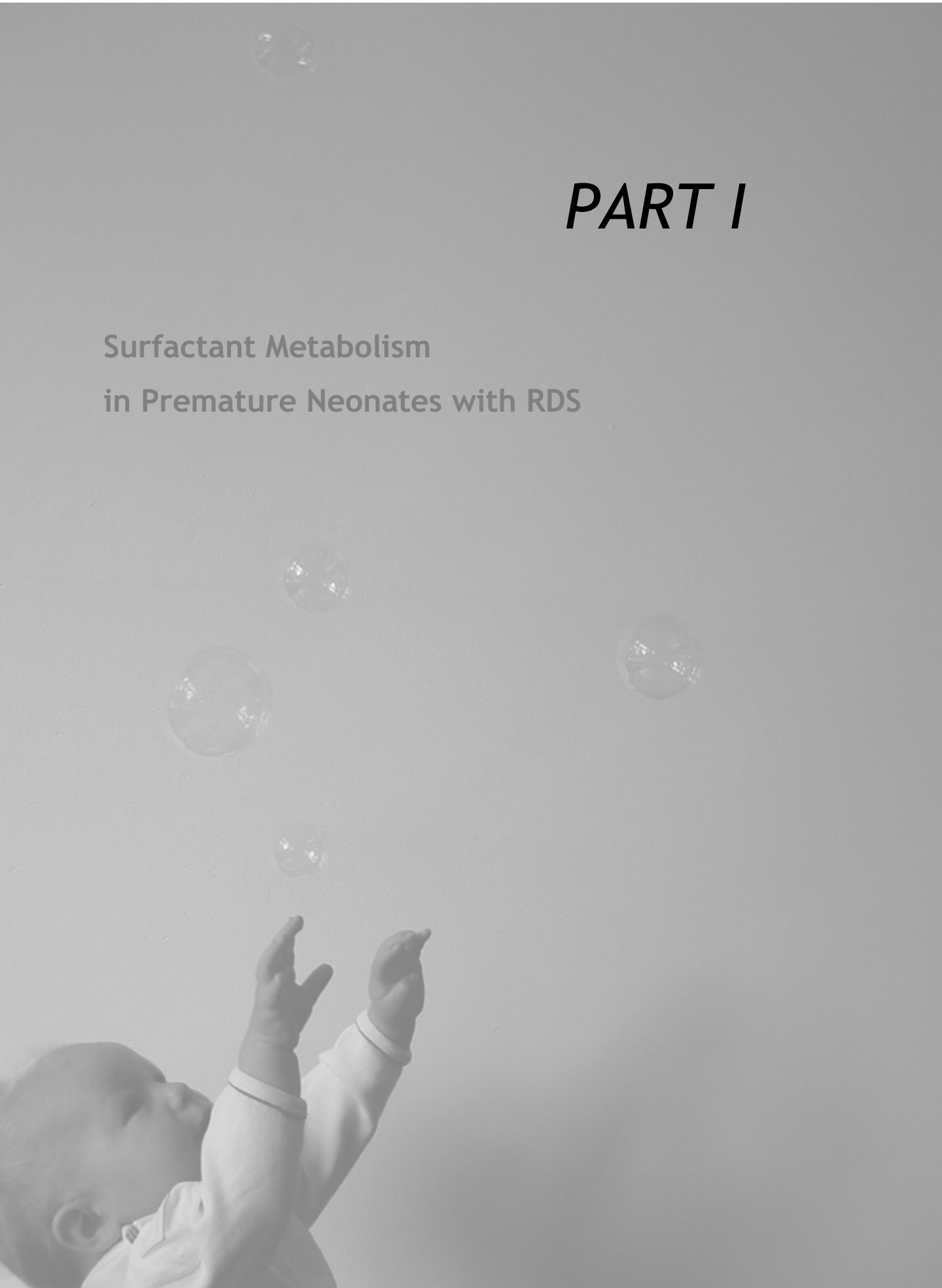
ECMO is a temporary support of heart and lung function using mechanical devices. The support is achieved by draining venous blood, removing carbon dioxide and adding oxygen through an artificial lung, and returning the blood to the circulation via a vein (venovenous) or artery (venoarterial) (Figure 2). The patients of the studies in this thesis were treated with venous-arterial ECMO. The right internal jugular vein and common carotic artery were cannulated.

Figure 2. *ECMO treatment in a fullterm neonate*



PART I

Surfactant Metabolism in Premature Neonates with RDS



CHAPTER 3

Endogenous Pulmonary Surfactant Metabolism is Not Affected by Mode of Ventilation in Premature Infants with Respiratory Distress Syndrome

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J Pediatr 2002; 140(6): 693-98

Abstract

In the current study we determined if high frequency oscillatory ventilation (HFOV) decreases surfactant production in preterm infants with respiratory distress syndrome (RDS). We randomized 19 infants < 28 weeks of gestational to either HFOV (n=8) or conventional ventilation (CV, n=11) at 24 hours of life. After a 24-hour continuous infusion of uniformly labeled carbon 13 glucose([U-$^{13}C_6$] glucose), we measured ^{13}C enrichment in surfactant phosphatidylcholine (PC) in tracheal aspirate samples using gas chromatography/mass spectrometry. We calculated fractional synthetic rate (FSR) of surfactant PC from labeled glucose and its half-life of clearance (T½). FSR did not differ between groups (4.7±2.7%/day CV versus 4.2±3.1%/day HFOV, *p*=0.7) Half-life was 79±18 hours in the CV group and 76±23 hours in the HFOV group (*p*=0.7). Neither degree of ventilatory support nor supplemental oxygen exposure correlated with surfactant metabolic indices. Three of 4 infants who died from RDS within the first month of life had a shorter half-life than 14 of 15 infants who survived. In conclusion, surfactant metabolism is similar in preterm infants ventilated with HFOV and CV. Shortened surfactant half-life may characterize a subset of preterm infants with lethal RDS.

Introduction

Pulmonary surfactant, a mixture of phospholipids and proteins synthesized by alveolar Type II cells, maintains alveolar expansion at end expiration. The phospholipid and protein components of the pulmonary surfactant are synthesized, assembled, secreted, degraded, and recycled in a complex metabolic cycle (17). The most abundant phospholipid in the pulmonary surfactant, phosphatidylcholine (PC) consists of a glycerol backbone to which choline and two fatty acids are attached. The principle surface-active phospholipid is dipalmitoylphosphatidylcholine (DPPC), which contains two palmitic acid chains. The palmitate in DPPC is derived either from circulating free palmitate or from de novo synthesis from precursors such as glucose and acetate. Of the many factors that influence pulmonary surfactant metabolism, physical stretch stimulates surfactant synthesis and secretion (233-235).

Previously, we noted that infants genetically deficient in surfactant protein-B exhibited better gas exchange and had less histologic evidence of surfactant accumulation in the airspaces when they were ventilated with high-frequency oscillatory ventilation (HFOV) (236). Because repetitive alveolar distension, such as occurs with conventional mechanical ventilation, stimulates surfactant synthesis and secretion, we speculated that HFOV, during which this alveolar excursion will be limited, decreased surfactant production in these surfactant protein-B-deficient infants. Studies evaluating the effect of HFOV on surfactant metabolism, however, have yielded conflicting results. Some have suggested no effect of HFOV on surfactant metabolism. For example, with intraperitoneal administation of radiolabeled palmitate, surfactant turnover was similar in adult rats with normal lungs ventilated with HFOV or conventional ventilation (CV) for 90 minutes (237). In other studies, no differences in surfactant phospholipid quantity or composition were found in nonhuman primates ventilated for 7 hours and 16 hours with CV or HFOV (238, 239). In contrast, studies using radiolabeled exogenous surfactant in preterm lambs and intravenous radiolabeled palmitate in adult rabbits suggested decreased surfactant secretion in response to HFOV (240, 241). Studies performed in preterm humans have also suggested that HFOV decreases surfactant production. For example, phospholipid and PC concentration in tracheal aspirates increased as infants were changed from receiving HFOV to CV (242). In addition, infants receiving HFOV had lower concentrations of surfactant protein-A in tracheal aspirates than infants receiving CV (243).

The studies in infants have been limited by techniques suitable for answering these questions. The composition of surfactant in tracheal aspirates reflects the steady state and cannot provide insights into the dynamic aspects of surfactant metabolism. Methods using stable isotope-labeled glucose of fatty acids have permitted evaluation of pulmonary surfactant phospholipid metabolism in humans in vivo (54, 55, 244). Therefore, to determine if HFOV decreased pulmonary surfactant production, we used stable isotope-labeled glucose to compare endogenous surfactant metabolism in premature infants with respiratory distress syndrome (RDS) who were ventilated with HFOV or CV.

Methods

Subjects

Infants <28 weeks gestational age admitted to the St Louis Children's Hospital neonatal intensive care unit (NICU) were eligible if they required mechanical ventilation from birth, had a chest radiograph consistent with RDS, and had indwelling arterial access as part of their routine clinical care. Exclusion criteria included known congenital infection, congenital anomalies, maternal diabetes mellitus, or imminent death. During the first 24 hours of life, the NICU team determined clinical management, including exogenous surfactant replacement. Exogenous surfactant replacement (Survanta, Abbott Laboratories, Chicago, IL) was given at a dose of 4 mL/kg/dose every 12 hours if fractional concentration of oxygen in inspired gas (FiO_2) was >0.3 to keep peripheral oxygen saturation >92%. Within the first 24 hours, after assent from the attending physician, the parents were approached for consent. We explained to the parents that available data did not show either HFOV or CV to be superior to one another, and that their infant would be randomized to one of these forms of ventilation. If the bedside team determined that the assigned mode of ventilation was not appropriate, the study would be discontinued. Once consent was obtained, the infant was randomly assigned to receive either CV (VIP Bird, Bird Products Co., Palm Springs, CA) or HFOV (Sensormedics Model 3100A, Sensormedics Critical Care Co, Yorba Linda, CA) starting at 24 hours of age. Infants who were already receiving HFOV as part of their routine care continued to receive it. Once an infant was randomly placed to treatment, the medical team determined medical care, including ventilatory and nutritional management. The Washington University Human Studies Committee approved the study.

Isotope infusion and sample collection

Once the infant was placed on the selected mode of ventilation, baseline tracheal aspirate and blood samples were collected. A 24-hour continuous intravenous infusion of [U-$^{13}C_6$] glucose (20 mg/mL, Campro Scientific, Veenendaal, The Netherlands and Cambridge Isotope Laboratories, Inc. Andover, Massachusetts, USA) dissolved in 5% or 10% dextrose and water, was started at 0.5 mL/kg/h to deliver approximately 0.05 mmol/kg/h of labeled glucose. The start of the study was defined by the start of the infusion of the labeled glucose. Blood samples were obtained every 6 hours during the infusion period. Samples were collected in an EDTA tube, immediately placed on ice, and directly centrifuged at 2500 rpm for 10 minutes. The plasma was stored at -70 C° until it was analyzed. Tracheal aspirates were collected in a standardized fashion every 12 hours as per current NICU suctioning protocol. Samples were collected for the length of time the infant received the assigned mode of ventilation of 14 days, whichever was shorter. The tracheal aspirates were frozen at –70 °C. Aspirates containing visible blood were not included.

Sample preparation and analysis

Details of the sample preparation and analysis were previously described (54, 55, 244). Briefly, plasma was delipidated and glucose was isolated and derivatized to an aldonitril pentacetate derivative (245). Lipids were extracted from tracheal aspirates according to

Bligh and Dyer (246) and surfactant PC was isolated using TLC. PC was derivatized and fatty acid methyl esters were extracted with hexane and stored at –20 °C. Enrichment of ^{13}C in palmitic acid, the most common fatty acid in surfactant PC, in tracheal aspirates and enrichment of ^{13}C-glucose in plasma were measured by gas chromatography-combustion interface-isotope ratio mass spectrometry (GC-CI-IRMS) (VG Isotech, Midlewich, Cheshire, UK) as previously described (54, 55, 244). The enrichment was expressed as atom percent excess (APE), which represents the increase in the percentage of ^{13}C atoms in total carbon dioxide from the combusted compounds above baseline enrichment (before infusion). Enrichments were corrected for the contribution of unlabeled carbon atoms added during derivatization.

Data collection and analysis

Clinical data, including ventilatory support, blood gas analyses, surgical and pharmacological interventions, and complications, were collected for the duration of the study. An integrated respiratory score was calculated for each infant as previously described (247). This score was determined by multiplying the average daily FiO_2 by the average daily mean airway pressure (MAP) and integrating the area under the curve for the total number of days in the study using Sigma Plot 2000 software (SPSS, Inc, Chicago, IL). We normalized the respiratory score for the study duration (range, 10-14 days) by dividing by the number of days each participant was in the study. Tissue-bound and alveolar surfactant were regarded as one pool. Time of appearance (T_{app}) is the time delay between the start of the infusion and the first appearance of ^{13}C enrichment in surfactant PC (reported in hours). Time of maximum enrichment (T_{max}) is the time that ^{13}C reaches its maximum enrichment (E_{max}). The half-life (T½, hours) of clearance of labeled endogenous PC was calculated from the monoexponential fitting of the downslope of the enrichment *versus* time curve. Fractional synthetic rate (FSR), which represents the percentage of the endogenous surfactant PC pool synthesized de novo from plasma glucose per day (%/day), was calculated by dividing the slope of the linear increase of the ^{13}C enrichment curve by the steady state enrichment of plasma glucose (54, 55, 244).

The study was designed to have sufficient power (ß=0.8) to detect a 30% difference in FSR at a significance level of 0.05. Normally distributed data are presented as means ± SD and non-normally distributed data are presented as median and range. Two-tailed Students' *t* tests were used for normally distributed data, Wilcoxon rank-sum tests were used for non-normally distributed data, and Fisher's exact tests were used for categorical data. All statistical analyses were performed using the SAS software, version 8.1 (SAS institute, Cary, NC).

Table 1. *Patient characteristics*

	CV n=11	HFOV n=8	*p*-value
Birth weight (g)	779±125	726±175	0.4
Gestational age (wk)	26 (24-28)	26 (24-27	1.0
Sex (M/F)	8/3	5/3	1.0
Race (white/black)	3/8	4/4	0.4
Apgar score at 5 minutes	7±2	6±2	0.4
Antenatal corticosteroids (n)	9	4	0.3
Doses of exogenous surfactant	2 (1-3)	2 (2)	1.0
FiO_2*	0.32±0.13	0.54±0.18	0.008
MAP*	6.9±1.3	9±1.5	0.005
OI*	3 (1.7-6.4)	5 (3.6-10.1)	0.01
Average daily respiratory severity score#	3.4±1.5	5.5±2.1	0.02

**At study start.*

#Total restiratory score (integrated MAP x FiO2) divided by the number of days in the study.

Results

Of the 33 families approached, 20 infants ultimately participated, 10 in each group. One infant randomized to HFOV did not tolerate it and was immediately changed back to CV. One additional infant in the HFOV group developed severe interstitial emphysema, which precluded regular tracheal aspirate sampling, and therefore was excluded. The clinical characteristics of the 19 infants are presented in Table 1. Both groups were similar in their demographic characteristics at the study entry; all infants received a similar amount of surfactant replacement. No infants received exogenous surfactant after 24 hours of life, and no infant required neuromuscular blockade during the period. There were no other differences between the 2 groups with regard to other complications, such as pulmonary or bloodstream infections, patent ductus arteriosus, surgical interventions, or corticosteroid, antibiotic, or vasopressor administration during the study period (Table 1).

There were no significant differences in the metabolic indices of endogenous surfactant synthesized from glucose between infants receiving HFOV and CV (Table 2). Specifically, FSR and T½ were similar between the two groups, suggesting no decrease in surfactant production or turnover in infants receiving HFOV. There were no correlations between surfactant metabolic indices and any respiratory indices (data not shown).

Four infants died from respiratory failure within 24 days of life (3 with HFOV, 1 with CV). Three of these infants had T½ shorter than 14 of the 15 survivors (Figure). These 4 infants also had a significantly higher respiratory score than did the survivors (median 6.6 [5-9.2] *versus* 3.6 [1.7-5.2] per day, p=0.01).

Table 2. *Endogenous surfactant PC metabolism: CV vs HFOV*

	CV	HFOV	*p*-value
T_{app} (h)	13 (9-23)	15 (10-28)	0.2
T_{max} (h)	70±24	87±22	0.1
E_{max} (APE)	0.19±0.08	0.23±0.07	0.2
T½ (h)	79±18	76±23	0.7
FSR (%/d)	4.7±2.7	4.2±3.1	0.7
Plasma enrichment (APE)	2.0±0.9	2.7±0.8	0.09

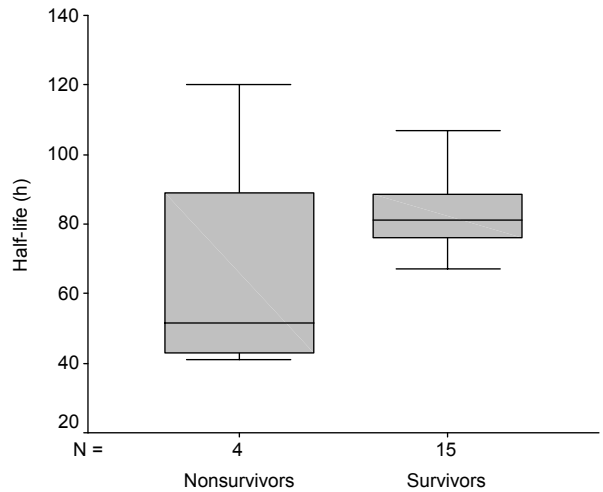
Discussion

Because alveolar stretch increases surfactant synthesis and secretion, we thought HFOV might decrease surfactant production, which could be detrimental in premature infants already deficient in pulmonary surfactant. We reasoned that if HFOV decreased surfactant production or pool size, it would be evident as changes in FSR, T½, or T_{max} . We found no differences in these tracer kinetic indices of surfactant metabolism, suggesting that the alveolar distension produced by HFOV did not decrease surfactant production.

This observation contrasts with the only previous studies in humans that relied on differences in surfactant composition to suggest differences in surfactant metabolism with HFOV and CV (242, 243). These studies are not comparable with ours in other respects. For example, Dargaville *et al*. (242) evaluated infants in the recovery phase of hyaline membrane disease from 1 to 77 days of age and 27 to 40 weeks of gestation, in contrast to our population of infants in the acute phase of illness. Gerdes *et al*. (243) evaluated the concentration of surfactant protein-A, which is under metabolic control separate from that of surfactant phospholipid (248). Ours is the first study to evaluate the dynamics of surfactant metabolism in premature infants receiving HFOV.

There may be several reasons that we did not detect differences in surfactant metabolism between the 2 groups of infants. First, we studied an extremely immature, critically ill population, in whom surfactant metabolism may already be sufficiently disrupted such that differences in metabolism might have been below the threshold of detection for this technique. Second, all infants received a similar amount of surfactant replacement, which, at 5 to 10 times the endogenous pool, could have masked small differences in metabolism (80). However, the FSR of 4.5% we obtained is similar to that of an earlier study in which differences in FSR (8.9, 3.3, 2.5, and 1.7%/day) were detected in infants who received 0, 1, 2, and 3 doses of exogenous surfactant, respectively (63). Thus, it is reasonable to assume that surfactant pool sizes were similar in the 2 groups of infants and that it is unlikely that the surfactant replacement masked differences in metabolism between the 2 groups. A third reason we might have been unable to detect differences between HFOV and CV is that we started our study at 24 hours of life. It is possible that alveolar stretch is a more important stimulus to surfactant production in the immediate transition from fetal to extra-uterine life and is less critical beyond 24 hours of life.

Figure. *The half-life of ^{13}C PC clearance of the 19 infants in the study*



Four who died of respiratory failure within 24 days of birth (nonsurvivors) and 15 who survived at least 6 months (survivors).

In our study, infants in the HFOV group had more severe lung disease, which itself could result in differences in surfactant metabolism. For example, Martini et al. (249) using stable isotope techniques similar to those used in this study, demonstrated decreases in PC synthesis and pool size in adult pigs with experimental thermal injury. In addition, Solimano et al. (240) found that MAP and alveolar surfactant pool size in preterm lambs were inversely related to severity of hyaline membrane disease. Finally, hyperoxia may disrupt PC synthesis and secretion as demonstrated in vitro in rabbit lung slices or cultured alveolar type II cells (250, 251). In any event, we would expect that the changes in surfactant metabolism associated with lung injury, if significant, would be similar to those we expected to find with HFOV, which would thereby accentuate, rather than mask, differences in surfactant metabolism. Furthermore, the lack of correlation between indices of endogenous surfactant metabolism with any of the ventilatory indices suggests that these differences in ventilatory support between the 2 groups did not influence our results.

Although we assume that we are measuring the metabolic indices of endogenous surfactant synthesized *de novo* from plasma glucose, we are actually measuring the net results of synthesis, secretion, catabolism, recycling, and the rate at which the surfactant ascends the tracheobronchial tree, the latter of which may be influenced by the mode of ventilation. Furthermore, our kinetic analysis makes no adjustment for ^{13}C tracer recycling through the glycolytic or gluconeogenic pathway. Distinguishing the magnitude of the contribution of specific steps is difficult in human infants because the tracheal aspirates are the only realistic samples that can be obtained. The recent development of stable isotope-labeled exogenous surfactant for estimating surfactant pool size will greatly enhance the interpretation of these data (62).

The intriguing observation of a shorter half-life of endogenous surfactant in premature infants who died of refractory respiratory failure suggests that there may be fundamental differences in surfactant metabolism in premature infants who do not survive. Understanding these fundamental differences will then permit the development of therapeutic strategies that are specific to the mechanism of metabolic disruption.

Acknowledgements

We thank F.S. Cole, MD, for his assistance in preparing this manuscript, Kristina Kloos, PhrmD, for tracer preparation, and the NICU staff at St. Louis Children's Hospital for their help during the study.

CHAPTER 4

Surfactant Phosphatidylcholine Half-Life and Pool Size Measurements in Premature Baboons Developing BPD

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Ped Res 2002; 52(5):724-29

Abstract

Because minimal information is available about surfactant metabolism in bronchopulmonary dysplasia (BPD), we measured half-lives and pool sizes of surfactant phosphatidylcholine in very preterm baboons recovering from respiratory distress syndrome (RDS) and developing BPD, using stable isotopes, radioactive isotopes, and direct pool size measurements. Eight ventilated premature baboons received ^{2}H-DPPC (dipalmitoylphosphatidylcholine) on d 5 of life, and radioactive ^{14}C-DPPC with a treatment dose of surfactant on d 8. After 14 d, lung pool sizes of saturated phosphatidylcholine (sat PC) were measured. Half-life of ^{2}H-DPPC (d 5) in tracheal aspirates was 28±4 h (mean±SEM). Half-life of radioactive DPPC (d 8) was 35±4 h. Sat PC pool size measured with stable isotopes on d 5 was 129±14 μmol/kg, and 123±11 μmol/kg on d 14 at autopsy. Half-lives were comparable to those obtained at d 0 and d 6 in our previous baboon studies. We conclude that surfactant metabolism does not change during the early development of BPD. More specifically, the metabolism of exogenous surfactant on d 8 is similar to that on the day of birth. Surfactant pool size is low at birth, increases after surfactant therapy, and is kept constant during the first two weeks of life by endogenous surfactant synthesis. Measurements with stable isotopes are comparable to measurements with radioactive tracers and measurements at autopsy.

Introduction

Since the introduction of surfactant therapy for the treatment of neonatal RDS, mortality and morbidity due to RDS have dramatically decreased (3). Most studies of surfactant metabolism have been performed in animals with radioactive tracers used to measure surfactant synthesis, half-life of surfactant, pool sizes, and influences of hormonal treatments (52, 252). Surfactant metabolism changes with lung injury and with development. Newborn animals have less *de novo* synthesis and a longer biological half-life of phosphatidylcholine (PC) than adult animals, but newborns also have higher rates of surfactant recycling (52). In adult animals, acute lung injury or oxygen exposure change surfactant composition and pool sizes (253).

Despite the progress in neonatal care, many very preterm infants develop BPD characterized by lung injury, inflammation, and an arrest in alveolar development (254). It is presently unclear whether the surfactant system is involved in the development of BPD. Most studies of surfactant metabolism in newborn animals and humans have been performed during the first days of life, during the acute phase of RDS. Almost no information is available about surfactant metabolism beyond the first week during the development of BPD (255). Infants who develop BPD have an altered phospholipid composition of surfactant (256, 257), and surfactant therapy in infants developing BPD resulted in a transient improvement of oxygenation (258). In ventilated preterm baboons developing BPD, the abnormal alveolar development and injury is similar to that found in humans (259). These very preterm baboons have a large increase in intracellular surfactant pool size during the first few days, but surfactant secretion to the alveolar space is very limited (59). Surfactant lipid and protein composition and surfactant function is abnormal in baboons developing BPD (59, 260). However, there is no information about the metabolism of the sat PC component of surfactant as the animals develop BPD.

We recently used stable isotopes to study the surfactant metabolism in premature human neonates (54, 62). However, a validation of the method using stable isotopes has not been performed because radioactive isotopes are medically and ethically not accepted in human newborns, and lung pool size of surfactant has only been measured in newborns who did not survive (261).

In the present study, we evaluated surfactant metabolism in very preterm ventilated baboons during the acute phase of RDS (d 1-3), the phase of recovery from RDS, and the development of BPD. We measured surfactant half-lives and pool sizes in each individual baboon during the first 2 weeks of life. We were specifically interested in the changes of the surfactant metabolism in the second week of life during the development of BPD. Furthermore, we compared the surfactant half-life and surfactant pool size measured with stable isotopes to measurements made with radioactive isotopes and direct measurements of pool size at autopsy.

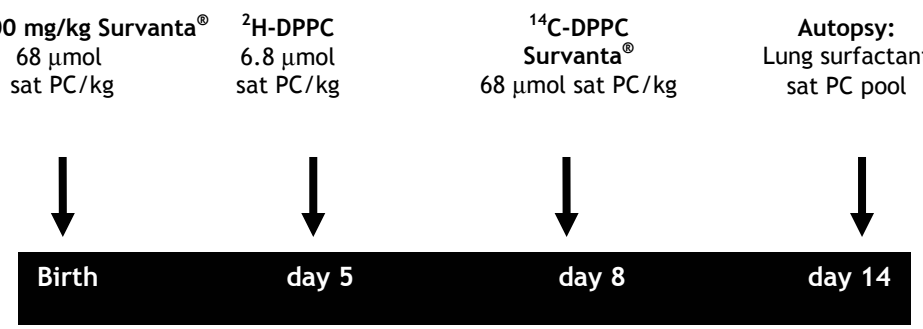
Methods

Study protocol

The animal studies were performed at the Southwest Foundation for Biomedical Research (San Antonio, TX), as described before and conformed to the American Association for Accreditation of Laboratory Animal Care guidelines (59).

We studied surfactant metabolism in 8 baboons (gestational age (GA) 124-127 d; full term at 185 d) using stable isotopes and radioactive isotopes with surfactant treatment at different times over a 14-d period of ventilation (Figure 1). The baboons received unlabeled surfactant at birth (100 mg/kg Survanta®, Abott Laboratories, Abott Park, IL, USA). At 120 h (d 5), they received a tracer dose of 5 mg/kg (6.8 μmol/kg) deuterium (stable isotope) labeled DPPC (^{2}H-DPPC, where 3 deuterium labels are present in the palmitoyl-groups) (L-3-phosphatidylcholine dipalmitoyl-D6, Doosan Serdary Research Laboratories, NJ, USA) intratracheally. The ^{2}H-DPPC was used to measure the disappearance of labeled PC palmitate from the tracheal aspirates and to calculate surfactant pool size. Day 5 was chosen because a 3-d interval is needed to reliably calculate surfactant half-life and pool size. At 192 h (d 8), these eight baboons received a treatment dose of 100 mg/kg (68 μmol sat PC/kg) Survanta® labeled with radioactive [^{14}C]choline-labeled DPPC (5 μCi ^{14}C-DPPC/kg birth weight). A 6-d interval from d 8 to d 14 was chosen to compare the surfactant half-life and pool size with our previous data (59). In that study, we measured the surfactant metabolism in the first 6 d of life. Over the interval from birth to d 6, most of the PC in the surfactant treatment given at birth was degraded. Tracheal aspirates were obtained every 12 h for 14 d as described before (59). At 336 h (d 14) the animals were killed with pentobarbital. Alveolar wash and lung homogenates were retrieved as described before (59).

Figure 1. *Schematic representation of the study protocol.*



Eight baboons received unlabeled surfactant at birth, exogenous ^{2}H-DPPC on d 5, and on d 8 they received exogenous ^{14}C-DPPC together with a treatment dose of Survanta®. After 14 d of ventilation, direct surfactant sat PC pool size measurements were performed at atopsy.

Analytical procedures

The tracheal aspirates, alveolar washes, and lung homogenates were processed as described before (262). The PC fraction of the tracheal aspirates was isolated by thin-layer chromatography and derivatives of PC palmitate were formed during incubation with 0.015 mL N-methyl-N-(tert-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA, Pierce, Onmilabo, Breda, The Netherlands) and 0.015 mL pyridine, to measure ^{2}H-enrichment. Analysis of radioactive isotopes in tracheal aspirates, alveolar washes, and lung homogenates were performed as described before (59). Fatty acid composition of surfactant PC in tracheal aspirates was measured by gas-chromatography (GC) (5890 series II, Hewlett-Packard, Amstelveen, The Netherlands (100).

Determination of enrichment of stable isotope

The ^{2}H-enrichment of surfactant PC-palmitate was measured with a Carlo Erba GC8000 gas chromatograph coupled to a Fisons MD-800 mass spectrometer (Interscience BV, Breda, The Netherlands). One μL of the derivative was injected on a fused silica capillary column of 25m x 0.22mm, coated with 0.11m HT-5 (SGE, Victoria, Australia). The enrichment was expressed as mol percent excess (MPE), which represents the molar percent of the labeled molecule (m+3 palmitate), corrected for baseline enrichment (before isotope infusion) (263).

Calculations

Calculations were performed as described previously (54, 62). As palmitic acid is by far the most abundant fatty acid in surfactant PC, calculations were performed for palmitic acid only.

The *half-life of ^{2}H-DPPC* and ^{14}C-DPPC was calculated by exponential curve fitting of the final monoexponential part ($y=a*e^{-kt}$) of the downslope of the curve of ^{2}H-enrichment and specific activity of ^{14}C *versus* time, respectively ($t½=\ln(2)/k$).

The *apparent lung surfactant pool size* was calculated by using the linear regression line representing the decay of the log-transformed ^{2}H-enrichment over time. Extrapolation back to the time of administration (t=120 h) represents the ^{2}H-enrichment at t=120, from which we calculate the dilution of the tracer. The endogenous apparent pool size was calculated by multiplying the amount of exogenous DPPC with the dilution factor. The endogenous apparent pool size was corrected for the amount of exogenous ^{2}H-DPPC administered, and expressed as μmol/kg sat PC by using the fatty acid composition of PC obtained by GC.

Data Analysis

Data are presented as mean±standard error of the mean (SEM). Differences between groups were analyzed by one-way ANOVA. Significance was accepted at $p<0.05$.

Results

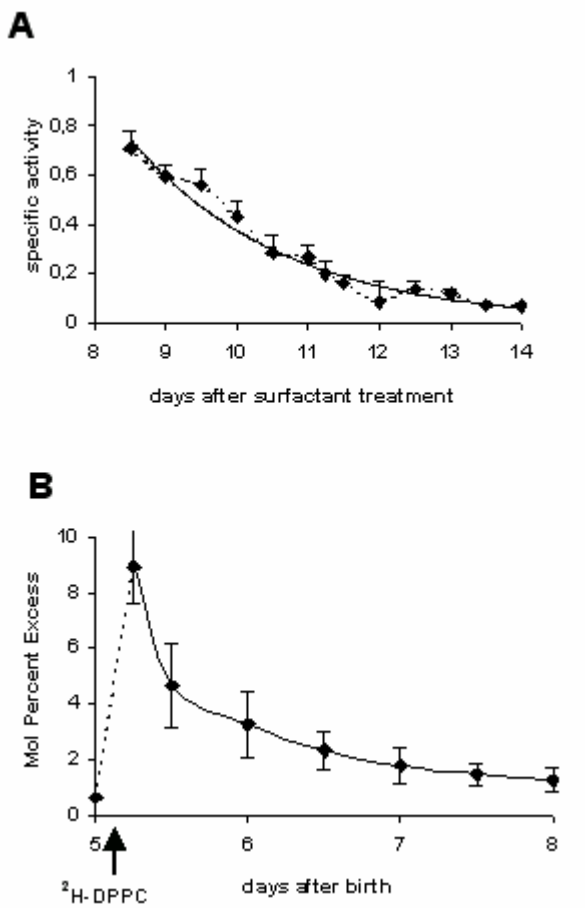
We studied 8 baboons with a mean birth weight of 386±11 g (4 females, 4 males).

On d 5, the baboons received a trace dose of ^{2}H-labeled DPPC (6.8 µmol/kg). The values of the ^{2}H-enrichment of PC palmitate in sequential tracheal aspirates *versus* time were used to calculate a half-life of 28±4 h (Figure 2B, Table 1). The half-life was calculated from the final mono-exponential part of the decrease in ^{2}H-enrichment in each baboon.

On d 8, the baboons received a therapeutic dose of radioactive surfactant (100 mg/kg Survanta®, 68 µmol sat PC/kg). The half-life, calculated by exponential curve fitting of the final mono-exponential part of the downslope of the curve of the specific activity of ^{14}C-DPPC in airway samples *versus* time curve, was 35±4 h (Figure 2A, Table 1).

We compared the results of these 8 baboons with our previous data for surfactant metabolism in similar premature baboons that were ventilated for 6 d (59, 262). The previous studies in very premature baboons (GA 125±2 d) were performed by the same investigators, using the same pre- and postnatal care. The half-life of ^{14}C-DPPC given on d 0 in the previous study is shown in

Figure 2. *Decay of the label in tracheal aspirates after endotracheal administration of labeled DPPC*



(A) Specific activities of sat PC from tracheal aspirates after treatment of baboons with 14C-DPPC labeled 100 mg/kg Survanta on d 8. Specific activities as CPM/µmol sat PC were normalized to the specific activity of the surfactant used to treat the preterm baboons. (B) Mean ± SEM ^{2}H-enrichment of surfactant PC-associated palmitate in sequential tracheal aspirates of eight baboons (125 GA) after ^{2}H-DPPC (stable isotope) administration at d 5 (t=120 h).

Table 1. The results show similar half-lives on d 0, d 5 and d 8 after birth.

The apparent lung sat PC pool size on d 5 was 129±14 μmol/kg (Table 1). The pool size was calculated from the monoexponential decrease in ^{2}H-enrichment, by using the linear regression line representing the decay of the log-transformed ^{2}H-enrichment over time. On d 14, the pool size of sat PC measured for the total lung was 123±11 μmol/kg (Table 1). Total lung pool size on d 0 and d 6 from previous experiments are shown for comparison (59). There was no significant difference between pool sizes on d 5, 6 and d 14 (one-way ANOVA, p=0.065).

Discussion

BPD was initially described as severe injury of the preterm lung resulting from mechanical ventilation and oxygen exposure (264). With the introduction of surfactant therapy and prenatal corticosteroids, BPD now primarily develops in very preterm infants weighing <1 kg, without severe RDS. This new BPD may be primarily caused by an arrest of lung development (265). We report here surfactant metabolism during the early phase of BPD in very premature baboons. Half-life and apparent lung sat PC pool size were calculated after endotracheal instillation of a tracer dose of deuterium labeled DPPC on d 5 after birth. On d 8 of life, half-life was measured after endotracheal radiolabeled DPPC together with a treatment dose of surfactant, and pool size measurements from whole lung were made on d 14. We show that although the baboons recover from RDS and develop the early phase of BPD, surfactant sat PC half-life and surfactant sat PC pool size do not change. It remains presently unclear whether the surfactant metabolism plays a role in the pathogenesis of BPD.

Table 1. *Surfactant half-lives and sat PC pool sizes*

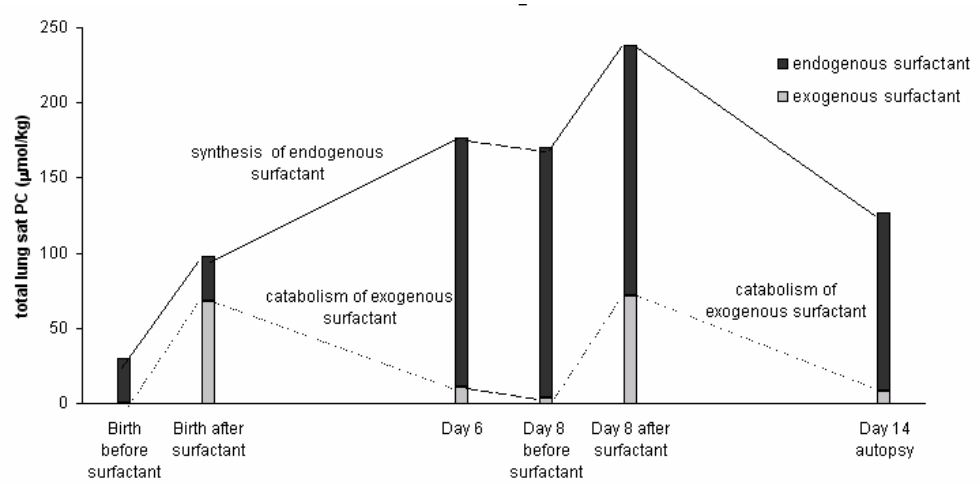
	Do* ^{14}C-DPPC/ autopsy	D5 ^{2}H-DPPC	D6* autopsy	D8 ^{14}C-DPPC	D14 autopsy
Half-life (h)	30	28±4	-	35±4	-
Pool size (μmol/kg sat PC)	30	129±14	166±11	-	123±11

*Half-life and sat PC pool size expressed as mean±SEM. On d 5, the baboons received ^{2}H-DPPC (stable isotope) endotracheally, on d 8 they received ^{14}C-DPPC (radioactive) with Survanta® (100 mg/kg) endotracheally, and on d 14 the baboons were killed. *The results are compared with results reported previously (59).*

Most studies of surfactant kinetics have been performed over a short time course. In the present study, we were able to evaluate surfactant metabolism during the first 2 weeks of life in the same animal. In our previous study, we measured surfactant synthesis in the

acute phase of RDS, and surfactant half-life and pool size were studied after treatment with surfactant at birth. By using a 14-d ventilated preterm baboon model, which shows abnormal alveolar development and injury similar to that found in humans (266), we were now able to measure surfactant kinetics during the development of BPD, more specifically on d 5, and after surfactant treatment on d 8. Our measurements in the preterm baboon are summarized in Figure 3. We measured a low sat PC pool size at birth, which was increased at d 6 after surfactant treatment. As 84% of the ^{14}C-DPPC given at birth was degraded by d 6 (59), the increase of the total sat PC pool size resulted from endogenous synthesis of surfactant. In the second week of life, the pattern of surfactant metabolism was similar: about 90% of the ^{14}C-DPPC administered at d 8 was degraded by d 14 (267), whereas the total lung sat PC pool size did not change significantly.

Figure 3. *An overview of the surfactant metabolism during the first two weeks of life in the very premature ventilated baboon*



At birth the baboons received 68 µmol/kg sat PC Survanta®, from which 84% was degraded at d 6 (results reported previously (11)). Pool size at d 6 was 166 µmol/kg sat PC, which indicate an increase of pool size by endogenous surfactant synthesis. In the second week of life during the early development of BPD, the endogenous surfactant pool size remains fairly constant and catabolism is similar to that in the first week after a gift of 68 µmol/kg sat PC Survanta®.

Sat PC pool size on d 5, measured with stable isotopes was 129 µmol/kg and was comparable to the total lung pool size measured at autopsy on d 14 (123 µmol/kg) in the same animal. The trend of a larger sat PC pool size at autopsy on d 6 in the previous study (166 µmol/kg) (59) compared to the pool size on d 5 in the current study, may be explained by measurements in different animals. It is also unclear to what extent an abnormal surfactant metabolism influences the measurements. In the previous study, we found very high tissue pools, but low alveolar pool sizes of surfactant, caused by a low secretion of *de*

novo synthesized sat PC into the alveolar space. No other studies have investigated the evolution of surfactant pool sizes in premature animals during a longer period. A study in term rats found a progressive decrease in total lung sat PC pool sizes corrected for weight during the first 100 days after birth (from ~125 µmol/sat PC/kg to ~5 µmol/sat PC/kg) (74).

Jackson *et al*. measured a total lung pool size of sat PC of ~45 µmol/kg in preterm monkeys with RDS at birth (79). However, these monkeys were not as premature as the baboons in the present study (83% *versus* 68% of the term gestational age) and they did not receive exogenous surfactant. Preterm lambs delivered at 132 days gestational age had total lung sat PC pool sizes of ~60 µmol/kg without surfactant treatment (268) and ~80 µmol/kg sat PC when treated with surfactant (269). In preterm neonates, using stable isotopes, we previously measured an apparent sat PC pool size of ~8 µmol/kg and ~22 µmol/kg before and after surfactant therapy, respectively (62). Hallman *et al*. showed an apparent pool size of ~12 µmol/kg in human preterm neonates with RDS (61). They used PG as label to measure surfactant pool size. With the same method Griese *et al*. calculated an apparent pool size of ~13 µmol/kg sat PC in preterm neonates with RDS (81). However, the pool size measurements in humans were made with a tracer together with one or more therapeutic doses of surfactant, whereas in the present study only a trace dose of surfactant was used on d 5. The pool sizes measured in the human studies probably represent the alveolar pool sizes. Their apparent pool size was comparable with the alveolar pool size found in other studies (~20 µmol/kg) (261). In the current study, the pool size measured with stable isotopes likely represents the total lung pool size as the small amount of tracer is quickly taken up by the lung cells in contrast to the large treatment doses.

The half-life of DPPC over the interval from d 5 to d 8, using stable isotopes, was 28±4 h, which was similar to the half-life measured with radioactive DPPC from d 8 onwards. Similar results were found at d 0, using radioactive DPPC, in our previous study (59). Thus, the half-life of surfactant did not change during the first two weeks of life in the very premature baboon. The same half-life of 35 h was found in preterm lambs using PG as tracer in alveolar washes (270). In one study in term newborn lambs, endotracheally injected ^{3}H-PC had a very long half-life of 6 d (58). Possibly, variations of half-lives are due to differences in maturity. In premature infants treated with amniotic fluid-derived surfactant containing PG, the half-life was 30 h (61). In another study in premature infants using PG and sphingomyelin (Sp) as markers, half-live of PG was 43 h and 105 h after treatment with Alveofact® and Survanta®, respectively, and the half-life of Sp was 97 h after treatment with Survanta® (81). The differences in half-lives could be explained by the different composition of the two surfactants and the different doses given. Comparable results were found in human premature neonates using stable isotopes (62). Preterm infants received two doses of surfactant labeled with ^{13}C-atoms, 5 h and 32 h after birth, respectively. The calculated half-life was 34 h and 17 h, respectively. All these studies in human neonates calculated half-life from the disappearance of the label (PG, Sp, and ^{13}C) from tracheal aspirates, as in the current study. The half-life of a trace amount of labeled DPPC (d 5) in the present study was not influenced by the administration of treatment

doses of surfactant (100 mg/kg) (d 8), which is in agreement with Hallman *et al*. (61), and with our previous results (63). However, in mice the half-life for ^{3}H-DPPC in total lung increased ~2-fold after the instillation of 45 µmol/kg sat PC compared with a trace dose (271). Nevertheless, further increase of the dose had no effect on the half-life.

As a secondary goal, we compared the method of DPPC half-life and sat PC pool size measurements using stable isotopes with that of radioactive isotopes and surfactant pool size measurements at autopsy in very premature ventilated baboons. The results obtained with the use of stable isotopes were comparable to the results obtained with radioactive tracers and tissue measurements of pool size. Estimation of surfactant half-life and pool size by the isotope dilution method is based on several assumptions. The distribution of the exogenous surfactant and the endogenous surfactant has to be similar, the phospholipid composition in the various surfactant compartments has to be uniform, the surfactant system has to be pulse labeled, there should be no endogenous synthesis of the label, and the pool size after exogenous surfactant has to be constant. It is reasonable to assume that most of the assumptions have been fulfilled as has been extensively discussed by Hallman *et al*. and by Torresin *et al*. (61, 62).

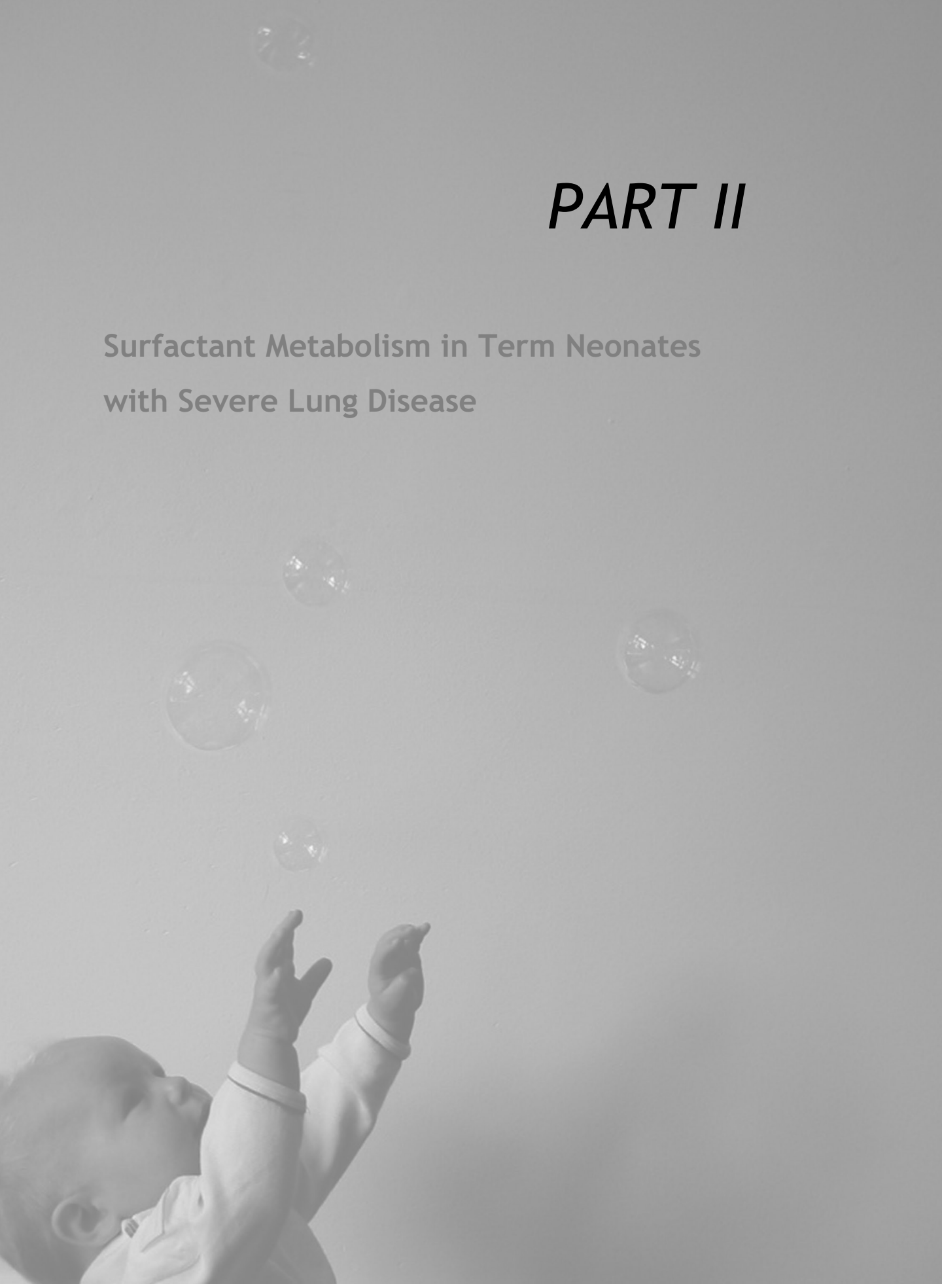
In conclusion, the metabolism of surfactant PC did not change during the recovery from RDS and the development of BPD. Very preterm baboons have low sat PC pool size at birth. Treatment with surfactant increased instantaneously the total lung sat PC pool size, which was kept relatively constant by endogenous surfactant synthesis. Half-lives of surfactant PC did not change during the first 2 weeks of life. We also showed that stable isotopes are a valid tool for studying surfactant metabolism *in vivo*. This method can be easily applied to human neonates, which will enable us to better understand the role of surfactant in different neonatal lung diseases.

Acknowledgments

The authors like to thank Wim van den Berg (Internal Medicine II, Erasmus University Rotterdam, The Netherlands), Jan Erik Bunt (Department of Pediatrics, Sophia Children's Hospital Rotterdam, The Netherlands) for excellent support and technical advice, Esther Wit and Lars de Zeeuw (medical students, Erasmus University, Rotterdam, The Netherlands) for technical assistance, and the BPD Resource Center at the Southwest Foundation for Biomedical Research.

PART II

Surfactant Metabolism in Term Neonates with Severe Lung Disease



CHAPTER 5

Surfactant Phosphatidylcholine Pool Size in Human Neonates with Congenital Diaphragmatic Hernia requiring ECMO

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J Pediatr 2003; 142:247-52

Abstract

We measured surfactant phosphatidylcholine (PC) pool size and half-life in human congenital diaphragmatic hernia (CDH) patients who required extracorporeal membrane oxygenation (ECMO). Surfactant PC pool size and half-life were measured by endotracheal administration of deuterium-labeled dipalmitoylphosphatidylcholine in 8 neonates with CDH on ECMO (CDH-ECMO), in 7 neonates with meconium aspiration syndrome on ECMO (MAS-ECMO), and in 6 ventilated infants (NON-ECMO). Lung PC pool size in the CDH-ECMO group was 73±17 mg/kg (mean±SEM), which was not significantly different from the MAS-ECMO (50±18 mg/kg) and the NON-ECMO group (69±38 mg/kg). Surfactant PC concentration in tracheal aspirates was not different between groups (~6 mg/mL). However, the percentage of palmitic acid in surfactant PC was significantly lower in the MAS-ECMO (56.3%) and the NON-ECMO (55.8%) group compared to the CDH-ECMO (67.6%) group. Surfactant PC half-life (~24 hours) was not different between the groups. A correlation was found between the surfactant PC half-life and the duration of ECMO. In conclusion, these data show no decreased surfactant PC pool size in high risk CDH patients who require ECMO. A shorter half-life of surfactant PC, indicating a faster turnover, may result in a faster improvement of the pulmonary condition during ECMO.

Introduction

Congenital diaphragmatic hernia (CDH) usually results in severe respiratory insufficiency that may lead to death because of pulmonary hypoplasia and persistent pulmonary hypertension. CDH lungs show some resemblance morphologically to the lungs of preterm infants with respiratory distress syndrome (RDS) (82). The lungs of neonates with RDS are surfactant-deficient and, in general, these patients respond to surfactant treatment.

Studies in animal and human models report conflicting data; it is unclear whether a primary surfactant deficiency is present in human CDH (100). In nitrofen-induced CDH rats and lambs with surgically induced CDH phospholipid, disaturated phosphatidylcholine (PC), and surfactant protein A (SP-A) in lung tissue are lower compared with controls (85, 87, 91-93). The scarce data in humans show no difference in amniotic lecithine:sphingomyeline ratio between CDH and control fetus (95). SP-A was lower in the amniotic fluid of fetuses with CDH who died or required extracorporeal membrane oxygenation (ECMO) (97). Autopsy studies in CDH infants showed a decrease of SP-A in the lungs (98, 99). Earlier, we showed that the concentration of surfactant PC in bronchoalveolar lavage from CDH patients is not different from control patients (100). Direct pool size measurements have only been done in autopsy studies of patients without CDH (261), and not in term-born patients with CDH. As a consequence, no data are available about the surfactant pool size and surfactant metabolism in human CDH patients.

We report on surfactant PC pool sizes and the half-life of surfactant PC in neonates with CDH who required ECMO. Surfactant PC pool size and half-life were measured *in vivo* with the use of stable isotopes.

Patients and Methods

Patients

The study was performed in the pediatric surgical and neonatal intensive care units of the Sophia Children's Hospital between October 1999 and April 2001. We studied 3 groups of neonates (Table 1): neonates with left-sided CDH who required veno-arterial ECMO (CDH-ECMO) (n=8), neonates with meconium aspiration syndrome (MAS) on ECMO (MAS-ECMO) (n=7), and a mixed group of ventilated term neonates without ECMO (NON-ECMO) (n=6). The diagnosis of the neonates in the NON-ECMO group were: CDH (n=2, one right-sided defect); MAS (n=2); right-sided cerebral medial infarction (ventilated because of apneic attacks and convulsions) (n=1); spina bifida; and Arnold Chiari malformation (ventilated because of apneic attacks) (n=1). Six CDH patients (1 NON-ECMO CDH patient) were diagnosed prenatally and were born in our hospital; 4 were postnatal referrals. One CDH-ECMO patient had a chromosomal disorder, and none underwent surgical repair before or during the study. Ventilator and arterial blood gas data before the start of ECMO are presented in Table 2.

Table 1. *Patients characteristics*

	CDH-ECMO n=8	MAS-ECMO n=7	NON-ECMO n=6
Male/Female	5/3	2/5	5/1
Gestational age (wk)	38.4±0.8	40.6±0.6	39.8±0.5
Birth weight (g)	3058±200	3267±184	3347±121
Apgar 5 min.	8.0 (5-9)	7.0 (1-8)	8.5 (5-10)
Age at start ECMO (h)	17±4	33±10	-
Age at start study (h)	38±5	59±12	100±30
Pneumothorax	3	2	0
Surfactant treatment	none	5 / 7	none
Duration of ECMO (h)	197±43	133±25	-
Time of ventilation (d)	22.5±2.7*	12.4±1.9	13.8±4.2
Supplemental O_2 (d)	36.5±14.2	16.9±2.1	15.5±4.5
Length of stay in ICU (d)	27±3	11±3	31±14
Survivors	3/8	7/7	6/6

Data are expressed as mean±sem, except Apgar score at 5 min., presented as median (range).
**Significantly different from MAS-ECMO and NON-ECMO group (p=0.046).*

Our institution uses a standardized protocol for treatment of CDH patients, consisting of: cardiac ultrasonagraphy, moderate fluid restriction (40-60 mL/kg body weight), no muscle paralysis, sedation with midazolam (0.1 mg/kg/h) and morphine (5-15 µg/kg/h), support of systemic blood pressure by dopamine and eventually norepinephrine, and continuous suctioning of double lumen nasogastric tube (272).

The entry criteria for ECMO were: gestational age of ≥34 weeks; birth weight of ≥2000 g; mechanical ventilation for <7 days; and alveolar arterial oxygen difference ($AaDO_2$) of >600 torr (>80 kPa) for 8 hours. A minimal partial pressure of oxygen in arterial blood (PaO_2) of ≥75 torr (≥10 kPa) was an additional criterion for CDH patients. During ECMO, ventilatory settings were usually reduced to peak inspiratory pressure of 12 to 16 cm H_2O, positive end-expiratory pressure of 5 to 6 cm H_2O, respiratory rate of 10 to 15 breaths/min, and fractional concentration of oxygen in inspired gas (FiO_2) of 0.25 to 0.3.

Study Protocol

The study protocol was approved by the local medical ethics committee, and patients were included after informed consent. The patients received a tracer dose of 5 mg/kg (1 ml/kg) deuterium-labeled dipalmitoylphosphatidylcholine (^{2}H-DPPC) (L-3-phosphatidylcholine dipalmitoyl-D6, Doosan Serdary Research Laboratories, Englewood Cliffs, NJ) endotracheally at the start of the study (t=0). The start of the study was within 24±3 hours after the onset of ECMO or after the onset of ventilation. The patient's head was placed in the midline. A 6-Fr catheter was passed down the endotracheal tube and

placed at the end of the tube. The ^{2}H-DPPC was administered through the catheter, and afterwards the neonate was immediately reconnected to the mechanical ventilator at pre-treatment settings. Tracheal aspirates were obtained every 4 to 6 hours during the time the infant was intubated, and immediately placed at -20°C, until further processing. No tracheal suctioning was done within 6 hours after ^{2}H-DPPC administration.

Procedure and determination of enrichment
The tracheal aspirates were processed as described before (54, 273). One µL of the derivative of deuterated PC palmitate was injected into a GC8000 gas chromatograph coupled to a Fisons MD-800 mass spectrometer (Interscience BV, Breda, The Netherlands). Enrichment was expressed as mol percent excess (MPE), representing the molar percent of the labeled molecule, corrected for baseline (natural) enrichment (before administration of the isotope).

Determination of composition and concentration
Fatty acid composition of surfactant PC and the amount of surfactant PC were analyzed on a gas-chromatograph (GC) (Hewlett-Packard, 5890 series II, Amstelveen, The Netherlands) (100). The concentration of surfactant PC in the epithelial lining fluid (ELF) was calculated by correcting for dilution of the ELF during endotracheal suction: dilution factor = $[urea]_{serum}/[urea]_{supernatant}$ (257).

Calculations
Calculations were performed as described before (54, 62, 273). The *half-life* of ^{2}H-DPPC was calculated by exponential curve fitting of the final mono-exponential part of the downslope of the curve of ^{2}H-enrichment over time. The *apparent lung PC pool size* was calculated as before (273), by using the linear regression line representing the decay of the log-transformed ^{2}H-enrichment over time, started at t=18 h till 4 times the half-life of each individual curve and expressed as mg/kg PC. We measured the enrichment of palmitic acid in surfactant PC. Palmitic acid accounts for ~70% of all fatty acids in surfactant PC. To calculate total PC pool size we therefore divided the pool size by the mean fraction of palmitic acid, measured in tracheal aspirates of the individual patients in the first 4 days of the study. Pool size was corrected for the amount of exogenous ^{2}H-DPPC administered.

Data analysis
Data are presented as mean±SEM. Differences between groups were analyzed by one-way analysis of variance, and correlation by Pearson correlation test. Significance was accepted at $p<0.05$.

Table 2. *Ventilator and arterial blood gas data before start of ECMO or highest ventilatory parameters in the NON-ECMO group*

		MAP (cm H_2O)	PIP or ΔP (cm H_2O)	PaO_2 (kPa)	$PaCO_2$ (kPa)	OI	$AaDO_2$ (mmHg)
CDH-ECMO	CV (n=1)	10.6	25	11.3	3.5	26.0±4.0	587±13
	HFO (n=7)	15.4±0.9	51.1±4.3	8.6±1.8	6.6±1.2		
MAS-ECMO	CV (n=4)	16.7±1.0	32.5±3.0	7.6±3.3	6.3±1.6	41.8±9.7	597±17
	HFO (n=3)	21.7±2.0	40.3±9.3	7.4±1.9	5.4±1.0		
NON-ECMO	CV (n=4)	9.3±2.0	19.1±3.6	11.7±3.6	6.1±0.7	8.3±2.0*	333±96*
	HFO (n=2)	15-16	32-56	10.3-21.4	5.1-5.6		

CV, conventional ventilation; HFO, high frequency oscillation; MAP, mean airway pressure; PIP, positive inspiratory pressure; ΔP, pressure amplitude on HFO; OI, oxygenation index (OI=(MAP x FiO_2)/PaO_2; Pa-aO_2, alveolar-arterial oxygen tension difference (Pa-aO_2 = PaO_2 - [(713 x FiO_2)-($PaCO_2$/0.8)].

Data are expressed as mean±SEM, except for the HFO ventilated NON-ECMO group where both values are given. In the NON-ECMO group, 1 MAS and 1 CDH patient received HFO.

**Signigicantly different from CDH-ECMO and MAS-ECMO ($p<0.05$).*

Results

The CDH-ECMO group was ventilated during a significantly longer time (p=0.046) compared with the MAS-ECMO and NON-ECMO group (Table 1). Oxygenation index (OI) and $AaDO_2$ were significantly different between the NON-ECMO group and the CDH-ECMO and MAS-ECMO group (p=0.032 and p=0.0001, respectively) (Table 2).

Surfactant PC pool sizes were not significantly different between the 3 groups (Table 3). The CDH-ECMO patients had a mean surfactant PC pool of 73±17 mg/kg, the NON-ECMO group had a surfactant PC pool of 69±38 mg/kg, and the MAS-ECMO group had a pool of 50±18 mg/kg. The NON-ECMO patients without significant lung disease had a surfactant PC pool size of 90 mg/kg (range: 26-155), whereas the patients with lung disease in the NON-ECMO group had a mean surfactant PC pool size of 59±46 mg/kg (no difference between groups). A correlation existed between the surfactant PC pool size and half-life (p=0.0001).

The mean surfactant PC half-lives of the different groups were not significantly different (Table 3). However, we found a correlation between the duration of ECMO and the half-life for both groups, the MAS-ECMO (p=0.046) and the CDH-ECMO (p=0.046) (Figure).

No correlation was found between the surfactant PC pool size or half-life and the following parameters: gestational age, birth weight, Apgar score at 5 minutes, age at start of the study, age at start of the ECMO therapy, OI, $AaDO_2$, time to extubation, and length of stay in the intensive care unit.

Survivors and nonsurvivors in the CDH-ECMO group were not different in surfactant PC pool sizes and half-life, as were the surfactant-treated and nonsurfactant-treated neonates in the MAS-ECMO group.

The mean surfactant PC concentration during the first 4 days of the study was the same for all groups. The CDH-ECMO group had a surfactant PC concentration of 6.5±2.3 mg/mL ELF, in the MAS-ECMO group the concentration was 6.4±3.8 mg/mL ELF, and 5.4±2.6 mg/mL ELF in the NON-ECMO group (Table 3). Fatty acid composition of surfactant PC was measured in tracheal aspirates during the first 4 days of the study. In the CDH-ECMO group, the percentage of palmitate was significantly higher compared with the MAS-group and the NON-ECMO group (p=0.029) (Table 3). Conversely the percentage of other fatty acids in PC were not significantly different between groups (data not shown).

Table 3. *Surfactant PC parameters measured in the 3 patient groups*

	Pool size (mg PC/kg)	Half-life (h)	PC concentration (mg/ml ELF)	Palmitic acid (%)
CDH-ECMO (n=8)	73±17	31.0±5.2	6.5±2.3	67.6±2.6*
MAS-ECMO (n=7)	50±18	21.9±6.4	6.4±3.8	56.3±2.1
NON-ECMO (n=6)	69±38	21.0±4.3	5.4±2.6	55.8±5.2

Data are expressed as mean±SEM.

**Significantly different from the MAS-ECMO and the NON-ECMO group (p=0.029).*

Discussion

We evaluated surfactant PC pool size in human neonates with CDH who require ECMO. Endotracheally administered ^{2}H-DPPC was used to measure surfactant PC pool size and half-life in human infants *in vivo*. The method was validated in premature baboons by comparing it with pool size measurements at autopsy and with half-life measurements with the use of radio-active isotopes (273). CDH patients receiving ECMO did not have a decreased surfactant PC pool size. We measured a mean total lung PC pool size of ~73 mg/kg, which was not significantly different from the MAS-ECMO (~50 mg/kg) and the NON-ECMO group (~69 mg/kg). Surfactant PC half-life was also not different between the 3 groups, and was ~31 hours for the CDH-ECMO patients.

Surfactant PC pool size has never been measured in human neonates with CDH who require ECMO. Most studies that investigated a possible surfactant deficiency in CDH measured surfactant phospholipid composition and PC concentrations in the lungs of animals or humans. There are only a few reports about the metabolism of surfactant *in vitro* in animal models (85, 87, 93). We measured a mean pool size of ~73 mg/kg surfactant PC in CDH patients receiving ECMO. Saturated PC pool sizes measured in term baboons are ~97 µmol/kg (59). Assuming that 60% of PC is saturated PC, this would become 162 mg/kg PC.

In 3-day-old rabbits, the surfactant PC pool size at autopsy in total lung homogenate was ~180 mg/kg (274). However, it is not clear how much of this PC is surfactant-associated and how much is associated with lung membrane PC. Pool size measurements in preterm infants with RDS, who received the tracer in combination with a treatment dose of surfactant (100 mg/kg) showed an endogenous surfactant PC pool size (before treatment) of ~10 mg/kg, which is much lower than we found in CDH patients receiving ECMO (62). Although CDH patients who require ECMO have the sickest lungs but similar pool sizes to our control group (~73 versus ~69 mg/kg PC), we expect that CDH patients who do not need ECMO have similar or even higher surfactant PC pool sizes. Alternatively, it is also possible that ECMO protects endogenous surfactant. In that case, CDH patients not receiving ECMO have lower surfactant PC pool sizes. Indeed, SP-A in tracheal aspirates of CDH patients also increases during ECMO, but the SP-A levels were still low compared to non-CDH patients receiving ECMO (108). Although our data suggest that surfactant PC pool size in CDH patients receiving ECMO is normal, a good comparison with an appropriate control group is very hard to make. Ideally, a control group would consist of nonventilated infants, but it is ethically not possible to obtain tracheal aspirates in these patients. However, from literature it is known that pool size of surfactant phospholipids in healthy term neonates is ~100 mg/kg (80). Our data certainly show that the degree of surfactant deficiency did not correlate with severity of disease, because patients receiving ECMO have similar surfactant PC pool size compared to NON-ECMO patients.

The results of the current study are in agreement with our results of an earlier study, where we found no difference in surfactant PC concentration in bronchoalveolar lavage fluid between CDH patients and controls (100). Surfactant PC concentrations during the first 4 days of the study were also not different between the groups, and were ~6 mg/mL ELF. Using the same method in a study in preterm infants, we found a lower surfactant PC concentration of 2.4 mg/mL ELF (244). Cogo *et al*. showed reduced levels of disaturated PC in ELF of CDH patients not receiving ECMO (~2.3 mg/kg disaturated PC) compared to controls (~4.6 mg/kg disaturated PC) (57). However, surfactant synthesis was not different between CDH patients and controls. It is not clear yet if surfactant concentrations measured in tracheal aspirates correlate with lung surfactant pool size. We did not find any correlation between surfactant concentration in ELF and surfactant PC pool size in our patients.

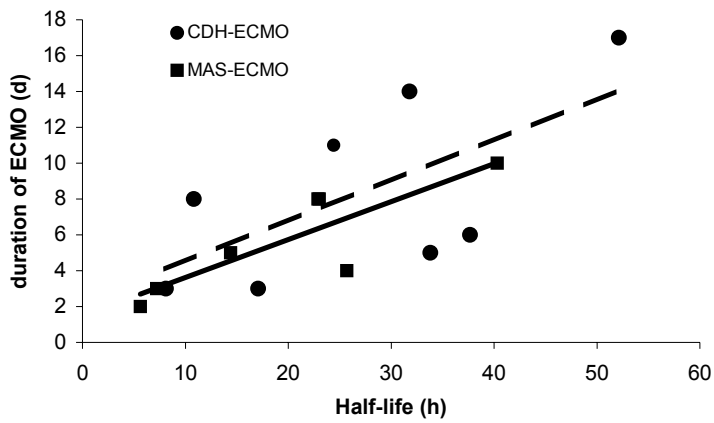
The results of surfactant PC half-life are comparable to the results in preterm humans with RDS (61, 62, 81) and preterm baboons (273). The half-life is also in agreement with that of CDH patients not requiring ECMO (~26 hours) (personal communication, P. Cogo, Padua, Italy).

A positive correlation was found between surfactant PC half-life and the duration of ECMO. Based on equal pool size and severity of disease at the start of the study, a possible explanation could be that a lower endogenous synthesis of surfactant leads to a slower dilution of the labeled exogenous surfactant in patients who required a longer duration of ECMO. A lower PC synthesis was also found in isolated type II pneumocytes from the lungs of the lamb model of CDH compared with normal (85, 87). We speculate that a surfactant

pool that is replaced by newly synthesized surfactant more rapidly results in a faster improvement of the clinical condition and a shorter duration of ECMO.

At the time of the study, our standard protocol did not include permissive hypercapnia before start of ECMO as was suggested by recent publications (275). Even so, using our approach of a more 'vigorous' ventilatory support, we did not find reduced surfactant PC pool sizes, rendering an additional argument against a primary surfactant deficiency in CDH cases in need for very intensive ventilatory support and ECMO. We believe that, taken into account, the discernible negative effects of artificial ventilation in the hypoplastic lungs of CDH (129), a secondary surfactant insufficiency is a common feature in CDH. This could possibly be avoided by protective ventilatory strategies such as permissive hypercapnia and high frequency ventilation.

Figure. *Correlation between the surfactant phosphatidylcholine (PC) half-life and the duration of extracorporal membrane oxygenation (ECMO)*



A positive correlation was found between surfactant PC half-life and the duration of ECMO (p=0.001). The correlation existed for both ECMO groups, CDH-ECMO (y=0.23x + 2.24, p=0.046) and MAS-ECMO (y=0.21x + 1.50, p=0.046).

In this study we observed neonates with MAS who required ECMO for comparison. Surfactant PC pool sizes in the MAS-ECMO group were not significantly decreased (~50 mg/kg). Surfactant inactivation seems to play a more important role in the pathophysiology of MAS. In vitro studies show an inhibition of surfactant function by meconium in a concentration-dependent manner (131). Surfactant inhibitors such as total protein and albumin, were elevated in lung lavage fluid of infants with MAS (143). We found a significant lower percentage of palmitic acid in surfactant PC in tracheal aspirates of the MAS-ECMO group compared with the CDH-group (56% versus 68%). This may indeed point to a less active surfactant that is also suggested by a decreased surfactant protein A and B in bronchoalveolar lavage fluid of rats with MAS (141). The percentage of surfactant PC

palmitate was also significantly lower in the NON-ECMO group compared with the CDH-ECMO group, which is because of the 2 MAS patients in the NON-ECMO group.

We used 2 groups for comparison with CDH patients in the current study. One group of neonates with developmentally normal lungs who require ECMO (MAS-ECMO group), and a NON-ECMO control group, consisting of infants with several diagnoses who did not require ECMO. It is difficult to include control patients without significant lung disease who are ventilated for at least 3 days to study surfactant metabolism.

In conclusion, surfactant PC pool size and half-life are not decreased in neonates with CDH who required ECMO compared with neonates with MAS who required ECMO and NON-ECMO patients. However, patients who needed a longer ECMO treatment had a longer surfactant PC half-life. Our results suggest no surfactant deficiency in CDH patients requiring ECMO, but does not exclude a surfactant inactivation secondary to the treatment.

Acknowledgments

The authors like to thank Wim van den Berg (Internal Medicine II, Erasmus University Rotterdam, The Netherlands), Jan Erik Bunt (Department of Pediatrics, Sophia Children's Hospital Rotterdam, The Netherlands) for excellent support and technical advice, Danielle Cordeiro (medical student, Erasmus University, Rotterdam, The Netherlands) for technical assistance, and the nursing staff, neonatologists and intensivists of the neonatal and surgical intensive care for their help and support with the study.

CHAPTER 6

Decreased Rate of Surfactant Phosphatidylcholine Synthesis in Neonates with Congenital Diaphragmatic Hernia during Extracorporeal Membrane Oxygenation

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Submitted

Abstract

Congenital diaphragmatic hernia (CDH) may result in severe respiratory insufficiency with a high morbidity despite recent advances in respiratory support and the use of extracorporeal membrane oxygenation (ECMO). The role of a disturbed surfactant metabolism in the pathogenesis of CDH is not clear yet. We therefore studied endogenous surfactant metabolism in the most severe CDH patients who require ECMO. Eleven neonates with CDH who required ECMO treatment (CDH-ECMO), and ten ventilated neonates without significant lung disease (controls) were studied. All infants received a 24-hr infusion with the stable isotope [U-^{13}C]glucose. The ^{13}C-incorporation into palmitic acid in surfactant phosphatidylcholine (PC) isolated from serial tracheal aspirates was measured. Mean PC concentration in epithelial lining fluid (ELF) was measured during the first four days of the study. Surfactant PC synthesis was decreased in CDH-ECMO patients compared to controls (2.4±0.33 versus 8.0±2.4 %/day, *p*=0.04). The control group had a higher maximal enrichment (0.18±0.03 versus 0.09±0.02 APE, p=0.04), and an earlier time of maximal enrichment (46.7±3.0 versus 69.4±6.6 hours, p=0.004) compared to the CDH-ECMO group, which reflects more and faster precursor incorporation in the control group. Surfactant PC concentration in ELF was similar in both groups. In conclusion, these results show that CDH patients who require ECMO have a decreased surfactant PC synthesis, which could serve as a rationale for the need for ECMO in this particular group of patients.

Introduction

Congenital diaphragmatic hernia (CDH) may result in severe neonatal lung 'disease' due to lung hypoplasia, pulmonary hypertension, and the sequelae of ventilatory support (129). Despite advances in respiratory support and the use of extracorporeal membrane oxygenation (ECMO), the morbidity of CDH remains high (276).

The presence of a surfactant deficiency in CDH is subject to ongoing debate. The scarce data in humans show no difference in amniotic lecithine/sphingomyelin ratio between CDH and control fetuses (95), but amniotic SP-A was reported to be lower in CDH fetuses who died or required ECMO (97). Moreover, autopsy studies in CDH infants showed decreased SP-A levels in the lungs (98, 99). Earlier, we showed that the concentration of surfactant PC in bronchoalveolar lavage from CDH patients was not different from control patients (100). And more recently, in a study using stable isotopes, we found no decreased surfactant PC pool size in high risk CDH patients who required ECMO (277).

In a previous study of our group, Cogo *et al*. studied the surfactant disaturated PC (DSPC) kinetics in CDH patients who did not require ECMO by using stable isotopes (57). Although the amounts of DSPC and SP-A in the tracheal aspirates of CDH patients were reduced, these patients had rates of endogenous surfactant DSPC synthesis comparable to control patients.

Several animal models, such as the surgically created CDH lamb model and the nitrofen-induced CDH rat model suggest decreased surfactant parameters in lung tissue (85, 87, 91-93). In vitro studies in isolated type II cells of the CDH lamb model show decreased incorporation of precursor in surfactant phosphatidylcholine (PC), suggesting decreased surfactant synthesis (85, 87).

The purpose of our present study was to study the surfactant PC kinetics *in vivo* in CDH patients who required ECMO with the use of stable isotopes. We hypothesized that the surfactant synthesis in these CDH patients on ECMO, who have "sicker" lungs compared to CDH patients who do not require ECMO, is decreased.

Patients and Methods

Patients

The study was performed in the pediatric surgical and neonatal intensive care units of the Sophia Children's Hospital between October 1999 and April 2001. We studied two groups of neonates (table 1): neonates with left sided CDH who required veno-arterial ECMO (CDH-ECMO) (n=11), and a group of ventilated term neonates without significant lung disease (control) (n=10). The diagnoses of the 10 ventilated control patients were varied and overall no primary pulmonary anomaly or sign of abnormal lung development was present: gastroschisis, cerebral infarction, small to moderate omphalocele (n=2), anal atresia, thymic cyst, spina bifida, pentalogy of Cantrell, and hypoxic ischemic encephalopathy (n=2). Apart from the two study groups, we studied two male CDH patients who did not require ECMO. The study was approved by the local medical ethics committee, and all patients were studied after written informed consent of parents.

Our institution uses a standardized protocol for treatment of CDH patients, consisting of: immediate endotracheal intubation at birth and insertion of a nasogastric tube for decompression of the stomach, cardiac ultrasound, fluid restriction (40-60 ml/kg body weight), no muscle paralysis, sedation with midazolam (0.1 mg/kg/h) and morphine (5-15 µg/kg/h), support of systemic blood pressure by dopamine and eventually norepinephrine, and continuous suctioning of double lumen nasogastric tube (272).

The entry criteria for ECMO were: gestational age of ≥34 weeks; birth weight of ≥2000 g; mechanical ventilation for <7 days; $AaDO_2$ of >600 torr (>80 kPa) for 8 hours; and OI> 25 for 4 hours. A minimal PaO_2 of ≥75 torr (≥10 kPa) was an additional entry criterium for CDH patients. During ECMO, ventilatory settings were routinely reduced to peak inspiratory pressure of 9-12 mm Hg (12-16 cm H_2O), positive end-expiratory pressure of ~4 mm Hg (5-6 cm H_2O), respiratory rate of 10 to 15 breaths/min, and FiO_2 of 0.25 to 0.3.

Isotope infusion and sample collection

All included patients received a 24-hour continuous intravenous infusion of [U-^{13}C]glucose (0.17 mg/kg/min, Campro Scientific, Veenendaal, The Netherlands and Cambridge Isotope Laboratories, Inc, Andover, Mass). The start of the isotope infusion was defined as the start of the study (t=0). Before and during the label infusion, 1 ml blood was drawn every 6 h for determination of ^{13}C-enrichment of plasma glucose. Samples were collected in an EDTA tube, immediately placed on ice, and centrifuged at 2500 rpm for 10 minutes. The plasma was stored at -70ºC until it was analyzed. Tracheal aspirates were obtained every 4 to 6 h during the time the infant was intubated, with a maximum of 2 weeks. The tracheal suctioning was performed during routine patient care and did not deviate form the normal clinical care. Tracheal aspirates were immediately placed at -20°C, until further processing.

Analytical procedure

The plasma and tracheal aspirates were processed as described before (54, 57). Briefly, plasma was delipidated and glucose was isolated and derivatized to an aldonitril pentacetate derivative (245). Organic extraction was used to isolate surfactant lipids in the tracheal aspirates (246), and surfactant PC was recovered by thin layer chromatography (278). Isotopic enrichments were measured by mass spectrometry, as described before (54). The ^{13}C-enrichments were expressed as atom percent excess (APE), which represent the increase in the percentage of ^{13}C atoms in total carbon dioxide from the combusted compounds above baseline enrichment (before infusion). Enrichments were corrected for the contribution of unlabeled carbon atoms added during derivatization.

Determination of composition and concentration

Fatty acid composition of surfactant PC and the amount of surfactant PC were determined by gas-chromatography (GC) (Hewlett-Packard, 5890 series II, Amstelveen, The Netherlands) (100). The concentration of surfactant PC in the epithelial lining fluid (ELF) was calculated by correcting for dilution of the ELF during endotracheal suction: dilution factor = $[urea]_{serum}/[urea]_{supernatant}$ (257).

Calculations

Calculations were performed as described before (54). *Time of first appearance (*T_{app}*)* is defined as the time delay between the start of the isotope infusion and the first appearance of the label in surfactant PC. *Time of maximal enrichment* (T_{max}) is the time where maximum enrichment is reached (E_{max}). *Half-life of surfactant PC* ($T_{½}$) was calculated from the downslope of the enrichment *versus* time curve. *Fractional synthesis time* (FSR) of palmitic acid in surfactant PC represents the percentage of the total PC-palmitate pool synthesized *de novo* from plasma glucose per day.

Data analyses

Data are presented as mean±standard error of the mean (SEM). The non-parametric Mann-Whitney U-test was applied to compare groups. The Spearman method was used to evaluate correlations between surfactant kinetic parameters, surfactant PC concentration and patient characteristics. Significance was accepted at a value of $p<0.05$.

Table 1. *Patients characteristics*

	CDH-ECMO n=11	**Control** n=10
Gestational age (wk)	38.2±0.8	39.0±0.4
Birth weight (g)	3077±150	3189±237
Apgar score at 5 min.	6.4±0.8	7.0±1.5
Male/Female	7/4	5/5
Pneumothorax (n)	5	1
Duration of ventilation (d)	28.3±5.1	25.6±9.9
Age at start study (h)	42.9±4.5	137.6±45.5*
Survivors (n)	5	8

**Significantly different p=0.03.*

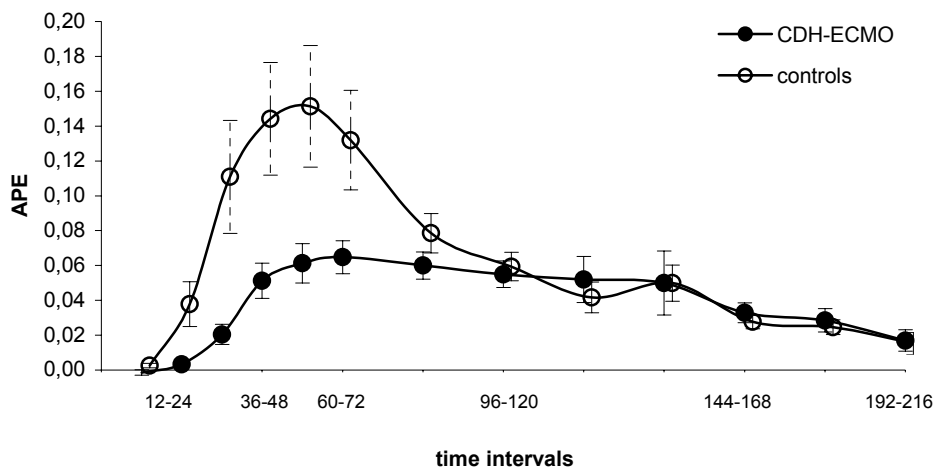
Results

Seven CDH-ECMO patients were diagnosed prenatally and were born in our hospital, four were postnatal referrals. The ECMO of the CDH patients was started 15.1±2.7 hours after birth, and had a duration of 226±34 hours. The time on ECMO before the start of the study was 27.8±4.2 hours. In five CDH-ECMO patients a pneumothorax was diagnosed, which in all cases occurred before the start of ECMO, sometimes even directly after birth. Six of the CDH-ECMO patients underwent a surgical repair of the diaphragmatic defect on day 20±6 of life, and in none of these CDH patients the surgical repair was performed before or during the study. All patients were successfully decannulated. The non-survivors in the CDH-ECMO group died at 28.2±5.5 days of life, due to rebound pulmonary hypertension which was resistant to maximal treatment. Unfortunately, none of the non-survivors un-

derwent an autopsy, as a consequence lung body weight ratio's are not available. Two patients (both patients with hypoxic ischemic encephalopathy) in the control group died because of severe cerebral damage at day 6, respectively day 15 of life. Clinical characteristics are described in Table 1. We studied two CDH patients who did not require ECMO, one was born in our hospital and was included 27.5 hours after birth before surgical repair of the defect, the other was diagnosed three days after birth and was included after repair of the defect (at 107 hours after birth).

Ventilatory parameters. Nine of the CDH patients were ventilated with high frequency oscillation (HFO) before ECMO treatment, two CDH patients were conventionally ventilated. Ventilation and oxygenation characteristics of the CDH patients before the start of ECMO were as follows: mean airway pressure (MAP) was 13.5±1.4 mm Hg (18.3±1.9 cm H_2O), oxygenation index (OI= (MAP x FiO_2)/PaO_2) was 40.6±8.1, and the alveolar-arterial oxygen gradient ($AaDO_2$= PaO_2 – [(713 x FiO_2)-($PaCO_2$/0.8)]) was 593.0±10.2 torr (79 kPa). The 10 control patients were conventionally ventilated for 26±10 days following surgery or because of apneic attacks. These patients had mild ventilatory settings: FiO_2: 0.26±0.03, MAP: 5.9±0.6 mm Hg (8.0±0.8 cm H_2O), ventilatory rate: 31±4/min., positive inspiratory pressure (PIP): 14.1±1.3 mm Hg (19.2±1.8 cm H_2O), positive end-expiratory pressure (PEEP): 3.2±0.1 mm Hg (4.4±0.2 cm H_2O).

Figure. ^{13}C-enrichment of palmitic acid in surfactant phosphatidylcholine in sequential tracheal aspirates



Neonates with congenital diaphragmatic hernia on extracorporeal membrane oxygenation (CDH-ECMO) (closed circles) and control patients (open circles) received a 24-h [U-^{13}C]glucose infusion as precursor for phosphatidylcholine-palmitate. Data are expressed as mean and standard error of mean of time intervals of 12 and 24 hours, with the 24 hour interval started at 72 hours.

Surfactant kinetic parameters. The ^{13}C-enrichment of plasma glucose was in steady state (E_{gluc}) in all infants between t=6 and 24 hours, and was similar in both groups (2.0±0.1 *versus* 2.2±0.3, p=0.70). The Figure shows the ^{13}C-glucose incorporation of surfactant PC palmitate in sequential tracheal aspirates in the CDH-ECMO patients and control patients. The control group had a higher maximal enrichment and an earlier time of maximal enrichment compared to the CDH-ECMO group, which reflects more and faster precursor incorporation in the control group (Table 2). The FSR, calculated from the linear increase of each individual patient is significantly lower in the CDH-ECMO patients compared to the control patients (2.4±0.33 *versus* 8.0±2.4 %/day, *p*=0.04). The incorporation of ^{13}C from the precursor glucose into surfactant PC palmitate in tracheal aspirates (T_{app}) started somewhat later in the CDH-ECMO patients (18.0±2.6 *versus* 11.7±1.1 hours, *p*=0.11), which was followed by a later T_{max} in the CDH-ECMO patients compared to the control patients (69.8±6.6 *versus* 46.7±3.0 hours, *p*=0.004). The E_{max} in the CDH-ECMO patients was lower than in controls (0.09±0.02 *versus* 0.18±0.03 APE, *p*=0.04). The half-life of label disappearance from surfactant PC was not different between groups (Table 2). No correlation between surfactant kinetic parameters and patient characteristics were found, except for surfactant half-life and gestational age which were correlated negatively in the CDH-ECMO group (p=0.03).

Table 2. *Surfactant kinetic parameters and surfactant phosphatidylcholine (PC) concentration in epithelial lining fluid (ELF) during the first four days of the study*

	CDH-ECMO n=11	**Control** n=10	***p*-value**
FSR (%/day)	2.4±0.3	8.0±2.4	0.04
T_{app} (h)	18.0±2.6	11.7±1.1	0.11
T_{max} (h)	69.8±6.6	46.7±3.0	0.004
E_{max} (APE)	0.09±0.02	0.18±0.03	0.04
Half-life (h)	69.0±10.3	63.4±10.7	0.79
Egluc (APE)	2.0±0.1	2.2±0.3	0.75
PC (mg/ml ELF)	6.6±1.9	12.8±2.6	0.78

Data are expressed as mean±standard error of mean (SEM). The non-parametric Mann Whitney U-test was applied to compare groups.

In the two included CDH patients who did not require ECMO treatment the T_{app} was 13 and 26 hours, maximal enrichment was reached at 39 and 43 hours, with a maximal enrichment of 0.17 and 0.23 APE, followed by a surfactant half-life of 56.8 hours. Surfactant half-life could only be measured in one of the two patients due to the early extubation. FSR in the two CDH patients not on ECMO was 7 and 14 %/day. PC concentration in ELF during the first four days of the study was 3.7 and 7.6 mg/ml.

The surfactant PC concentration in ELF during the first four days of the study was equal in both groups (Table 2). No correlations were found between the surfactant PC concentration and the surfactant PC kinetic parameters, nor between the surfactant PC concentration and the OI, $AaDO_2$, and the gestational age.

Surfactant PC concentration in ELF during the first four days of the study was not different between the survivors and non-survivors of the CDH-ECMO group (9.5±3.6 *versus* 4.5±1.8 mg/ml ELF, p=0.27). There were also no differences in surfactant kinetic parameters between the survivors and non-survivors of the CDH-ECMO group. However, gestational age was lower in the non-survivors compared to the survivors of the CDH-ECMO group (37.1±0.6 *versus* 39.6±0.5 weeks, p=0.02).

Discussion

In the present study we report a significantly lower rate of surfactant PC synthesis in human CDH patients who require ECMO compared to control patients. The surfactant PC synthesis was studied *in vivo* with the use of stable isotopes, by measuring incorporation rates of precursor into surfactant PC (2.4±0.3 versus 8.0±2.4 %/day, p=0.04). The time of maximal enrichment (T_{max}) was reached later in the CDH-ECMO group and these patients also had a lower maximal enrichment (E_{max}) compared to the controls. Surfactant PC half-life was not different between the two groups. We measured surfactant PC concentration in ELF during the first 4 days of the study, which was not significantly different between the groups.

As in the present study, in vitro studies also found decreased PC synthesis (85, 87, 279). Type II cell cultures from rat fetuses with severe CDH showed a reduced activity of phosphocholine cytidyltransferase, which regulates the *de novo* synthesis of PC (279). There was a tendency of a decreased incorporation of choline into PC in isolated type II cells of CDH lambs compared to control lambs (85, 87). In another study in lambs the ipsilateral CDH lungs had a lower PC synthesis rate compared to the contralateral lungs (85). Recently, Bohlin *et al*. measured surfactant synthesis in term infants using ^{13}C-acetate as precursor (56). They found a decreased FSR in term infants with severe respiratory failure, similar to the FSR of preterm infants with respiratory distress syndrome, suggesting that a lower FSR might be reflective of severely injured and dysfunctional lungs. The FSR we found in our CDH patients is also similar to the FSR in preterm infants with RDS (~2.7 %/day), after ^{13}C-glucose infusion as precursor (54).

Seven CDH-ECMO patients, two control patients, and one CDH patient who did not require ECMO were also included in another study, in which we calculated total lung surfactant pool size (277). By multiplying the FSR by the pool size, we were able to calculate the 'absolute synthesis rate' (ASR). The mean ASR of 7 CDH-ECMO patients was 2.7±0.8 mg/kg/d, which is comparable with the ASR of premature infants (2.7±0.8 mg/kg/d) (63). In the two control patients the ASR was 11.2 and 11.5 mg/kg/d, and in the CDH patient who did not require ECMO the ASR was 5.7 mg/kg/d.

Our group recently studied surfactant disaturated PC (DSPC) synthesis and kinetics in infants with CDH who did not require ECMO (57). FSR in CDH patients was not different from

control subjects (~22 *versus* 17%/day), neither was the T_{app}, T_{max} and half-life of surfactant. However, the concentration of DSPC in ELF and surfactant protein-A in tracheal aspirates were significantly lower in CDH patients compared to controls. The most plausible explanation for the difference in surfactant synthesis is that the CDH infants in the present study required ECMO, and therefore have more severely hypoplastic lungs compared with CDH patients who do not require ECMO. In fact, in two CDH patients who did not require ECMO we found a higher FSR of ~11 %/day after infusion of labeled glucose. Also these sicker infants require more intensive ventilation before start of the ECMO which may cause even more damage of the lungs (129). By comparison, our CDH-ECMO patients were ventilated with a MAP of ~13 mm Hg (18 cm H_2O) before the start of ECMO, whereas the CDH patients who did not require ECMO had a MAP of ~8 mm Hg (11 cm H_2O) (57). The amount of pulmonary hypoplasia and lung damage due to the resulting vigorous ventilatory support could play a role in the differences in surfactant synthesis that we found.

An alternative explanation of the decreased surfactant synthesis in CDH patients who require ECMO is that these patients had severe pulmonary hypertension which necessitated ECMO treatment. It could be speculated that reduced blood flow through the lungs due to severe pulmonary hypertension provides less substrate for surfactant synthesis. In addition, during ECMO treatment the lung blood flow is also significant lower. Earlier, we found an increased surfactant synthesis in premature infants who had a clinical relevant persistent ductus arteriosus, a situation which leads to an increased lung blood flow (63). Furthermore, ECMO is known to result into increased vascular permeability and cytokine release which could influence surfactant kinetics (280).

From animal studies it is known that physical stretch of the alveoli stimulates surfactant synthesis and secretion (235). Physical stretch during ECMO treatment is reduced because low ventilatory settings (PIP 9-12 mm Hg) are used as part of the concept of "lung rest", and could possibly decrease surfactant synthesis. However, in a recent study in preterm infants we found no difference in surfactant synthesis ventilated with high frequency oscillation compared to conventional ventilation, which argues against the influence of stretch *in vivo* on surfactant metabolism (281).

Another explanation of the different results obtained by Cogo *et al*. is that these infants received [U-^{13}C]palmitic acid instead of [U-^{13}C]glucose we used. As our studies are the only one available in literature, it is unknown whether the use of another tracer determines the difference in results. Alternatively, CDH lungs could utilize other precursors (i.e. palmitate) preferentially, and it may be that the *de novo* synthesized fatty acids are a minor contributor to overall surfactant production in CDH lungs. Cogo *et al*. found lower concentrations of DSPC in ELF and SP-A in tracheal aspirates of CDH patients compared to controls (57). If a lower concentration of DSPC in ELF of CDH patients reflects a lower surfactant pool size, this could be associated with a higher FSR. The concentration of PC in ELF in the present study was not different during the first four days between the CDH patients on ECMO and controls (Table 2). We recently showed that surfactant PC pool size in CDH patients requiring ECMO was not decreased when compared with the pool sizes of neonates with meconium aspiration syndrome requiring ECMO and neonates who did not require ECMO (277).

The PC concentration in ELF of the CDH-ECMO patients during the first four days of the study was 6.6 mg/ml (Table 2), which is comparable with the data we found earlier in CDH patients who were on ECMO (~6.5 mg/ml), and with the results of CDH patients who did not require ECMO (~4 mg/ml) (100, 277, 57). In preterm infants, using the same method, a lower surfactant PC concentration was found (2.4 mg/ml) (244).

We found no significant difference of the PC concentration in ELF during the first four days of the study between the CDH-ECMO and the control group (p=0.08), which is in agreement with our earlier results (100, 277). Lower DSPC concentrations were found in human CDH patients who were not on ECMO (57). In comparison, in the lamb model lower concentrations of phospholipids and a lower percent of PC in CDH lungs were found compared to normal (84-87). Phospholipids and DSPC concentration were decreased in rats with CDH (92, 93).

Surfactant PC concentration in ELF during the study was not significantly lower in the non-survivors of our CDH-ECMO group, though in the literature a correlation between a surfactant component and survival have been mentioned (97, 144). SP-A concentrations in amniotic fluid were lower in CDH patients who died or required ECMO postnatally (97), and a lack of increase in SP-A concentrations was associated with unsuccessful weaning from ECMO (144). In our recent study we found that patients who needed a longer ECMO treatment had a longer surfactant PC half-life after administration of labeled surfactant (277). The non-survivors in the CDH-ECMO group had a shorter gestational age compared to the surviors (p=0.02). This may refer to a delayed maturation of the hypoplastic lung to play a role in the clinical outcome of CDH.

A limiting factor of our study is that it is not possible to determine the exact role of ECMO treatment on surfactant kinetics. As we already discussed above the lower blood flow through the lungs, the low ventilatory pressures, the increased vascular permeability and the release of cytokines during ECMO treatment could influence the surfactant synthesis. Another confounder may be that the control patients were significantly older at start of the study (~43 versus ~138 hours, p=0.03). This is because it is almost impossible to include newborn infants who are ventilated without significant lung disease. Nevertheless, we found no correlation between the age at start of the study and the surfactant kinetic parameters. In a study in which labeled acetate was given to term controls with and without respiratory failure no correlation was found between the age at start of the study and the FSR (Personal communication K. Bohlin). These data suggest that the age at start of the study is of no importance for the FSR in the age range of our patients.

In conclusion, the surfactant synthesis in neonates with CDH on ECMO is decreased compared to ventilated control patients. However, surfactant PC concentration in ELF during the first four days of the study and surfactant PC pool size was not decreased in CDH patients who required ECMO (277). We speculate that the severely disturbed morphology of the lungs of CDH patients who require ECMO increases the predisposition to develop lung damage. We cannot rule out that the amount of ventilatory support before ECMO treatment contributes to the morphological changes of the lung which are reflected into a decreased surfactant synthesis in the CDH patient on ECMO.

Acknowlegdments

We thank Kajsa Bohlin (Huddinge University Hospital, Karolinska Institute, dep Pediatrics, Stockholm, Sweden) for support. Roel Venrooij (medical student, Erasmus MC, Rotterdam, The Netherlands) for technical support, Wim van den Berg (Internal Medicine, Erasmus MC-Dijkzigt, Rotterdam, The Netherlands) for technical support, and the nursing staff, neonatologists, and intensivists of the neonatal and surgical intensive care units of the Erasmus MC-Sophia for their help and support during the study.

CHAPTER 7

Surfactant Phosphatidylcholine Metabolism in Human Neonates with Meconium Aspiration Syndrome

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Submitted

Abstract

Meconium directly inhibits surfactant function. However, the role of meconium on endogenous surfactant synthesis and clearance is not clear. We studied surfactant phosphatidylcholine (PC) metabolism in 11 neonates with meconium aspiration syndrome (MAS) who required extracorporeal membrane oxygenation (ECMO) with the use of stable isotopes. For comparison we studied 10 term neonates ventilated for non-pulmonary indications, and 6 neonates with persistent pulmonary hypertension (PPHN) who required ECMO. All patients received a 24 h [U-^{13}C]glucose infusion as precursor for palmitic acid in surfactant phosphatidylcholine. In the MAS group, the maximal ^{13}C-incorporation in PC was half of that in controls (0.09±0.01 versus 0.18±0.03 APE, *p*=0.027). There was a tendency of a lower surfactant synthesis in the MAS group (3.3±0.7%/day) and PPHN group (2.6±0.3%/day) compared to controls 8.0±2.4%/day, *p*=0.058), which was also reflected by a significantly lower PC concentration in the MAS group (4.4±2.6 mg/ml) and PPHN group (3.6±2.0 mg/ml) compared to controls (12.8±2.6 mg/ml, p=0.01). Endogenously synthesized surfactant had a similar half-life in all groups, ranging from 63 to 98 hours. We conclude that in at least the 'sickest' meconium aspiration patients who need ECMO, disturbed surfactant synthesis and concentrations play a role in the pathophysiology of MAS.

Introduction

Meconium aspiration syndrome (MAS) is a severe respiratory disorder in the term neonate. The exact pathophysiology is not yet clearly understood, but consists of mechanical obstruction of the airways by meconium, followed by a sometimes very severe inflammatory response and chemical pneumonitis. Aspiration of meconium inhibits surfactant function directly, it increases the minimum and maximum surface tension, and lowers the rate of the surface spreading of surfactant (130-134).

Studies about the surfactant concentration and composition in MAS are scarce. Analyses of bronchoalveolar lavage (BAL) fluid from 8 infants with MAS receiving mechanical ventilation revealed no difference in phospholipid and surfactant protein-A (SP-A) content compared to control subjects (143). In MAS patients who required extracorporeal membrane oxygenation (ECMO) surfactant total phospholipids, phosphatidylcholine (PC) and SP-A in tracheal aspirates increased during the ECMO treatment (144-146). Cleary *et al*. found decreased SP-A and SP-B levels in the large aggregates of surfactant in a rat model of MAS (141). However, phospholipid levels did not change significantly after meconium installation in either the lung tissue or BAL of animal models (141, 142).

The effect of surfactant therapy in neonates with MAS has been studied by several groups (6, 8, 152-155). Surfactant therapy showed an improvement in oxygenation and a decrease in ECMO requirement.

To our knowledge, only one study investigated the surfactant metabolism in the presence of meconium (147). This *in vitro* study showed that meconium in low concentrations (1%) increases the PC secretion by type II cells, but had no effect on surfactant synthesis.

Surfactant metabolism has never been studied in human neonates with MAS. In fact, the presence of a surfactant deficiency in MAS is still a matter of ongoing discussion. We studied the surfactant PC metabolism *in vivo* by stable isotopes techniques, in a group of neonates with the severest form of MAS, those who required ECMO.

Patients and Methods

Patients

The study was performed in the pediatric surgical and neonatal intensive care units of the Sophia Children's Hospital between October 1999 and April 2001. We studied three groups of neonates (Table 1): neonates with meconium aspiration syndrome who required veno-arterial ECMO (n=11), a group of neonates with persistent pulmonary hypertension of the newborn (PPHN) who required ECMO (n=6), and a group of ventilated term neonates without significant lung disease (control) (n=10). Patients of the PPHN group required ECMO for idiopathic PPHN (n=4), and pneumonia/sepsis (n=2). PPHN was established by cardiac ultrasound. The diagnoses of the 10 ventilated control patients were non-pulmonary: gastroschisis, cerebral infarction, omphalocele (n=2), anal atresia, thymic cyst, spina bifida, pentalogy of Cantrell, and hypoxic ischemic encephalopathy (n=2). Apart from the three study groups, we studied two patients with MAS who did not require ECMO. The study was approved by the local medical ethical committee and informed consent was

approved by the local medical ethical committee and informed consent was obtained at start of the study.

The entry criteria for ECMO were: gestational age ≥34 weeks; birth weight ≥2000 g; mechanical ventilation <7 days; and $AaDO_2$ >600 torr (>80 kPa) for 8 hours. During ECMO, ventilatory settings were usually reduced to peak inspiratory pressure of 12-16 cm H_2O, positive end-expiratory pressure of 5-6 cm H_2O, respiratory rate of 10-15 breaths/min, and FiO_2 of 0.25-0.3.

Isotope infusion and sample collection

All patients received a 24-hour continuous intravenous infusion of [U-^{13}C]glucose (0.17 mg/kg/min, Campro Scientific, Veenendaal, The Netherlands). The start of the study (t=0) was defined by the start of the isotope infusion. Before and during the label infusion, 1 ml blood was drawn every 6 h for determination of ^{13}C-enrichment of plasma glucose. Tracheal aspirates were obtained every 4 to 6 h during the time the infant was intubated, with a maximum of 2 weeks. The tracheal suctioning was performed during routine patient care and did not deviate form the normal clinical care.

Analytical procedure

The plasma and tracheal aspirates were processed as previously described (54, 57). Briefly, plasma glucose was isolated and derivatized to an aldonitril pentacetate derivative (245). Organic extraction was used to isolate surfactant lipids in the tracheal aspirates (246), and surfactant PC was recovered by thin layer chromatography (278). Isotopic enrichments were measured by gas chromatography mass spectrometry, as described before (54). The ^{13}C-enrichments were expressed as atom percent excess (APE).

Determination of composition and concentration

Fatty acid composition of surfactant PC and the amount of surfactant PC were analyzed on a gas-chromatograph (GC) (Hewlett-Packard, 5890 series II, Amstelveen, The Netherlands) (100). The concentration of surfactant PC in the epithelial lining fluid (ELF) was calculated by correcting for dilution of the ELF during endotracheal suction: dilution factor = $[urea]_{serum}/[urea]_{supernatant}$ (257), and is given as a mean value for the first 4 days of study.

Calculations

We measured the first appearance of ^{13}C in surfactant PC (T_{app}), the time of maximal enrichment (T_{max}), the maximum enrichment (E_{max}), the half-life of surfactant PC ($T_{½}$), and the fractional synthesis time (FSR) of palmitic acid in surfactant PC, which represents the percentage of the total PC-palmitate pool synthesized *de novo* from plasma glucose per day (54).

Data analyses

Data are presented as mean±standard error of the mean (SEM). One-way ANOVA was applied to compare groups (Post Hoc Tuckey), Spearman correlation test was used to evalu-

ate correlations between surfactant parameters and patient characteristics. Significance was accepted at a value of $p<0.05$.

Table 1. *Patients characteristics*

	MAS n=11	PPHN n=6	Control n=10
Gestational age (wk)	40.2±0.7	38.1±0.9	38.7±0.5
Birth weight (g)	3345±175	3412±202	3189±237
Apgar score at 5 min.	6.0±1.0	6.5±1.2	7.0±1.5
Male/Female	6/5	3/3	5/5
Time of ventilation (d)	13.8±2.5	16.2±2.8	25.6±9.9
Age at start study (h)	58.3±8.7	47.8±6.3	137.6±45.5
Survivors (n)	11	5	8
Age at start of ECMO (h)	27.9±6.5	16.0±4.3	-
Duration of ECMO (h)	156±27*	248±28	-
Oxygenation Index	41±7	40±13	-
$AaDO_2$	588±17	604±11	-

Results reported as mean ± standard error of mean (SEM). Oxygenation index ($OI=(MAP \times FiO_2)/PaO_2$; $AaDO_2$, alveolar-arterial oxygen tension difference ($AaDO_2=PaO_2 - [(713 \times FiO_2)-(PaCO_2/0.8)]$.
**Significantly different from the PPHN group ($p=0.035$).*

Results

Patients characteristics

Patients characteristics are depicted in Table 1. Four of the MAS patients on ECMO received surfactant treatment as rescue therapy (100 mg/kg Survanta®), and one of the four surfactant treated infants received two doses of surfactant. Surfactant treatment was given before the start of the study. As comparison we studied a group of ventilated neonates without significant lung disease and a group of patients with PPHN who required ECMO. Four patients of the PPHN group received surfactant, one of these received two doses of surfactant before start of the study. The three groups were comparable with regard to the patients characteristics, except for duration of ECMO, which was longer in the PPHN group compared to the MAS group on ECMO ($p=0.035$). The two additional MAS patients who did not require ECMO (not in Table 1) were both male and had comparable gestational age and birth weight as the other patient groups. The age at start of the study was ~50 hours, and they were ventilated for ~8 days. One of the MAS patients who did not require ECMO was treated with two doses of surfactant before the start of the study.

Ventilation characteristics

Seven patients of the MAS group were conventionally ventilated before the start of ECMO, four patients were ventilated with high frequency oscillation after failure of conventional ventilation. The mean airway pressure (MAP) of the MAS patients pre-ECMO was 20.1±0.6 cm H_2O. All patients of the PPHN group were conventionally ventilated with a pre-ECMO MAP of 16.3±1.3 cm H_2O. Pre-ECMO Oxygenation index (OI) and alveolar-arterial oxygen tension difference ($AaDO_2$) are reported in Table 1. The 10 control patients were conventionally ventilated following surgery or because of apneic attacks. These patients had mild ventilatory settings: FiO_2: 0.26±0.03, MAP: 8.0±0.8 cm H_2O, and a positive inspiratory pressure (PIP): 19.2±1.8 cm H_2O. The two MAS patients not on ECMO were conventionally ventilated with a MAP of 13 and 14 cm H_2O, an OI of 12 and 14, and a $AaDO_2$ of 543 and 573.

Surfactant kinetics

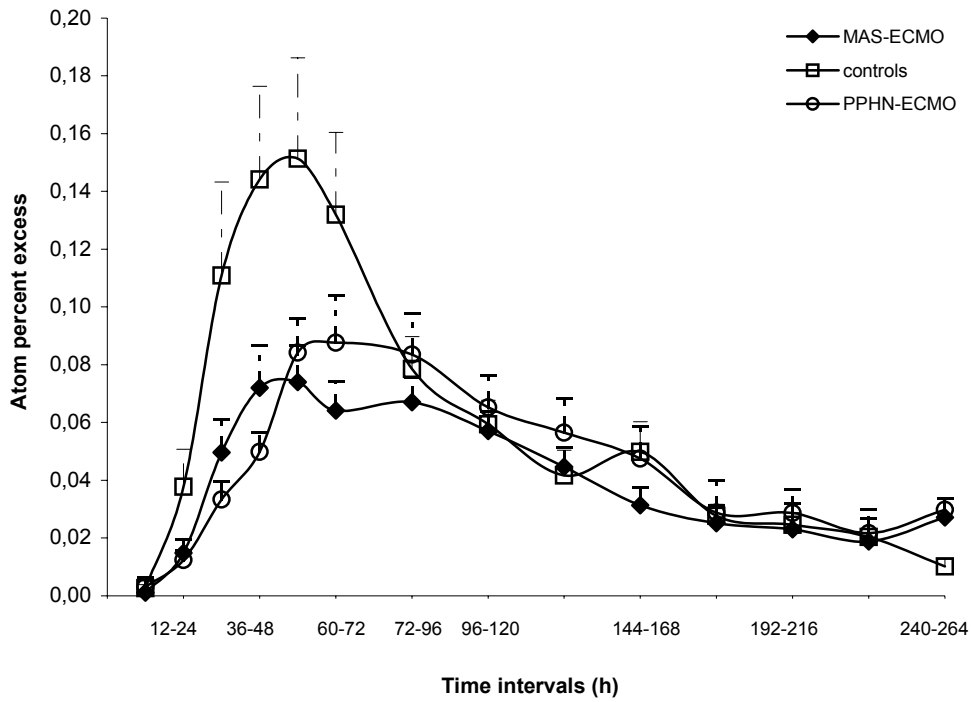
The ^{13}C-enrichment of plasma glucose (E_{gluc}) was in steady state in all infants between t=6 and t=24, and was similar in all groups (Table 2). Figure shows the ^{13}C-glucose incorporation in surfactant PC palmitate in sequential tracheal aspirates in the three study groups. The kinetic parameters of surfactant are reported in Table 2. The T_{app} was similar in the groups and was ~12 hours, followed by a T_{max} which was significantly longer in the PPHN-group compared to controls (p=0.017). From Figure and Table 2 it is also clear that E_{max} was significantly lower in the MAS group compared to controls (p=0.027). The surfactant PC half-life was not different between groups. There was a trend to a lower FSR in the MAS and PPHN group compared to the control group (p=0.058). The two MAS patients who did not require ECMO treatment had the following surfactant kinetic parameters: E_{gluc} 2.4 and 3.0 APE, T_{app} 8 and 16 hours, T_{max} in both patients 49 hours, E_{max} 0.05 and 0.08 APE, half-life 43 and 76 hours, and FSR 1.3 and 2.1 %/day. If these two patients were included, then the MAS patients had a significantly lower FSR compared to the control group (p=0.035).

The surfactant PC concentration in ELF during the first four days of the study was significantly lower in the MAS and PPHN group compared to the controls (p=0.011, Table 2). No correlations were found between the surfactant PC concentration and the surfactant kinetic parameters, OI, $AaDO_2$, and gestational age. The two MAS patients not on ECMO had PC concentrations of 0.9 and 9.5 mg/ml ELF. The patients with a PC concentration of 9.5 mg/ml received two doses of surfactant before the start of the study.

In the MAS group the duration of ECMO was shorter in the patients who were treated with surfactant before the start of ECMO (p=0.023). This relation between surfactant treatment and duration of ECMO was not observed in the PPHN group.

Fatty acid composition of PC (C14:0: myristic acid ~5%, C16:0: palmitic acid ~63%, C16:1ω7 ~3.5%, C18:0 ~7%, C18:1ω9 ~7%, C18:2ω6: linoleic acid ~3%) in tracheal aspirates during the first four days was not different between groups.

Figure. *^{13}C-enrichment of palmitic acid in surfactant phosphatidylcholine (PC) in sequential tracheal aspirates*



MAS patients (closed triangles), control patients (open squares) and PPHN patients (open circles) received a 24-h [U-^{13}C]glucose infusion as precursor for PC-palmitate. Enrichment is represented as Atom Percent Excess (APE). Data are expressed as mean and standard error of mean (SEM) of time intervals of 12 and 24 hours, with the 24 hour interval started at 72 hours.

Discussion

This is the first report on surfactant metabolism in human neonates with MAS who require ECMO. With the use of stable isotopes we studied surfactant PC palmitate metabolism *in vivo* in three patient groups. In the MAS patients who required ECMO the maximal enrichment (E_{max}) was lower compared to controls (Table 2 and Figure). The MAS patients as well as the PPHN patients tended to have a lower surfactant synthesis (FSR) compared to the controls (p=0.058). The suggestion of a lower surfactant PC synthesis in the ECMO groups was reflected by lower PC concentrations in ELF during the first four days of the study (~4 mg/ml in MAS and PPHN group versus ~12 mg/ml in controls). Furthermore, if 2 MAS patients who did not require ECMO were included, then the FSR was significantly lower in the MAS group compared to controls (p=0.035).

Table 2. *Surfactant kinetic parameters and surfactant phosphatidylcholine (PC) concentration in epithelial lining fluid (ELF) during the first four days of the study*

	MAS	PPHN	Control	*p*-value
	n=11	n=6	n=10	
E_{gluc} (APE)	2.0±0.2	2.0±0.2	2.2±0.3	0.798
T_{app} (h)	12.6±1.5	13.0±2.0	11.7±1.1	0.815
T_{max} (h)	50.4±4.2	64.1±3.4*	46.7±3.0	0.017
E_{max} (APE)	0.09±0.01*	0.11±0.01	0.18±0.03	0.027
FSR (%/day)	3.3±0.7	2.6±0.3	8.0±2.4	0.058
Half life (h)	68.5±11.8	98.1±27.2	63.4±10.7	0.305
PC (mg/ml ELF)	4.4±2.6*	3.6±2.0*	12.8±2.6	0.011

*Data are expressed as mean±standard error of mean (SEM). Differences between groups were analyzed by one-way ANOVA. *Different from controls.*

In isolated type II cells of rats, meconium in low concentrations (1%) has no effect on surfactant PC synthesis, though the secretion of surfactant PC is increased (147). The same study showed that higher meconium concentrations were toxic to the cultured type II cells, though the effect of these higher concentrations on surfactant synthesis is not known. The chemical pneumonitis caused by the aspiration of meconium could influence surfactant synthesis by damage to the type II cells. Moreover, the release of cytokines due to the inflammation has inhibitory effects on surfactant synthesis (282).

The PC concentration in ELF during the first four days of the study was decreased in the MAS group on ECMO (~4 mg/ml). In animal studies total phospholipid and dipalmitoyl PC levels in either lung tissue or bronchoalveolar lavage were not different after meconium installation compared to controls (141, 142). In bronchoalveolar lavage from infants with MAS who did not receive ECMO, disaturated PC (DSPC) concentration (~2 mg/ml) was not different from control patients (143). The lower PC concentration in the present study could be explained by more severe lung disease. In MAS patients who required ECMO total phospholipid and DSPC concentration in tracheal aspirates were lower at the beginning of ECMO, and increased significantly during ECMO treatment (145). Unfortunately, in that study no control group was included. In a recent study in which we measured surfactant PC pool size in MAS patients who required ECMO, we found no differences in pool size compared to CDH patients who required ECMO and non-ECMO patients (277). However, the MAS-ECMO patients tended to a lower surfactant PC pool size compared to the two other groups (80 versus 118 mg/kg).

Four MAS patients on ECMO, two control patients, and one MAS patient not on ECMO of the present study were also included in another recent study (277), in which we measured total lung surfactant PC pool size. In the four MAS patient on ECMO we were able to calculate “absolute synthesis rate” (ASR). By multiplying the FSR by the pool size, we found an ASR of 1.3±1.0 mg/kg/d surfactant PC synthesized from glucose. In the two control pa-

tients, the ASR were higher (11.2 and 11.5 mg/kg/d). The MAS patient who did not receive ECMO had an ASR of 7 mg/kg/d, which was not due to surfactant therapy, as this patient did not receive any surfactant treatment. ASR in premature infants was calculated to be 2.7±0.8 mg/kg/d (63). The MAS-ECMO patients seem to have a lower ASR, which means that the tendency of a lower FSR, which we found in the present study, is not caused by a higher dilution of the precursor due to a higher surfactant PC pool size.

Besides a suggested disturbed surfactant metabolism, also surfactant inactivation plays a role in MAS. Several proteins, lipids and free amino acids can inactivate surfactant in several ways (253). In the present study we did not measure surfactant activity by dynamic surface pressure measurements, nor did we measure surfactant inhibitors, like albumin in the tracheal aspirates. We analyzed the fatty acid composition of surfactant PC in tracheal aspirates during the first four days, which was not different compared to controls.

We found that MAS patients who were treated with surfactant before the start of ECMO had a shorter duration of ECMO compared to non-treated MAS patients. This is similar to the findings in a randomized study in ECMO treated patients (146). The clinical efficacy of exogenous surfactant therapy in MAS has been suggested by several non-randomized studies (6, 8, 152, 153) and two randomized controlled trials (154, 155). From these studies it could be suggested that surfactant treatment in MAS is most effective when given in the early phase of the respiratory failure and at a high dose.

The question whether the disturbed surfactant metabolism is a result of the underlying condition or a consequence of ECMO treatment remains unanswered. It seems clear that patients who require ECMO have more damaged lungs compared to patients who do not require ECMO treatment. The sick lungs will be even more damaged due to the intensive ventilation prior to the ECMO treatment. We studied a group of patients with PPHN who required ECMO. These patients also show a trend of a lower FSR compared to the controls, which suggests a role of ECMO on surfactant metabolism. However, two patients with MAS who did not require ECMO had a FSR in the same range as the ECMO treated patients (1.3 and 2.1 %/day). In another study, the surfactant PC and SP-A in tracheal aspirates increase in patients on ECMO, suggesting no detrimental effect of ECMO (144-146).

During ECMO the lung blood flow is lower, and it could be speculated that a reduced blood flow through the lungs provides less substrate for surfactant synthesis. We found an increased surfactant synthesis in premature infants who had a clinically relevant persistent ductus arteriosus, a situation which leads to an increased lung blood flow (63). From animal studies it is known that physical stretch of the alveoli stimulates surfactant synthesis and secretion (235). As physical stretch is low during ECMO treatment due to the low ventilatory settings as part of the concept of “lung rest”, this could possibly decrease surfactant synthesis. A result of ECMO treatment is an increase in capillary permeability, which together with a release of cytokines change surfactant function. This is even more plausible in an ECMO population in whom lung damage before ECMO may contribute to capillary protein leakage.

In summary, infants with MAS have normally developed lungs. Due to the direct effect of meconium and the inflammatory response in the lung, surfactant function in MAS is inhibited. In this study a disturbed surfactant synthesis and concentration is suggested at least in the sickest MAS patients who required ECMO. Further studies have to reveal the effect of ECMO on surfactant metabolism.

Acknowlegdments

The authors express their sincere thanks to Darcos Wattimena and Wim van den Berg (Internal Medicine, Erasmus MC, Rotterdam, The Netherlands) for support and technical advice, to Aaron Hamvas (Washington University/St. Louis Children's Hospital, St. Louis, USA) for support, to Esther van Emmen (medical student, Erasmus MC, Rotterdam, The Netherlands) for technical support, and to the nursing staff, neonatologists, and intensivists of the neonatal and surgical intensive care units of the Erasmus MC-Sophia for their help and support during the study.

CHAPTER 8

Surfactant Phosphatidylcholine Metabolism in Human Neonates with Surfactant Protein-B Deficiency

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Submitted

Abstract

Neonates with inherited surfactant protein-B (SP-B) deficiency develop a lethal respiratory insufficiency after birth. Because there are major ultrastructural changes in the SP-B deficient lung, disorganized tubular myelin, lamellar bodies, and SP-C, we expected a severely disturbed surfactant phosphatidylcholine (PC) metabolism. Surfactant PC metabolism was measured *in vivo* using stable isotopes. Four SP-B deficient infants and 10 controls received a 24 h [U-^{13}C]glucose infusion. One SP-B deficient infant and 5 controls received a 24 h [U-^{13}C]palmitic acid infusion. Isotopic enrichment was measured in palmitic acid of surfactant PC. Fractional synthesis rate (FSR) of surfactant PC after [U-^{13}C]glucose was 4.7±2.4%/day. Maximal enrichment was reached at 48.4±8.3 hours, followed by a half-life of 65.4±24.8 hours. Controls had similar data. After [U-^{13}C]palmitic acid the FSR was 65% in SP-deficiency and only 17.0±11.0% in controls. Time of maximal enrichment was 36 versus 51.0±9.5 hours and half-life was 35.7 versus 43.0±6.0 hours in the SP-B deficient patient and controls, respectively. PC content in tracheal aspirates were also not different between SP-B patients and controls. Despite lethal respiratory insufficiency, aberrant surfactant, and severe structural changes of the lung, the overall surfactant PC kinetics seem not disturbed in SP-B deficiency.

Introduction

Surfactant protein B (SP-B) is a hydrophobic peptide with several functions related to alveolar surface tension reduction and metabolism of pulmonary surfactant (23). SP-B enhances the rate of adsorption and surface spreading of the phospholipids, it plays a role in the formation of the surface active layer of surfactant, and in the organization and turnover of pulmonary phospholipids. Furthermore, SP-B counteracts the inhibition of surfactant function by plasma proteins, and contributes to the recycling of surfactant from the alveolar space (22). In humans the SP-B is encoded by a 9.5 kb gene that contains 11 exons on chromosome 2, and is expressed exclusively in the epithelium of the lung (23). Mature SP-B is detectable in the human lung after 19 weeks of gestation, and is found in the amniotic fluid after 31 to 33 weeks of gestation (179).

Inherited SP-B deficiency is a lethal disease only identified in term neonates, to date. It was first recognized in 1993 by Nogee *et al* (24), and it is characterized by the absence of SP-B, an aberrant form of SP-C (174), and altered phospholipid composition (283). Ultrastructural studies show disorganized lamellar bodies and absence of tubular myelin (25). The most common mutation described to date is a net 2 base-pair insertion at codon 121 in exon 4 of the SP-B gene (121ins2), causing a frameshift and premature stop signal followed by termination of the translation. Also other mutations have been identified resulting in total or partial SP-B deficiency (181-187). Clinically, neonates with SP-B deficiency present with severe progressive respiratory distress which does not respond to surfactant therapy, corticosteroid administration, or extracorporeal membrane oxygenation (ECMO) (177). The infants will die in the first few weeks of life, unless a lung transplantation is performed.

Because of the ultrastructural changes in SP-B deficient lungs, the altered surfactant phospholipid composition, and the severity of the lung disease, we hypothesized that the production and clearance of surfactant phosphatidylcholine (PC) would be disrupted. We therefore studied the surfactant PC metabolism *in vivo* in neonates with a congenital SP-B deficiency and in a control group with the use of stable isotopes (54, 64).

Methods

Subjects

The SP-B deficient infants were included in different neonatal intensive care units; one infant was studied in the Sophia Children's Hospital in Rotterdam (The Netherlands), one in the St. Louis Children's Hospital (USA), one in the A.Z. Sint-Jan Hospital in Brugge (Belgium), one in the Universitätsklinikum der RWTH Aachen (Germany), and one in the University Hospital of Padua (Italy). The diagnoses of the 15 ventilated control patients varied and overall no primary pulmonary anomaly or sign of abnormal lung development was present: gastroschisis, cerebral infarction, omphalocele, anal atresia, thymic cyst, spina bifida, pentalogy of Cantrell, and hypoxic ischemic encephalopathy. The study was approved by the local medical ethical committee and informed consent was obtained at start of the study.

Isotope infusion and sample collection
Four SP-B deficient patients and 10 controls received a 24-hour intravenous infusion of [U-^{13}C]glucose (0.17 mg/kg/min, Campro Scientific, Veenendaal, The Netherlands). One SP-B deficient patient and 5 controls received a 24 hour intravenous infusion of [U-^{13}C]palmitic acid (Martek Biosciences, Columbia MD), as previously described (64). The start of the study (t=0) was defined by the start of the isotope infusion. Before and during the label infusion, 1 ml blood was drawn every 6 h for determination of ^{13}C-enrichment of plasma glucose or plasma palmitic acid. Tracheal aspirates were obtained every 4 to 6 h during the time the infant was intubated, with a maximum of 2 weeks. The tracheal suctioning was performed during routine patient care and did not deviate form the normal clinical care. Tracheal aspirates were immediately placed at -20°C, until further processing.

Analytical procedure
The plasma and tracheal aspirates were processed as described before (54, 57). Briefly, plasma glucose was isolated and derivatized to an aldonitril pentacetate derivative and plasma palmitic acid was derivatized to a methyl-ester (245). Organic extraction was used to isolate surfactant lipids in the tracheal aspirates (246), and total surfactant PC was recovered by thin layer chromatography (278). In the patients who received labeled palmitic acid disaturated PC (DSPC) was purified from tracheal aspirates (54, 57). Isotopic enrichments were measured by gas chromatography mass spectrometry, as described before (54, 57, 64). The ^{13}C-enrichments were expressed as atom percent excess (APE) when [U-^{13}C]glucose was administered, and mol percent excess (MPE) when [U-^{13}C]palmitic acid was administered.

Determination of composition and concentration
Fatty acid composition of surfactant PC and the amount of surfactant PC were analyzed on a gas-chromatograph (GC) (Hewlett-Packard, 5890 series II, Amstelveen, The Netherlands) (100). The concentration was determined by original sample volume and expressed as µmol per tracheal aspirate (µmol/TAS).

Calculations
We measured time of first appearance of enrichment *(*T_{app}*)*, time of maximal enrichment (T_{max}), maximal enrichment (E_{max}), and half-life of surfactant (DS)PC ($T_{½}$). Fractional synthesis rate (FSR) of palmitic acid in surfactant PC represents the percentage of the total (DS)PC-palmitate pool synthesized *de novo* from plasma glucose respectively plasma palmitic acid per day (54).

Data analyses
Data are presented as mean±standard error of the mean (SEM). Differences between groups were analyzed by Student T-test. Significance was accepted at $p<0.05$.

Table 1. *Clinical parameters of patients*

	^{13}C-Glucose		^{13}C-Palmitic Acid	
	SP-B deficient n=4	Controls n=10	SP-B deficient n=1	Controls n=5
Gestational age (wk)	39.4±0.5	38.7±0.5	40	38.4±2.4
Birth weight (g)	3435±221	3171±231	3400	2700±600
Male/Female	3/1	5/5	0/1	3/2
Age at start study (d)	44.5±15.3	5.4±1.7	30	96.3±73.4
Surfactant treatment	4/4	0/10	1/1	0/8
Survivors	1/4	8/10	0/1	4/5

Data expressed as mean±SEM.

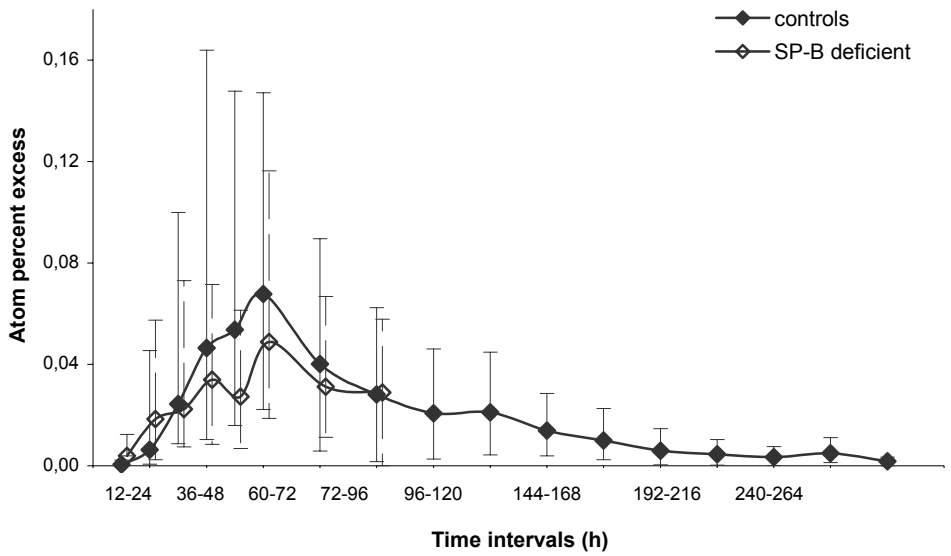
Results

All SP-B deficient patients had a 121ins2 mutation (the most common mutation), and all except two died during the neonatal period (at 36±8 days of life). One survivor underwent a lung transplantation at four months of age and another one died at the age of 6 months. Three patients (all patients with hypoxic ischemic encephalopathy) in the control group died at 6, 15 and 25 days of age because of severe cerebral damage.

Clinical parameters are described in Table 1. All SP-B deficient patients were ventilated from birth to death or lung transplantation. One SP-B deficient patient was treated with extracorporal membrane oxygenation (ECMO) during 10 days, starting at day 8 after birth. This patient was studied during the time he was on ECMO. The 10 control patients who received labeled glucose were conventionally ventilated for 26±10 days following surgery or because of apneic attacks. These patients had mild ventilatory settings: FiO_2: 0.26±0.03, mean airway pressure (MAP): 8.0±0.8 cm H_2O, ventilatory rate: 31±4, positive inspiratory pressure (PIP): 19.2±1.8 cm H_2O, positive end-expiratory pressure (PEEP): 4.4±0.2 cm H_2O. The 5 control patients who received labeled palmitic acid were conventionally ventilated for 21.2±10.0 days, with the following settings: FiO_2: 0.24±0.3, mean airway pressure (MAP): 3.2±0.2 cm H_2O.

The Figure shows the ^{13}C-glucose incorporation of surfactant PC palmitate in sequential tracheal aspirates in the SP-B deficient patients and control patients. ^{13}C-enrichments were corrected for the mean plasma glucose enrichments of each patient. Both curves show the same course of incorporation of the ^{13}C label. We found no significant difference in enrichment at each time interval between the SP-B and the control group. The curve of the SP-B deficient patients is shorter because in three of the four patients treatment was stopped soon after the diagnosis became clear.

Figure. *^{13}C-enrichment of palmitic acid in surfactant phosphatidylcholine (PC) in sequential tracheal aspirates, corrected for plasma glucose enrichment*



SP-B deficient patients (open triangles) and control patients (closed triangles) received a 24-h [U-^{13}C]glucose infusion as precursor for PC-palmitate. Data are expressed as median and interquartile ranges of time intervals of 12 and 24 hours, with the 24 hour interval started at 72 hours.

Table 2 reports the metabolic indices of endogenous surfactant synthesized from glucose. There were no differences in T_{max}, $T_{½}$, and FSR between the SP-B deficient patients and the control patients. The incorporation of ^{13}C from the precursor glucose into surfactant PC palmitate in tracheal aspirates started at 9.4±2.7 hours in the control group (=T_{app}). The T_{app} of the SP-B deficient patients could not be calculated reliably. Also the metabolic indices of endogenous surfactant synthesized from palmitic acid are shown in Table 2. The SP-B deficient patient who received ^{13}C labeled palmitic acid as precursor had a FSR of 65%/day, whereas the 5 controls had a FSR of 17.0±11.0%/day.

E_{max} corrected for the precursor enrichment (= plasma glucose enrichment) in the patients who received labeled glucose, was not different between groups (Table 2 and Figure). The mean plasma glucose enrichment for the 4 SP-B deficient patients was 1.2±0.5 APE and 2.3±0.4 APE for the 10 control patients (p=0.1). If we did not correct for the plasma glucose enrichment E_{max} was significant lower in the SP-deficient patients compared to the control patients (0.05±0.01 versus 0.18±0.04 APE, p=0.04). This could be explained by a lower plasma glucose enrichment in SP-B deficient patients possibly due to a higher non-labeled glucose administration compared to the control group.

Mean PC amount in tracheal aspirates of SP-B deficient patients and control patients did not reach statistical significance, but the mean value in the SP-B deficient group was about 40% of the value of the controls (0.23±0.19 versus 0.62±0.17 µmol/TAS, p=0.22).

Fatty acid composition of PC from tracheal aspirates was also not different between the two groups, except for stearic acid (C18:0) which was significantly higher in the SP-B deficient group (table 3). Mean DSPC in tracheal aspirates of the SP-B deficient patient who received labeled palmitic acid was 0.21 µmol/ml, whereas in the 5 controls mean DSPC was 2.3±0.2 µmol/ml.

Table 2. *Metabolic indices of endogenous surfactant synthesized from glucose and palmitic acid*

	^{13}C-Glucose			^{13}C-Palmitic Acid	
	SP-B deficient n=4	Controls n=10	*p*-value	SP-B deficient n=1	Controls n=5
FSR (%)	4.7±2.4	7.8±2.4	0.46	65.0	17.0±11.0
T_{max} (h)	48.4±8.3	48.4±8.3	0.81	36.0	51.0±9.5
E_{max} (APE/MPE)	0.06±0.03*	0.10±0.03*	0.39	0.20	0.22±0.05
Half-life (h)	65.4±24.8	63.4±10.7	0.93	35.7	43.0±6.0
T_{app} (h)	-	-	-	13.9	8.5±2.5

** E_{max} is corrected for plasma glucose enrichment (maximum enrichment of surfactant PC palmitate / glucose plasma enrichment). No significant differences were found in surfactant kinetic indices between the two groups.*

Discussion

SP-B deficiency leads to a disorganized intracellular structure of the pulmonary type II cell, including a lack of mature lamellar bodies and absence of tubular myelin (25, 175). Furthermore, surfactant phospholipid composition is altered (283). These structural and compositional abnormalities suggest multiple effects on surfactant synthesis, secretion and catabolism. We studied the endogenous surfactant PC metabolism *in vivo* in SP-B deficient human neonates using stable isotopes. SP-B deficient patients who received an infusion of labeled glucose had a FSR of endogenous surfactant PC palmitate of ~4.7 % per day, with a maximal surfactant PC palmitate enrichment at ~48 hours, and a half-life of disappearance of label of ~65 hours. These data indicate that in SP-B deficient patients the PC synthesis from plasma glucose and PC half-life are not markedly different from control patients without lung disease.

This is the first study that describes the surfactant PC metabolism *in vivo* in human neonates with an inherited SP-B deficiency. Only two studies of the surfactant metabolism in SP-B deficiency have been performed, one in animals (190), and one in human lung tissue obtained at transplant (194). Our results are comparable with findings in SP-B knock-out mice (190). These mice die within minutes after birth, have poorly compliant lungs, and express the aberrant SP-C. Ultrastructural examination of the lungs revealed an absence

of lamellar bodies and tubular myelin (189). However, phospholipid content in lungs from SP-B knockout mice was not altered compared to normal mice. Neither was the incorporation of labeled choline and labeled palmitic acid in lung saturated PC of SP-B deficient fetuses after injection of the labels in pregnant mice (190).

Beers *et al*. investigated the phospholipid content, composition and synthesis in lung tissue and lavage fluid acquired at transplantation or postmortem from 10 SP-B deficient infants (194). They found elevated phosphatidylinositol and percent disaturated PC in lung tissue, and decreased levels of phosphatidylglycerol in both lavage and lung tissue compared to normal lungs. As in our study, no difference between SP-B deficient infants and controls was found in percentage of palmitic acid in lung lavage.

Table 3. *The mean concentration of phosphatidylcholine (PC) and the fatty acid composition of PC in tracheal aspirates (TAS) from the patients who received ^{13}C-glucose*

	SP-B deficient n=3	**Controls** n=7	***p*-value**
PC (µmol/TAS)	0.23±0.19	0.62±0.17	0.22
C16:0 (%)	61.0±1.1	58.8±4.4	0.75
C18:0 (%)	17.9±0.7	5.7±1.2	0.0002
C18:1ω9 (%)	8.3±3.0	10.5±2.9	0.67
C18:2ω6 (%)	3.6±1.3	4.4±1.1	0.67

Data are expressed as mean±SEM. C16:0 (palmitic acid), C18:0 (stearic acid), C18:1ω9 (oleic acid), C18:2ω6 (linoleic acid).

Beers also studied the synthesis of phospholipids by determining the rate of incorporation into phospholipids after incubation of explanted lung tissue with three different labeled lipid precursors: choline, glycerol and acetate (194). The incorporation rate of choline into PC and acetate into phospholipids was increased in lung tissue from SP-B deficient infants compared to lung tissue of infants with chronic lung disease, but was not different when compared to normal lungs. The incorporation rate of glycerol into PC was not different between groups. In this study, infants with chronic lung disease who underwent lung transplantation were used as comparison because they had undergone the same therapeutic interventions as the SP-B deficient patients. The difference in incorporation could also be due to a lower surfactant synthesis in patients with chronic lung disease. At least, they found no decreased incorporation in SP-B deficient lung tissue.

We also studied one SP-B deficient patient who received labeled palmitic acid. This patient had a much higher FSR compared to the 5 control patients (65 % versus ~17.0 %). Unfortunately, we only had the opportunity to study one SP-B deficient patient with labeled palmitic acid as precursor. This makes it difficult to compare the data with the data of the controls. A possible explanation for the high FSR could be a low surfactant pool size in

the SP-B deficient patient. This is also suggested by the lower DSPC concentration in tracheal aspirates compared to the controls (0.015 mg/ml versus 0.17 mg/ml). The higher FSR could also be due to the kind of label used. Beers *et al*. showed a higher incorporation rate in SP-B deficient tissue when choline and acetate were used, but not when glycerol was used as precursor (194). Nevertheless, these additional data show also no impaired surfactant synthesis in SP-B deficiency.

The method we used to study the surfactant metabolism *in vivo* was first described by Bunt *et al* (54). Surfactant kinetics in preterm infants with respiratory distress syndrome after prenatal corticosteroids and postnatal surfactant administration were studied. The FSR in these preterm infants was ~2.7 %/day and half-life was ~113 hours. Preterm infants seem to have a slower surfactant metabolism compared to our SP-B deficient patients and controls.

Our study has certain limitations. One is that the SP-B deficient patients were significantly older at start of the study compared to the control patients (~45 versus ~5 days, p=0.001). It would have been more appropriate to include age-matched control patients. It is however very difficult to find infants of one or two months of age, without significant lung disease, who are ventilated for several days. The control patients in our study were included during the first week of life, except two patients who were included at day 11 and day 18 of life. These two patients also had a higher FSR of ~21 %/day. In a study in which labeled acetate was given to term controls with and without respiratory failure no correlation existed between the age at start of the study and the FSR (Personal communication K. Bohlin). From this we suggest that the age at start of the study is of no importance in relation to the FSR.

The number of included SP-B deficient patients in the present study is low. But with a gene frequency of approximately one 121ins2 mutation per 1000-3000 individuals (284), the incidence of a SP-B deficiency is rare. This low incidence of the disease is also the reason why our four SP-B deficient infants were studied in four different clinical centers. Besides the rarity of the disease it was also difficult to include SP-B deficient neonates in the study because in most cases treatment was stopped the moment the diagnosis was known.

In conclusion, although normal lamellar bodies and tubular myelin are absent in inherited SP-B deficiency, the overall surfactant PC palmitate synthesis from plasma glucose and its half-life seem not disturbed. Surfactant PC fractional synthesis rate is not decreased in SP-B deficient neonates, although no information is available on absolute synthesis. Probably, other factors such as failure in the formation of a surface active film in the alveoli, a disturbed recycling and uptake of surfactant, and a disturbed metabolism of surfactant proteins contribute to the clinical course of this disease.

Acknowledgements

The authors express their sincere thanks to Dr. P. van Laer (Pediatrics, St. Jan hospital, Brugge, Belgium) and Prof. Dr. H. Hoernchen (Kinderklinik, Universitätsklinikum der RWTH, Aachen, Germany) for their help and support with the inclusion of SP-B deficient patients, and to the nursing staff of all the hospitals that cooperated with this study for their help and support.

CHAPTER 9

Summary, General Discussion & Future Perspectives

Summary and interpretation of the studies

Surfactant metabolism was measured in premature infants and baboons with respiratory distress syndrome, and term infants with severe lung disease. Understanding of the metabolism of surfactant is of importance for the development of therapeutic strategies that are specific to the mechanism of metabolic disruption. The method employing the safe non-radiating stable isotopes to study surfactant metabolism was developed recently by us (54, 62). The endogenous surfactant metabolism was studied by a 24-h infusion with the stable isotope [U-^{13}C]glucose as a precursor for palmitic acid of surfactant PC. During the period of intubation, tracheal aspirates were collected to obtain serial time points to measure incorporation of the stable isotope ^{13}C into surfactant PC palmitate. Fractional synthesis rate (FSR), which is the percentage of the total surfactant pool synthesized from glucose per day, and half-life of endogenous surfactant were calculated.

In preterm infants with RDS, surfactant metabolism studied with the these methods is slow (232). Prenatal corticosteroids and surfactant therapy were found to be stimulating for the endogenous surfactant synthesis in preterm infants. Physical stretch is another factor which stimulates surfactant synthesis and function (235). During conventional ventilation, repetitive alveolar distension stimulates surfactant synthesis and secretion. In contrast, during high frequency oscillation (HFO) this alveolar excursion may be limited, which could result in a lower surfactant synthesis and secretion. We studied surfactant metabolism in premature infants with RDS, randomized for either HFO or conventional ventilation (**Chapter 3**). FSR from glucose did not differ between groups (4.7 *versus* 4.2%/day), neither did the half-life of endogenous surfactant (~79 *versus* ~76 hours). The infants ventilated with HFO appeared to have more severe lung disease, which could be due to the way of randomization at 24 hours of age. Infants who were already receiving HFO as part of their routine care continued to receive it, infants on CV at 24 hours were randomized to HFO or CV. The fact that we did not detect differences in surfactant metabolism between the two groups could be explained by several reasons. One of these reasons could be the sicker lungs of the infants in the HFO group. However, if a lower surfactant synthesis is expected in the HFO group due to less alveolar stretch, this lower synthesis would be even lower in sicker lungs. It could be that HFO has a protective role on surfactant metabolism, because it combats the overexpansion of the alveoli. And, in this way may mask the decreased surfactant synthesis due to the severe lung disease. The study also showed that premature infants who died of refractory respiratory failure seem to have a shorter half-life of endogenous surfactant.

We measured surfactant half-lives and pool sizes in very premature baboons during the first two weeks of life (**Chapter 4**). As there is almost no information available about the surfactant metabolism during the development of bronchopulmonary dysplasia (BPD), we were especially interested in the changes of the surfactant metabolism in the second week of life (i.e. during the development of BPD). In this study we administered stable isotope labeled dipalmitoylphosphatidylcholine (^{2}H-DPPC, 5 mg/kg) endotracheally in very premature baboons on day 5 of life, and on day 8 we administered radioactive labeled

DPPC. This made it possible to measure the half-life of exogenous surfactant and surfactant pool size. By using a baboon model, we were able to compare the half-life and pool size obtained by our stable isotope method to measurements made with radioactive isotopes and direct measurements of pool size at autopsy. The results of this study were compared with previous data for surfactant metabolism in similar premature baboons ventilated for 6 days. The half-life of exogenous surfactant and the surfactant pool size did not change during the recovery from RDS and the development of BPD in very premature baboons. In addition, stables isotopes were shown to be a valid method for studying half-life of exogenous surfactant and surfactant pool size.

The method of administration of ^{2}H-DPPC to measure the half-life of exogenous surfactant and surfactant pool size was also used in term neonates with severe lung disease (**Chapter 5**). Surfactant PC pool size was ~73 mg/kg in neonates with CDH who required ECMO. Neonates with MAS who required ECMO had a surfactant PC pool size of ~50 mg/kg, which was not significantly different from the CDH patients. We also studied a varied group of patients who were not on ECMO, which had a comparable surfactant PC pool size (~69 mg/kg). In addition, we measured PC concentrations in epithelial lining fluid (ELF), which were similar in all groups. The results of this study showed that the lungs of CDH and MAS patients were not surfactant deficient. The half-life of exogenous surfactant was similar in all groups (~24 hours), and a correlation (positive) was found between the half-life and the duration of ECMO. From this correlation we speculated that a surfactant pool that is replaced by newly synthesized surfactant more rapidly results in a faster improvement of the clinical condition and a shorter duration of ECMO. The relation between a shorter half-life of surfactant and a faster improvement is in contradiction with what we found in chapter 3. However, in chapter 3 we measured the half-life of endogenous surfactant (^{13}C-glucose), whereas in this study we measured the half-life of exogenous surfactant (^{2}H-DPPC). We found no correlation between the two half-lives (endogenous *versus* exogenous), when measured in the same patient. When a plasma precursor (^{13}C-glucose) is used to label endogenous surfactant, it takes about 3 days before the maximal enrichment is reached and the labeled surfactant is started to disappear. Whereas when exogenous labeled surfactant is used as label, the disappearance of the labeled exogenous surfactant can directly be measured. In the intermediate 3 days after infusion of the plasma precursor, the surfactant metabolism could have changed. The fact that the half-life of endogenous surfactant is longer can be explained by ongoing synthesis of labeled surfactant from intermediate precursor pools (e.g. palmitic acid, acetate).

The MAS patients who require ECMO have relatively low surfactant pool sizes even though five of the seven patients received surfactant treatments that contained more than 50 mg/kg PC. However, most surfactant treatments were given in the first 24 hours of life, whereas the age of start of the study was ~59 hours. As the half-life of the exogenous surfactant is ~22 hours in the MAS patients, more than 50% of the administered surfactant treatment already disappeared by the time the study was started.

The endogenous surfactant metabolism was measured in CDH patients (n=11) and MAS patients (n=11) on ECMO, and compared with ventilated term control patients without significant lung disease (n=10) (**Chapter 6 & 7**). Results and comparison with other patients and precursors are shown in Table 1. Surfactant PC synthesis was decreased in CDH patients on ECMO compared to controls (~2.4 *versus* ~8.0 %/day). The maximal enrichment was lower (~0.09 *versus* ~0.18 atom percent excess (APE)), and the time of maximal enrichment was reached later (~69 *versus* ~47 hours) in CDH patients on ECMO. The half-life of endogenous surfactant was similar in both groups (~65 hours), as was the surfactant PC concentration in ELF. These data reflect less surfactant synthesis in the CDH group.

In the study of the MAS patients, we included a group of term neonates with persistent pulmonary hypertension (PPHN) who required ECMO (n=6). In MAS patients on ECMO the maximal incorporation of ^{13}C in PC was half of that in controls (~0.09 *versus* 0.18 APE). And there was a tendency of a lower FSR in the MAS patients (~3.3 %/day) and PPHN patients (~2.6 %/day) compared to controls (~8.0 %/day), which was also reflected by a lower PC concentration in ELF in the MAS (~4 mg/ml) and PPHN (~4 mg/ml) patients compared to controls (~13 mg/ml). The half-life of endogenous surfactant was similar in all groups, ranging from 63-98 hours. In conclusion, a disturbed surfactant synthesis and concentration is suggested in at least the sickest MAS patients who require ECMO.

The decreased FSR with the normal surfactant pool size (chapter 5) in CDH patients who require ECMO seems contradictionary, but could be explained by the fact that these severely sick patients use other precursors (e.g. palmitic acid) than glucose for the synthesis of surfactant. Another explanation could be that CDH patients have originally a normal FSR, which however decreases due to damage of type II cells by intensive ventilation. As it takes some time before a decreased FSR leads to a decreased surfactant pool size, we did not yet find a decreased pool size in the CDH patients.

We were able to study the surfactant metabolism in 5 patients with an inherited surfactant SP-B deficiency (**Chapter 8**). This rare and lethal neonatal lung disease was originally described by Nogee *et al.* (24). The lungs of the SP-B deficient neonate show major ultrastructural changes, disorganized tubular myelin and lamellar bodies, and pro SP-C, but no mature SP-C. Because loss of SP-B has such an impact on the stucture and functioning of type II cells of the lung, we expected a severely disturbed surfactant PC metabolism. However, we found no difference in surfactant PC metabolism in SP-B deficient patients compared to controls (Table 1). Our results are in accordance with studies in SP-B knockout mice and in vitro studies in autopsy lungs of SP-B infants (190, 194). In one SP-B deficient patient we used another precursor (^{13}C-palmitic acid instead of ^{13}C-glucose) to study the surfactant metabolism. The FSR in this patient, after infusing ^{13}C-palmitic acid was 65 %/day, which is much higher compared to the FSR from the control patients (~17 %/day, n=5). It could be that the relative contribution of precursors for surfactant synthesis changes in states of disease or congenital malformation. CDH patients not on ECMO had a similar FSR as control patients after ^{13}C-palmitic acid infusion (~22 *versus* ~17 %/day) (57).

However, after ^{13}C-glucose infusion in CDH patients on ECMO, these CDH patients had a significant lower FSR from glucose compared to controls (~2.4 *versus* ~8.0 %/day).

All the SP-B deficient infants received various treatments, including exogenous surfactant administration and dexamethason. From earlier studies it is known that exogenous surfactant treatment and dexamethason have stimulating effects on surfactant synthesis (63, 244, 262, 285). It is therefore possible that we measured an overestimation of the 'actual' FSR. However, because these SP-B deficient infants were also intensively ventilated, the effect of an overestimated FSR due to the treatment with exogenous surfactant and dexamethason could be counteracted.

Limitations of the studies

The randomization of the infants to either HFO or conventional ventilation was suboptimal. Randomization was started at 24 hours after birth, but infants who already received HFO as part of their routine care continued to receive it. Furthermore, the CDH and MAS patients were not randomized to receive ECMO treatment, which is ethically not possible. Moreover, because infants have to be intubated for at least three days for reliable calculations of surfactant kinetic parameters, only the sickest infants were included in the study.

As this kind of studies are expensive and laboratory analyses intensive, it is difficult to study large series. Especially, inclusion of control patients is difficult, as these patients have to be ventilated for at least three days without having significant lung disease. This is also the reason why we were not able to include age-matched control infants in the study about surfactant metabolism in SP-B deficient patients (Chapter 8).

We used tracheal aspirates to collect alveolar surfactant PC. Theoretically, a more accurate method could be bronchoalveolar lavage (BAL), but this method cannot be used for frequent sampling in the studied population. However, the PC concentrations we measured in tracheal aspirates of CDH patients on ECMO and control patients did not differ from the PC concentrations measured in BAL fluid of CDH patients on ECMO and ventilated control patients (100). Furthermore, it is known that the phospholipid composition in tracheal aspirates and BAL are similar, just like the ^{13}C enrichment of palmitic acid of surfactant PC was similar in tracheal aspirates and BAL (262). And as all tracheal aspirates were handled similarly in all studies, any accuracy would have occurred in all patient groups probably to the same extent. Loss of surfactant during the handling of the tracheal aspirates does not influence the measurement of ^{13}C or ^{2}H enrichment in surfactant PC palmitate. Enrichment is measured by mass spectrometry by direct measurement of the ratio of labeled versus unlabeled palmitic acid in surfactant PC. This ratio is not influenced by sampling size or loss during preparation.

In the studies of this thesis we only studied the palmitic acid metabolism of surfactant PC. Surfactant PC composes of different components (i.e. glycerol, choline, phosphate, and different fatty acids) which probably have different metabolism. Other components of surfactant, such as some surfactant proteins are also important for proper functioning of

surfactant. However, in none of our studies the metabolism of surfactant proteins was studied.

We used ^{13}C-glucose as precursor to study the metabolism of endogenous surfactant PC palmitate (Chapter 3, 6, 7 & 8). Incorporation of ^{13}C molecules derived from labeled plasma glucose and palmitic acid was measured. Palmitic acid in the type II cell can also be synthesized from other substrates like glycogen, intracellular palmitic acid, ketone bodies, glycerol and lactate. The surfactant PC synthesis (FSR) we measured by infusing ^{13}C-glusose is therefore probably an underestimation. Moreover, calculation of the 'true' FSR is only possible from the enrichment of the direct precursor, which is acetylCoA. The enrichment of acetylCoA in the type II cell cannot be measured, and therefore we calculated FSR from plasma precursor enrichments.

Most of our included patients were on ECMO during the study. The exact effect of ECMO on the surfactant metabolism could not be distinguished in the present studies. During ECMO the lung blood flow is lower, and it could be speculated that a reduced blood flow through the lungs provides less substrate for surfactant synthesis. Secondly, as physical stretch is low during ECMO treatment due to the low ventilatory settings as part of the concept of "lung rest", this could possibly decrease surfactant synthesis (235). And finally, a result of ECMO treatment is an increase in capillary permeability (280), which together with a release of cytokines change surfactant function (282, 286). This is even more plausible in an ECMO population in whom lung damage before ECMO may contribute to capillary protein leakage. But overall, it seems clear that patients who require ECMO have more damaged lungs compared to patients who do not require ECMO treatment, which could be the reason of the disturbed surfactant metabolism. And sick lungs will be even more damaged due to the intensive ventilation prior to the ECMO treatment. Unfortunately, we did not have enough CDH and MAS patients who did not require ECMO to use as 'control' for ECMO treatment. In a study by Cogo et al. surfactant metabolism was studied in CDH patients not on ECMO (57). After infusion of ^{13}C-palmitic acid they found no difference in surfactant synthesis compared to control patients. These contradictory results could be due to the use of another precursor, to the fact that these infants did not require ECMO, and therefore had less severe lung disease, or to the influence of ECMO on surfactant metabolism. But also in patients who are not on ECMO, the role of the treatment and ventilation before the start of the study on the surfactant metabolism could not be elucidated.

Another important confounder is the use of feeding. The patients were not standardized for the start of the parenteral and/or enteral feeding, nor for the composition of the feedings. A recent publication suggests an effect of the dietary fatty acid composition on pulmonary function (287). Increased unsaturated fatty acids in the diet of critically ill patients could affect surfactant function detrimentally. Most of the included patients received Intralipid® as parenteral feeding. Intralipid® could be a substrate of surfactant synthesis, and if so, it would dilute the precursor pool and give an underestimation of the FSR.

It is difficult to interpret the half-life of surfactant. The half-life measures the decrease in amount of tracer relative to the pool with which it is mixing. In the lung, there are pri-

marily two pools, the intracellular pool and the alveolar pool. The half-life measurements is a composite of the loss of the tracer due to catabolism within the lung and the dilution of the tracer due to synthesis of newly unlabeled surfactant. In the newborn lung recycling of surfactant is very efficient and exceeds 90% in animal studies (47, 274). Efficient recycling of surfactant can contribute to a longer surfactant half-life. In our studies it was not possible to discriminate between newly synthesized surfactant and recycled surfactant.

The method we used to calculate surfactant pool size (chapter 4 & 5) needs more standardization. The *apparent lung PC pool size* was calculated by using the linear regression line representing the decay of the log-transformed ^{2}H-enrichment over time. The start and the length of the enrichment *versus* time curve used for pool size calculation is decisive for the calculation of the surfactant pool size. We tried to standardize the method in the study of the term infants with severe lung disease by defining the start of the curve at 18 hours after the administration of the label, and the length at four times the half-life of each individual curve. The method of surfactant pool size measurement is derived from the compartment model which has extensively been described by *Jacobs et al* (47). Three-day-old rabbits were injected endotracheally with radioactive labeled DPPC. They described a rapid initial fall of the specific activity in the alveolar wash after the delivery of the labeled DPPC, followed by a more gradual decay, and finally by a long slow decay. The initial rapid phase represents the deposition of the label in the large airways, and the distribution from the large airways to the alveoli. The second phase corresponds to the disappearance of the label from the alveolar space to the intracellular lamellar bodies. Finally, the third slow phase reflects the disappearance of the label from the total lung. However, in the ^{2}H-enrichment versus time curves of the baboons and the term infants with severe lung disease it was difficult to distinguish the two slower phases, which represent the metabolism of surfactant. Therefore, it was not quite clear what we measured: the alveolar surfactant pool, the total surfactant pool, or a mix of both. By extrapolating the tracer dilution from measurements beginning 18 hours after tracer administration, we probably measuring an estimate of the total lung surfactant pool because of the assumption that recycling will have resulted in mixing of the alveolar and tissue pools by 18 hours. The fact that we could not distinguish between the two metabolic phases, could suggest a disturbance of mixing of the label by recycling between the alveolar and tissue pools.

Measurements of surfactant half-life and pool size by the isotope dilution method (Fick principle) are based on several assumptions. One of these assumptions is that the lung is in steady state for surfactant metabolism. However, the lungs and surfactant pools are likely to be changing as a normal developmental process if for no other reasons.

Table 1. Surfactant kinetic parameters in term human infants with and without severe lung disease studied with different precursors

	Term controls			**Term infants with severe lung disease**								
				CDH		MAS	Term patients with varied lung disease				SP-B deficiency	
Precursors	*^{13}C-GLU*	*^{13}C-PA*	*^{13}C-AC*	*^{13}C-GLU**	*^{13}C-PA*	*^{13}C-GLU**	*^{13}C-GLU**	*^{13}C-PA*	*^{13}C-LA*	*^{13}C-AC*	*^{13}C-GLU*	*^{13}C-PA*
	n=10	n=5	n=7	n=11	n=12	n=11	n=6	n=8	n=8	n=10	n=4	n=1
T_{app} (h)	12	9	7	18	8	13	13	9	10	11	-	14
T_{max} (h)	47	51	35	70	52	50	64	49	46	53	48	65
Half-life (h)	63	43	28	69	59	69	98	68	59	68	65	36
FSR (%/day)	8	17	15	2	22	3	3	34	50	3	5	65
references	*This thesis*	(57)	(56)	*This thesis*	(57)	*This thesis*	*This thesis*	(64)	(64)	(56)	*This thesis*	*This thesis*

^{13}C-GLU, [U-^{13}C]glucose; ^{13}C-PA, [U-^{13}C]palmitic acid; ^{13}C-LA, [U-^{13}C]linoleid acid; ^{13}C-AC, [1-$^{13}C_1$]acetate. All labels were administered via intravenous infusion during 24 hours. T_{app}, time of first appearance of the label, T_{max}, time of maximal enrichment, FSR, fractional synthesis time. CDH, congenital diaphragmatic hernia; MAS, meconium aspiration syndrome. *Patients on ECMO.

Directions for future research

As we did not have age-matched control patients to compare with SP-B deficient patients, it is relevant to study the effect of age on surfactant metabolism. Two of our control patients were included at day 11 and day 18 of life, and had a higher FSR of ~21 %/day. However, in a study in which labeled acetate was given to term controls with and without respiratory failure no correlation existed between the age at start of the study and the FSR (56). Surfactant metabolism should be measured in term control patients, shortly after birth, in the second week, and for example at 3 months of age.

Moreover, surfactant metabolism could be measured twice in the same patient. Bohlin *et al*. performed two studies 3 weeks apart (at age 24 and 45 days) in one control patient (56). They found nearly identical enrichment curves and metabolic indices (FSR 25.4 and 25.2 %/day, half-life 25.0 and 25.4 hours). To study the course of surfactant metabolism during ECMO, a patient who requires ECMO could be studied twice, at the beginning of the ECMO run and one week after the start of ECMO, or just after ECMO treatment is ended. In this way the effect of ECMO on surfactant metabolism could be studied.

All our patients were already ventilated for some days before the start of the study. Especially in sick lungs, ventilation can cause even more damage and may disturb surfactant metabolism. A way to measure the effect of CDH on surfactant metabolism without the influence of therapy or ventilation, is to measure surfactant metabolism in lung tissue acquired postmortem from CDH patients who died at birth. This kind of study was also performed by Beers *et al*. in lungs from SP-B deficient infants (194). But in that case we have to keep in mind that we are studying the worst cases of CDH, *in vitro*.

The study of the metabolism of the specific surfactant proteins would contribute to a better understanding of the overall surfactant metabolism. The surfactant proteins play a critical role in the formation and functioning of the surfactant monolayer as well as in the regulation of the surfactant metabolism (16). Decreased SP-A levels have been found in tracheal aspirates of human neonates with CDH (57, 99) and infants with pneumonia (209). Recently, we were able to demonstrate a secondary SP-B deficiency in MAS patients, either caused by degradation of SP-B or by disturbed SP-B metabolism (Personal communication, F. van Iwaarden, Rotterdam).

We studied the metabolism of endogenous surfactant PC by using labeled glucose. Our data give valuable information but methodological problems remain. As I already pointed out in the summary and interpretation of the studies we used plasma enrichments instead of direct precursor enrichments to calculate FSR. With the use of multiple isotopomer distribution analysis (MIDA) it is possible to determine isotopic enrichment of the direct precursor pool (56, 288). But also this technique does not account for the incorporation of unlabeled palmitic acid from plasma into surfactant PC. Furthermore, the use of a more direct precursor could be used to reflect more directly surfactant metabolism (e.g. linoleic acid, palmitic acid, choline, glycerol).

Metabolic measurements are just one part of understanding surfactant function. In future studies we also should study alterations in surfactant aggregate forms and surfactant function, which could be more disturbed even though surfactant metabolic measurements are unchanged (289).

CHAPTER 10

References

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Samenvatting

Surfactant is een stofje in de longen dat ervoor zorgt dat de longblaasjes (= alveoli) kunnen ontplooien bij de inademing en open blijven bij de uitademing. Via de longblaasjes wordt zuurstof vanuit de lucht naar de bloedbaan getransporteerd, en koolstofdioxide vanuit het bloed via de lucht afgevoerd. Indien er te weinig surfactant in de long aanwezig is, zullen de longblaasjes samenvallen en daardoor zal de opname van zuurstof en de afgifte van koolstofdioxide ernstig belemmerd worden. Een tekort aan surfactant is de oorzaak van het Respiratoir Distress Syndroom (RDS), dat vaak optreedt na de geboorte van te vroeg geboren kinderen.
Surfactant bestaat hoofdzakelijk uit vetten (90%), voornamelijk (70-80%) fosfatidylcholine (FC) en eiwitten (10%). Er bestaan 4 surfactant eiwitten, SP-A, SP-B, SP-C en SP-D, met ieder een specifieke functie. Surfactant wordt in de type II cel van de long geproduceerd en opgeslagen als lamellaire structuren in de cel. Na uitscheiding van surfactant naar het longblaasje, vespreidt het zich als een zeer dunne laag over het totale longoppervlak. Deze dunne laag verlaagt de oppervlaktespanning tussen lucht en het longblaasje waardoor het longblaasje open blijft. Na enkele ademteugen wordt surfactant afgebroken en grotendeels door de type II cel weer opgenomen en hergebruikt, een klein deel wordt afgevoerd.
Tijdens het laatste trimester van de zwangerschap is er een geleidelijke toename van de hoeveelheid surfactant in de foetale longen. Bij de geboorte worden grote hoeveelheden surfactant uitgescheiden in de longblaasjes, zodoende kunnen de longen zich direct na de geboorte voldoende ontplooien en kan een adequate gasuitwisseling plaatsvinden. De hoeveelheid surfactant in de longen verandert met de leeftijd; naarmate men ouder wordt, daalt de hoeveelheid surfactant in de longen.
Studies in dieren tonen een langzamere aanmaak en afbraak (=metabolisme) van surfactant in pasgeborenen en te vroeg geborenen in vergelijking met oudere dieren. In pasgeborenen speelt hergebruik (recycling) van surfactant een grote rol, terwijl in oudere dieren meer aanmaak van nieuw surfactant plaatsvindt. Het metabolisme van surfactant werd recent bestudeerd in te vroeg geboren kinderen met behulp van stabiele isotopen. Deze studies toonden ook dat de aanmaak en afbraak van surfactant in deze groep kinderen langzaam verloopt.

Naast RDS, dat voorkomt bij te vroeg geboren kinderen, zijn er een aantal longziekten bij pasgeborenen (neonatale longziekten) waarbij een tekort aan surfactant ook een rol zou kunnen spelen. Enkele neonatale longziekten, zoals congenitale hernia diafragmatica (CDH), meconium aspiratie syndroom (MAS), surfactant proteïne B (SP-B) deficiëntie en neonatale pneumonie (= long ontsteking) worden in **Hoofdstuk 1** besproken. Het hoofdstuk geeft een overzicht van de literatuur over het metabolisme van surfactant in relatie tot de bovengenoemde ziektebeelden.
Neonaten met CDH hebben een gat in het middenrif, dat al vroeg in de zwangerschap is ontstaan. Ten gevolge van het defecte middenrif komt een deel van de buikorganen in de borstholte te liggen waardoor de long onvoldoende ruimte heeft om zich adequaat te ont-

wikkelen. Het is echter nog steeds niet duidelijk of deze neonaten met 'onrijpe' longen ook een tekort aan surfactant hebben.
Bij MAS is er sprake van aspiratie (= verslikking) van vruchtwater met meconium (= eerste ontlasting), waardoor er meconium in de longen terechtkomt. Meconium heeft een remmend effect op de functie van surfactant. Het is echter nog de vraag of er naast deze inactivatie van surfactant ook een tekort aan surfactant een rol speelt in MAS.
SP-B deficiëntie is een aangeboren genetische afwijking waarbij er geen SP-B (1 van de 4 surfactant eiwitten) wordt aangemaakt in de long. Daarnaast is er ook een verstoring van de intracellulaire structuren in de type II cel van de long. Het gevolg is dat deze kinderen na de geboorte ernstige ademhalingsproblemen krijgen. Kinderen met een SP-B deficiëntie zullen overlijden mits er een longtransplantatie zal worden uitgevoerd. De rol van het genetische defect op het metabolisme van surfactant is bestudeerd in muizen, maar studies in levende mensen zijn nog niet verricht.
Een pneumonie in pasgeborenen komt regelmatig voor, met name in te vroeg geboren kinderen. Het kan worden veroorzaakt door een bacterie, virus of een schimmel. Studies laten zien dat pneumonie een effect heeft op de functie van surfactant. Tevens daalt de concentratie van surfactant in de longen indien er sprake is van een pneumonie. Echter, de meeste studies zijn verricht in dieren of volwassen mensen. Over de rol van een neonatale pneumonie op het metabolisme van surfactant in pasgeboren kinderen is weinig bekend.

Eerder onderzoek van het metabolisme van surfactant in te vroeg geboren kinderen en het bovenbeschreven literatuur overzicht hebben aanleiding gegeven tot de vraagstellingen die in **hoofdstuk 2** beschreven zijn:

1. Heeft beademing met hoge frequentie (high frequency oscillation) een remmend of stimulerend effect op de aanmaak van surfactant bij te vroeg geborenen met RDS?
2. Hoe verloopt de aanmaak en afbraak (= metabolisme) van surfactant en de hoeveelheid surfactant in de longen (= pool size) gedurende de ontwikkeling van bronchopulmonaire dysplasie (= chronische long ziekte die soms ontstaat na RDS)?
3. Hoeveel surfactant hebben pasgeboren kinderen met verschillende longziekten?
4. Is het metabolisme van surfactant in pasgeboren kinderen met congenitale hernia diafragmatica, pasgeboren kinderen met een meconium aspiratie syndroom en pasgeborenen met een surfactant proteïne B deficiëntie verschillend ten opzichte van pasgeboren kinderen zonder longziekte?

De meeste kinderen beschreven in dit proefschrift zijn zo ziek dat ze aan een 'long machine' liggen. Dit machine wordt in de studies 'extracorporeal membrane oxygenation' (ECMO) genoemd. Met behulp van een pomp wordt bloed via een canule uit het kind langs een 'kunst-long' geleid en van zuurstof voorzien. Na verwarming stroomt het zuurstofrijke bloed weer naar het lichaam. Op deze wijze wordt het kind van zuurstof voorzien, wordt koolstofdioxide afgevoerd en hebben de zieke longen tijd te herstellen.

Het metabolisme van surfactant is bestudeerd met behulp van stabiele isotopen. Stabiele isotopen zijn atomen met 1 extra neutron in hun kern, waardoor ze als het ware 'gemerkt' zijn. Het zijn veilige stoffen, die niet vervallen zoals gebeurd bij radio-actieve isotopen. In dit proefschrift hebben we gebruik gemaakt van 2 verschillende soorten stabiele isotopen: het stabiele isotoop van waterstof en het stabiele isotoop van het koolstof-atoom. De toediening van deze 2 verschillende isotopen gebeurde op verschillende wijzen. Ten eerste, gelabeld dipalmitoylfosfatidylcholine (= belangrijkste actieve component van surfactant) (^{2}H-DPPC) werd via de luchtpijp toegediend. Na toediening mengt ^{2}H-DPPC zich in het longblaasje met het aanwezige ongelabelde surfactant en zal worden opgenomen door de type II cel waar menging met het intracellulaire surfactant zal plaatsvinden. Uiteindelijk zal het label geleidelijk uit de longen verdwijnen. Door de verdwijning van het label te meten, kan de half-waardetijd van ^{2}H-DPPC en de pool size (totale hoeveelheid) van fosfatidylcholine worden berekend.
De verdwijning van het label (^{2}H) uit de long werd gemeten in trachea-aspiraten. Een trachea-aspiraat wordt verkregen door slijm uit de beademingsbuis te zuigen, iets wat routinematig regelmatig wordt gedaan in beademde kinderen om verstopping van de beademingsbuis te verkomen. Uit het trachea-aspiraat wordt surfactant fosfatidylcholine geïsoleerd. Met behulp van speciale apparatuur kan de verhouding tussen het gelabelde en ongelabelde fosfatidylcholine worden gemeten.
De andere methode is toediening van stabiele isotopen in de vorm van een 24-uurs infuus met [U-^{13}C]glucose, om vervolgens de inbouw van het ^{13}C-isotoop in surfactant te vervolgen. Het ^{13}C-isotoop in de glucose wordt door de longen gebruikt om de vetzuren van surfactant fosfatidylcholine te maken. Als dit ^{13}C wordt aangetoond in surfactant, dan is het surfactant 'verrijkt'. De snelheid van inbouw en verdwijning van ^{13}C is een maat voor de surfactant aanmaak en verdwijning. Eenvoudig gesteld geldt dat hoe meer ^{13}C in surfactant fosfatidylcholine gemeten wordt, des te hoger de aanmaak van surfactant fosfatidylcholine is. In deze methode werd surfactant fosfatidylcholine eveneens geïsoleerd uit trachea-aspiraten.

Eerdere studies toonden dat surfactant therapie na de geboorte en toediening van corticosteroïden voor de geboorte van het kind (= prenataal) een stimulerende invloed hebben op de eigen (= endogene) productie van surfactant. Fysieke rek van de longblaasjes is ook een stimulus voor de surfactant productie. Tijdens normale (= conventionele) beademing vindt er herhaaldelijke uitzetting van de longblaasjes plaats. Deze uitzetting zorgt voor stimulatie van de productie van surfactant en uitscheiding naar de longblaasjes. Echter, tijdens high frequency oscillation (HFO), is de stretch van de longblaasjes veel kleiner. Dit zou kunnen resulteren in een verminderde surfactant productie. Het effect van HFO beademing op het surfactant metabolisme in te vroeg geboren kinderen hebben we bestudeerd (**Hoofdstuk 3**). De fractionele synthese snelheid (FSR) van surfactant was niet verschillend tussen de kinderen die conventioneel beademd werden en kinderen die middels HFO beademd werden (4.7 *versus* 4.2%/dag). Een fractionele synthese snelheid van 4.5%/dag betekent dat ongeveer 4.5% van de totale hoeveelheid surfactant FC per dag uit glucose wordt aangemaakt. De half-waardetijd van endogeen surfactant was ook niet ver-

schillend tussen beide groepen (~79 *versus* ~76 uren). Echter, bij kinderen die te vroeg waren geboren en die overleden ten gevolge van respiratoir falen leek de half-waardetijd van surfactant korter te zijn.

Hoofdstuk 4 beschrijft een studie in te vroeg geboren bavianen. In deze bavianen is de half-waardetijd van surfactant en de 'pool size' (= de hoeveelheid surfactant in de longen) in de eerste 2 weken na de geboorte bestudeerd. De meeste studies over het metabolisme van surfactant zijn uitgevoerd direct na de geboorte. Er is weinig informatie over het metabolisme van surfactant in de weken na de geboorte en/of tijdens de ontwikkeling van bronchopulmonale dysplasie (BPD). BPD is een chronisch ziektebeeld van de long dat soms ontstaat nadat een te vroeg geboren kind RDS heeft doorgemaakt. De precieze oorzaak van BPD is nog onbekend, evenals het antwoord op de vraag of surfactant een rol speelt in de ontwikkeling van RDS naar BPD. De te vroeg geboren bavianen kregen ^{2}H-DPPC via de luchtpijp op dag 5 na de geboorte en op dag 8 kregen ze radio-actief gelabeld surfactant via de luchtpijp toegediend. De half-waardetijd van het toegediende (exogeen) surfactant werd berekend en op dag 5 werd tevens een surfactant pool size berekend. De half-waardetijd verkregen met behulp van de stabiele isotopen methode was vergelijkbaar met de half-waardetijd verkregen na toediening van radio-actief surfactant (ongeveer 30 uur), en toonde geen verandering in de 2^{e} levensweek. De surfactant pool size op dag 5 was vergelijkbaar met de pool size op dag 6 uit een eerdere studie in bavianen en eveneens met de pool size bij autopsie op dag 14. Dus, de half-waardetijd en de pool size van surfactant veranderen niet in de tweede week na de geboorte tijdens de ontwikkeling van BPD.

In **hoofdstuk 5** wordt ^{2}H-DPPC toegediend aan pasgeboren kinderen met een ernstige longziekte en een normale zwangerschapsduur. Er werden 3 groepen bestudeerd: een groep van kinderen met CDH aan ECMO (CDH-ECMO), een groep kinderen met MAS aan ECMO (MAS-ECMO) en een groep kinderen met verschillende diagnoses, niet aan ECMO (NON-ECMO). De half-waardetijd en de pool size van surfactant waren gelijk in de 3 groepen. Waarschijnlijk hebben kinderen met CDH of MAS aan ECMO geen tekort aan surfactant in hun longen. Daarnaast werd er in deze studie ook een relatie gevonden tussen de surfactant half-waardetijd en de duur van ECMO. Kinderen waarbij de hoeveelheid surfactant in de longen sneller vervangen werd (= kortere half-waardetijd), toonden een snellere klinische verbetering en hadden dus een kortere ECMO duur.

Hoofdstuk 6 beschrijft de studie waarin het surfactant fosfatidylcholine metabolisme in kinderen met CDH wordt gemeten. Elf kinderen met CDH aan ECMO en 10 controle kinderen (= kinderen die wel beademd werden, maar geen longziekte hadden) kregen gedurende 24 uur een infuus met gelabeld glucose. De inbouw van het label in surfactant werd vervolgd. De CDH-ECMO kinderen toonden een lagere surfactant aanmaak ten opzichte van de controle groep (FSR ~2.4 *versus* ~8.0%/dag). De surfactant half-waardetijd was in beide groepen gelijk (~65 uur). De surfactant fosfatidylcholine concentratie in de longvloeistof liet geen significante verschillen zien tussen de 2 groepen (~7 versus ~13 mg/ml). Het feit

dat kinderen met CDH wel een lagere productie van surfactant hebben maar geen lagere surfactant concentratie in de longen kan op verschillende manieren verklaard worden. Ten eerste kan het zo zijn dat hele zieke kinderen andere bouwstoffen in plaats van glucose gebruiken voor de productie van surfactant. Wij meten alleen de productie van surfactant uit glucose. Een andere verklaring is dat de FSR in kinderen met CDH bij de geboorte normaal is, maar dat de FSR daalt door beschadiging van de type II cel ten gevolge van de intensieve beademing die deze kinderen nodig hebben voordat ze aan ECMO gaan. Omdat het enige tijd duurt voordat de verlaagde FSR leidt tot een verlaagde surfactant concentratie in de long, vinden wij nog geen verlaagde pool size in kinderen met CDH aan ECMO.

Het surfactant fosfatidylcholine metabolisme in kinderen met MAS wordt beschreven in **hoofdstuk 7**. In deze studie zijn er behalve 11 kinderen met MAS aan ECMO en 10 controle kinderen, ook 6 kinderen met een zogenaamde persisterende pulmonale hypertensie (PPHN) aan ECMO, bestudeerd. Deze PPHN groep is een soort 'controle' groep om uit te sluiten dat de resultaten die we vinden niet het gevolg zijn van behandeling met ECMO. In zowel de MAS als de PPHN groep leek er een tendens tot een lagere FSR in vergelijking met de controle groep (~3.3 en ~2.6 *versus* ~8.0%/dag), echter de verschillen waren niet significant. De surfactant fosfatidylcholine concentratie in de longvloeistof was wel significant lager in de MAS en de PPHN groep ten opzichte van de controle groep (~4 *versus* ~13 mg/ml). Het lijkt er dus op dat de kinderen met MAS een verstoord surfactant metabolisme en concentratie hebben. Echter het is moeilijk na te gaan in hoeverre dit veroorzaakt wordt doordat de kinderen aan ECMO liggen. Behandeling met ECMO zou kunnen leiden tot een lagere FSR, doordat tijdens ECMO de bloeddoorstroming van de longen lager is dan normaal. Door minder aanvoer van bloed naar de longen, is er minder aanbod van bouwstoffen (o.a. gelabeld glucose) voor de productie van surfactant. Tijdens ECMO wordt het bloed door een 'kunst-long' van zuurstof voorzien en zijn de longen in een 'rust-toestand'. Hierdoor is er tijdens ECMO minder rek van alveoli en daardoor minder surfactant productie. Een derde verklaring is dat er tijdens behandeling met ECMO een vergrote doorlaatbaarheid van de bloedvatwand bestaat, waardoor er in de long stoffen kunnen komen die de functie van surfactant remmen, of surfactant afbreken.

Hoofdstuk 8 beschrijft het surfactant fosfatidylcholine metabolisme in kinderen met een SP-B deficiëntie. SP-B deficiëntie is een ziektebeeld dat nog niet zo lang geleden ontdekt is en dat zeldzaam is. Vandaar dat er in deze studie maar 4 kinderen met dit ziektebeeld bestudeerd zijn. Ook in deze studie zijn de resultaten vergeleken met die van 10 controle kinderen. Er werd geen verschil gevonden in het surfactant fosfatidylcholine metabolisme van kinderen met SP-B deficiëntie en dat van controle kinderen. Deze resultaten komen overeen met de bevindingen van studies in dieren. Daarnaast hebben we 1 kind met een SP-B deficiëntie en 5 controle patiënten in plaats van gelabeld glucose, gelabeld palmitaatzuur gegeven. In dit kind werd een hoge FSR gevonden (63 %/dag *versus* 17 %/dag in controle kinderen). Dit zou verklaard kunnen worden door het gebruik van andere bouwstoffen voor de aanmaak van surfactant in zieke kinderen.

Dankwoord

De klus is geklaard en ik ben trots op mezelf dat het gelukt is. Het was geen makkelijke klus, en het ging zoals bij ieder onderzoek gepaard met de nodige dalen. De grootste tegenslag was dat het apparaat dat de benodigde resultaten moest leveren kapot ging op het moment dat ik alle resultaten nodig had. Dit leidde tot veel frustraties en verlies van mijn motivatie. Daarnaast waren er ook privé nogal wat dalen die overbrugd moesten worden. Dat maakte het extra moeilijk, maar het geeft nu des te meer voldoening.

Vele mensen hebben me tijdens het project bijgestaan en geholpen:

Deze studie had niet kunnen plaatsvinden zonder alle kinderen en hun moedige ouders. Nu ik zelf moeder ben, zie en met name voel ik veel beter wat deze ouders hebben moeten doormaken. Je kind zo ziek aan al die 'slangen' zien liggen moet je een angstig en machteloos gevoel geven. Lieve kinderen en ouders bedankt voor jullie dappere medewerking.

De verpleging van de chirurgische en neonatale intensive care wil ik bedanken voor de hulp bij het verzamelen van de trachea-aspiraten en het bloed, en voor jullie ondersteuning van de studies.

I would like to thank the Sophia Foundation for Medical Research for making this project financially possible.

Mijn copromoter, Dr. Luc Zimmermann wil ik hartelijk bedanken. Beste Luc, je hebt me vanaf het begin meegesleept naar alle congressen. Het waren voor jou drukke jaren met veel veranderingen, maar als ik om aandacht schreeuwde, had je altijd tijd voor me. Het maakte niet uit hoe laat, in het weekend, in het ziekenhuis of thuis. Jouw kennis en je vertrouwen in mijn kunnen hebben er mede toe bijgedragen dat ik de eindstreep heb gehaald. Jij gaat Rotterdam verlaten en je weer richting het zuiden begeven naar de stad waar ik vandaan kom. De komende jaren is het mijn beurt om jou iets te leren, namelijk 'het carnaval vieren'.

Prof. Dr. D. Tibboel, mijn promoter wil ik bedanken voor zijn steun en inzet. Dick, je was de stok achter mijn deur. Je was actief betrokken bij de inclusie van patiënten in de studie. Wanneer er weer een mogelijk kandidaatje voor de studie werd opgenomen, liet je me dat *direct* even weten. Manuscripten werden snel door jou nagekeken en je hield nauwlettend de voortgang van de studie in de gaten.

Aaron Hamvas, MD, dear Aaron, it has been a pleasure to work with you. Thank you for reading my manuscripts, your quick answers and your clear comments. I feel very honored that you will come to Holland to participate in the discussion of my thesis.

I also would like to thank Kajsa Bohlin, for 'borrowing' your control patients. I wish you good luck finishing your thesis. I would like to thank Assaad Merchak for working together at the HFO versus CV paper. Although I regret we never met, our collaboration by email went smoothly.

Prof. Dr. V.P. Carnielli and P. E. Cogo, dear Virgilio and Paola, thanks for your cooperation and for reviewing my papers. I am very grateful that you helped me out of distress by measuring all my samples after the machinery broke down in Rotterdam. You both are always busy with working, I hope that you also can enjoy doing nothing (*dolce far niente*).

Prof. dr. H.A. Büller en Prof. dr. J.C. de Jongste wil ik hartelijk danken voor de bereidheid in de kleine commissie zitting te nemen, en voor de beoordeling van het manuscript. Prof. dr. C. Blanco dank ik voor zijn komst naar Rotterdam en zijn bijdrage aan de oppositie. Prof. dr. F.W.J. Hazebroek dank ik voor zijn deelname aan de grote commissie.

Alan Jobe, MD, PhD, dear Alan, it has been a honour to work with you. Your never ending interest in my studies and your enthusiasm about doing research, stimulated me. Steven Seidner, MD, PhD, dear Steven, thank you for giving me the opportunity to participate in the baboon study.

Alle samples heb ik verwerkt en geanalyseerd in het wetenschappelijk laboratorium Kindergeneeskunde. Zonder de hulp van Ingrid Luijendijk was me dat niet gelukt. Ingrid, je hebt me wegwijs gemaakt in het wereldje van het lab en alle bloedsamples voor de studies bewerkt en geanalyseerd. Samen hebben we onze frustraties gedeeld en ook af en toe een lekker recept. Bedankt!
Verder wil ik Darcos Wattimena bedanken voor zijn kennis van die verschrikkelijk gevoelige apparatuur. Ad de Bruijn wil ik bedanken voor zijn bijdrage aan de 'surfactant meetings' en de eiwit bepalingen in trachea-aspiraten. Freek van Iwaarden, Freek jij bracht wat afleiding in het lab met je ratten operaties en je imposante proefopstellingen om surfactant eiwitten te isoleren. Succes verder in Maastricht.

Het Algemeen Klinisch Chemisch Lab van het Sophia wil ik bedanken voor de snelle bepalingen van de ureumconcentratie in de trachea-aspiraten.

Afgelopen jaren hebben enkele studenten me geholpen bij het uitvoeren van de studies en het bewerken van de trachea-aspiraten: Esther Wit, Roel van Venrooij, Lars de Zeeuw, Danielle Cordeiro, Esther Emmen & Willem de Jong. Bedankt allemaal, en succes met jullie verdere opleiding!

Een belangrijke factor in het AIO leven zijn de andere AIO's/arts-onderzoekers. Bedankt voor de gezelligheid, de goede tips, de borrels, etentjes, de Roparun en de AIO-weekenden. De groep is inmiddels zo groot geworden dat ik me er niet meer aan waag iedereen bij naam te noemen. Maar enkele wil ik speciaal noemen:
De oud-AIO's en aanstaande collega's Theo, Dick, Laurens, Hettie, Annemarie, Inge, Saskia, Carola & Nico (mijn zwangerschapsmaatje). Mijn oud-kamergenoten: Hannerieke, Leon, Lizette & Geertien, alias 't Pompe team'. De andere Limbo's: Joep & Ward (v'r zien us, en es v'r us neet zien dan schikke v'r us unne Rommedoe, en dan ruuke v'r us!). De mensen die het Sophia hebben verlaten: Esther, Sacha, & Sophie. En de achterblijvers: Barbara (keep smiling), Edwin (zet 'm op) & Jessie (collega, buurvrouw en noodoppas). De moeders onder de AIO's: Venje, José, Clementine & Karin. En niet te vergeten de 'aanstaande moeder' Marieke!

Mijn paranimfen, Hestien Vreugdenhil en Carine Janssen. Lieve Hestien, mijn kamergenoot, psycholoog en nu 'paardennimf'. Dank je voor je steun en luisterend oor en niet te vergeten je rood-wit zoute snoepjes. Lieve Carine, dezelfde achternaam en beiden Limburgse 'roots', maar toch geen familie. Fijn dat je naast me staat. Lieve nimfjes, laten we er een geweldige dag van maken!

Mijn vriendinnetjes Viv, Gon & Im. Wat een bijzondere vriendschap hebben we toch! We kennen elkaar al zo lang, we hebben zoveel meegemaakt met elkaar, leuke en minder leuke dingen. Laten we vooral zo doorgaan.

Mijn allerliefste ouders, bedankt voor jullie onvoorwaardelijk vertrouwen en jullie steun. Jullie hebben mij de kans gegeven, gestimuleerd en de kracht gegeven om dit te bereiken. Ik hou van jullie.
Lieve Alan en Marijke, mijn broer(tje) en (aanstaande?) schoonzusje, we zien elkaar veel te weinig, ik hoop dat dat de komende tijd gaat veranderen.

Aller, allerliefste Jan Erik, het waren woelige jaren. Het huisje, boompje,… kindje heeft zich bij ons in een razend tempo ontwikkeld. Naast de dieptepunten hebben we ook hele mooie momenten gekend, met als hoogtepunt de geboorte van Thomas. Laten we de komende jaren genieten van elkaar, Thomas, van alles! Bedankt voor je vertrouwen en je liefde, ik hou van je.
Lieve, lieve Thomas, mamma is klaar en kan nu nog meer van je genieten. Deze zomer gaan we samen spelen, naar het strand, naar de dierentuin, en met name heel veel plezier maken. Knuffel van mamma, x-je.

Daphne

Curriculum Vitae

Daphne Janssen was born on March 19, 1971 in Geleen. She completed secondary school (VWO, Albert Schweitzer scholengemeenschap, Geleen) in 1989. In 1990 she attended Medical School at the University of Maastricht until May 1997. During her medical study she spent six weeks in a hospice (house for dying people) in Cheltenham, United Kingdom. After her medical training she worked during one year as a resident at the pediatric department of the Catharina Hospital, Eindhoven. In March 1998 she started working as a research fellow (Assistent In Opleiding, AIO) at the division of Neonatology of the Sophia Children's Hospital. Under the supervision of Dr. Luc Zimmermann and Prof. Dr. Dick Tibboel she studied the surfactant metabolism in term neonates, which lead to the current thesis. From Oktober 2002 to May 2003 she worked as a physician in a medical daycare center for infants with medical and/or behavioral problems (MOI, De Kleine Plantage, Rotterdam). In Oktober 2003 she will start her training in pediatrics at the Erasmus MC-Sophia Children's Hospital, Rotterdam (head Prof. Dr. H.A. Büller).

She is living together with Jan Erik Bunt, and they have a wonderfull son Thomas.

List of Publications

1. **DJ Janssen**, D Tibboel, VP Carnielli, E van Emmen, IH Luijendijk, JLD Wattimena, LJ Zimmermann. Normal surfactant phosphatidylcholine pool size in human neonates with congenital diaphragmatic hernia.
   J Pediatr 2003; 142(3): 247-52

2. **DJ Janssen**, VP Carnielli, PE Cogo, SR Seidner, IH Luijendijk, JLD Wattimena, AH Jobe, LJ Zimmermann. Surfactant phosphatidylcholine half-life and pool size measurements in premature baboons developing BPD.
   Pediatr Res 2002; 52(5): 724-25

3. A Merchak, **DJ Janssen**, K Bohlin, BW Patterson, LJ Zimmermann, VP Carnielli, A Hamvas. Endogenous pulmonary surfactant metabolism is not affected by mode of ventilation in preterm infants with respiratory distress syndrome.
   J Pediatr 2002; 140(6): 693-8

4. **DJ Janssen**, LJ Zimmermann, A Hamvas, PE Cogo, IH Luijendijk, DJ Wattimena, VP Carnielli, D Tibboel. Decreased rate of phosphatidylcholine synthesis in neonates with congenital diaphragmatic hernia during extracorporeal membrane oxygenation.
   Submitted

5. **DJ Janssen**, VP Carnielli, PE Cogo, K Bohlin, IH Luijendijk, JEH Bunt, D Tibboel, LJ Zimmermann. Surfactant phosphatidylcholine metabolism in human neonates with meconium aspiration syndrome.
   Submitted

6. **DJ Janssen**, PE Cogo, A Hamvas, K Bohlin, U Merz, D Lecoutere, DJ Wattimena, IH Luijendijk, JEH Bunt, D Tibboel, VP Carnielli, LJ Zimmermann. Surfactant phosphatidylcholine in human neonates with surfactant protein B deficiency.
   Submitted

7. JE Bunt, VP Carnielli, **DJ Janssen**, D Wattimena, W Hop, P Sauer, LJ Zimmermann.
   Treatment with exogenous surfactant stimulates endogenous surfactant synthesis in premature infants with respiratory distress syndrome.
   Crit Care Med 2000; 28 (10): 3383-88

8. WA Dik, LJ Zimmermann, BA Naber, **DJ Janssen**, AH van Kaam, MA Versnel. Thrombin contributes to bronchoalveolar lavage fluid mitogenicity in lung disease of the premature infant.
   Pediatr Pulmonol 2003; 35(1): 34-41

Abbrevations

ASR	Absolute fractional synthesis
BAL	Bronchoalveolar lavage
BPD	Bronchopulmonary dysplasia
CDH	Congenital diaphragmatic hernia
DPPC	Dipalmitoyl phosphatidylcholine
DSPC	Disaturated phosphatidylcholine
ECMO	Extracorporeal membrane oxygenation
E_{gluc}	Mean plasma glucose enrichment
E_{max}	Maximal enrichment
FSR	Fractional synthesis rate
GBS	Group B streptococci
LPS	Lipopolysaccharide
L/S	Lecithin/Sphingomyelin
LysoPC	Lysophosphatidylcholine
MAS	Meconium aspiration syndrome
PC	Phophatidylcholine
PE	Phosphatidylethanolamine
PG	Phospatidylglycerol
PI	Phosphatidlylinositol
PPHN	Persistent pulmonary hypertension of the newborn
RDS	Respiratory distress syndrome
SP-B	Surfactant protein-B
T_{app}	Time of first appearance
TAS	Tracheal aspirate
T_{max}	Time of maximal enrichment
TRH	Thyrotropin-releasing hormone