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TESI DI DOTTORATO

STABLE ISOTOPE TRACERS TO ESTIMATE SURFACTANT METABOLISM IN NEWBORNS WITH RESPIRATORY DISTRESS SYNDROME

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Chapter 3

Investigation Of The Preferential Metabolic Precursors For Surfactant Dspc Biosynthesis In Preterm Newborns With Respiratory Distress Syndrome In First Days Of Life, With Low Energy Intake

Chapter 4

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ABSTRACT

Aims of the thesis are to describe new applications of stable isotope tracers, applied to study pulmonary surfactant kinetics in human newborns.

My PhD project was initially focused on the most suitable metabolic substrate to measure surfactant DSPC synthesis and more specifically on the difference between *de-novo* synthesis an remodeling synthesis using free fatty acids derived from the circulating blood. In the second part a newly synthesized stable isotope tracer, $^{2}H_{3}$-U^{13}C PA-DPPC, was used to investigate the different kinetics of the sn-1 and sn-2 palmitate moieties in infants with different lung diseases.

RIASSUNTO

Obiettivo di questa tesi è stato l'applicazione di traccianti marcati con isotopi stabili come per lo studio della sintesi e della cinetica del surfattante polmonare in neonati.

Il primo studio ha interessato la ricerca del substrato metabolico preferenziale per la sintesi della fosfatidilcolina disatura (DSPC) del surfattante e più specificatamente sulla differenza tra il processo di sintesi "*de-novo*" e di rimodellamento a partire dagli acidi grassi liberi plasmatici.

Nel secondo studio abbiamo utilizzato un nuovo tracciante di sintesi, $^{2}H_{3}$-U^{13}C PA-DPPC, per investigare la cinetica delle due molecole di acido palmitico diversamente marcate della DSPC del surfattante, in neonati con differenti malattie polmonari.

Chapter 1

Lung And Pulmonary Surfactant

INTRODUCTION

The lung alveolar system is the largest surface of our body that is exposed to the environment, covering up to 120 m^2. The human respiratory tract consists of a remarkable, highly branched, tubular structure that leads to ~300 million alveolar sacs, creating an extensive surface area through which oxygen and carbon dioxide are exchanged blood within the alveolar capillaries. The alveolus is the primary site of gas exchange from the blood in mammalian lungs. It consists of an epithelial layer and extracellular matrix surrounded by capillaries and has a radius of about 0.1 mm, and a wall thickness of about 0.2 μm. The alveolar surface is formed by two types of epithelial cells, pneumocytes I and pneumocytes II, or alveolar cells types I and II, respectively (Figure 1).

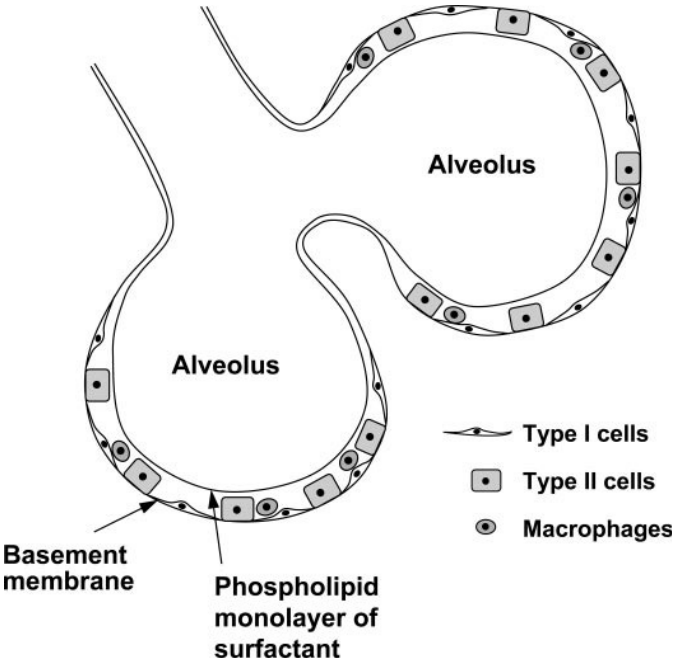


Figure 1. Cell types present in alveolus.

Type I cells make up 95% of the alveolus, while the Type II cells account for the remaining 5%[1]. Type I cells are flattened cells, which are unable to divide, contain just a few organelles, and serve as a thin barrier between blood and the air. Another cell type present in alveoli are macrophages, which remove particles and microorganisms coming with the air and are responsible for the catabolic mechanism of surfactant in the lung[2]. For a schematic representation of the localization of lung cell types see Figure 2.

Type II cells participate in clearance and in repair by proliferating and migrating to damage areas. Type II cells are able to divide and to differentiate into type I cells. They participate in defence responses by expressing various receptors (in particular, Toll-like receptors). Type II cells are involved in the lung cytokine/chemokine network by secreting and responding to an array of cytokines and chemokines[3,4,5,6]. They regulate transmigration of monocytes across the epithelial layer and possibly participate in T cell activation. However, the main, and the most extensively studied, functions of type II cells are the synthesis and secretion of the lung surfactant[7].

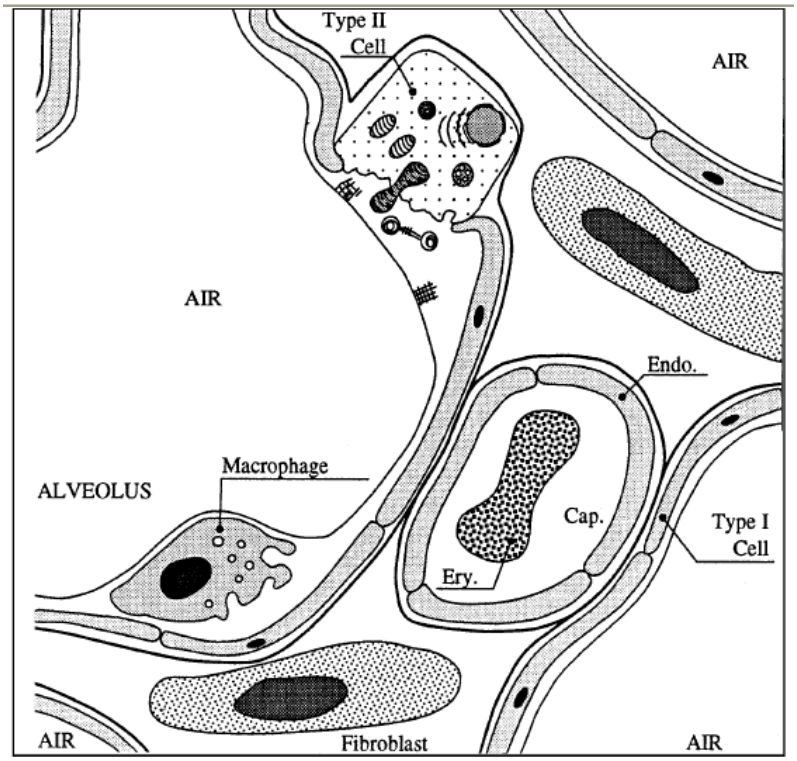


Figure 2. Schematic drawing of lung tissue.

Pulmonary surfactant, a complex of lipids and proteins, lines the alveolar capillary membrane and plays an essential role in normal lung function. The alveolar surface, lined by type II and type I alveolar epithelial cells, is in direct contact with respiratory gases, creating collapsing forces at the air-liquid interface. Thus the alveoli have an innate tendency to collapse because of their spherical shape, small size and the contribution of water vapour to surface tension [8]. To maintain inflation, these surface forces are mitigated by the presence of pulmonary surfactant that is synthesized and stored in lamellar bodies until it is secreted into the alveolar surface by type II epithelial cells. Its major physiologic function is to reduce alveolar surface tension, confer stability to alveoli, and maintain the alveolar surface free of liquid to facilitate gas exchange preventing collapse (Table 1). The type II cell plays a critical role in surfactant production and in repair of the lung following injury, and it is the progenitor cell for type I epithelial cells, which comprise the majority of the gas-exchange region of the alveolus.

Table 1. Surfactant functions.

Related to surface forces	Not related to surface forces
Maintainace of high surface area	Specific and not-specific defense to airways agents
Increase pulmonary compliance during inspiration	Barrier against external pathogens
Alveoli stabilization during expiration	Antibatteric/antiviral activity
Airways stabilization	Smooth muscle stretching
Anti-edema effect	
Anti-adesion agent	
Facilitate mucociliary transport	
Particle removal	

Alveolar surfactant is organized into an abundant and highly ordered material called tubular myelin, as well as multi-lamellated and smaller, vesicular, protein-lipid structures that can be isolated from the lung. These two surfactant forms are determined by the presence or absence of surfactant proteins and lipids, by the physical forces generated during the respiratory cycle, and by selective processes mediating uptake or degradation. Surfactant multilayers, derived from highly lamellated and tubular myelin forms, spread over the surface of the alveolus and reduce surface tension.

A deficiency of surfactant causes a disturbance of alveolar gas exchange and is one of the hallmark features of respiratory distress syndrome (RDS), the most common lung disorder among infants born prematurely. Addition of exogenously

administered surfactant has demonstrated efficacy for both prophylactic and rescue approaches[9].

RDS in newborns is characterized by respiratory failure, decreased pulmonary compliance, alveolar collapse with reduced residual functional capacity, few gas exchange and widespread interstitial edema. Currently RDS incidence in preterm decreases with gestational age. It is estimated that 10% of preterm infants, in developed countries, are afflicted with RDS[10].

Exogenous surfactant replacement therapy in preterm newborns with RDS ameliorated respiratory function and prognosis, but the whole effect is influenced by dose, time of administration and by the type of surfactant used[11].

PULMONARY SURFACTANT COMPONENTS

Pulmonary surfactant is a multi-component complex of several phospholipids (PL), neutral lipids, and associated proteins as depicted in Figure 3. Lipids constitute the major part (approximately 90%) of pulmonary surfactant; among these 80-90% are represented by PLs, whereas cholesterol comprises the largest amount of neutral lipids (10-20%). Phosphatidylcholine (PC) makes up the major part (80%) of the PLs and around 60% of PC molecules contains two saturated fatty-acid moieties. This di-saturated PC (DSPC) contains mainly palmitic acid and is defined as 1,2-dipalmitoyl-sn-3-phosphocholine dipalmitoyl phosphatidylcholine (DPPC). The anionic phosphatidylglicerol (PG) accounts for approximately 8%; other lipids are phosphatidylethanolamine (PE, ±10%), phosphatidylinositol (PI, ±7%), phosphatidylserine and sphingomielin in small quantities (less than 2%)[12].

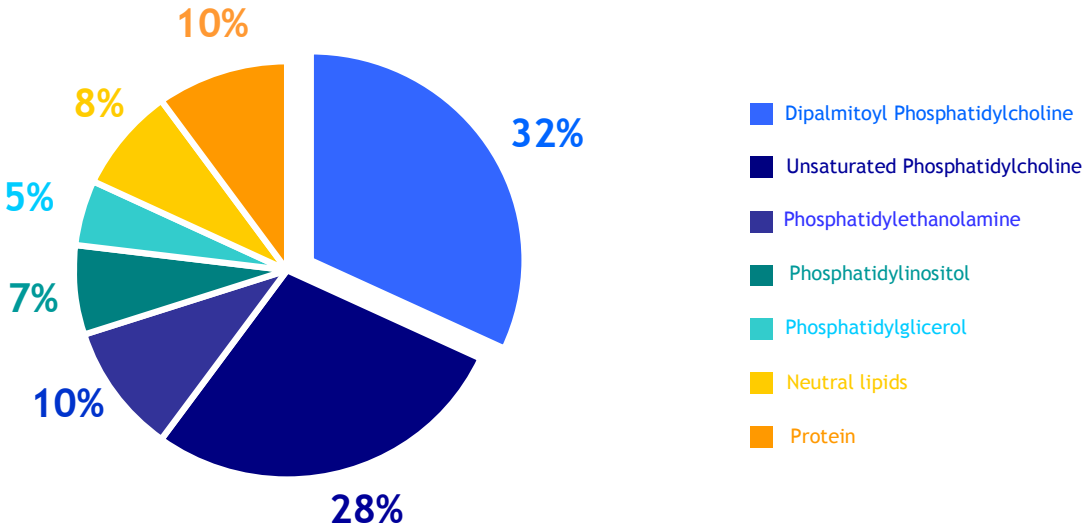


Figure 3. Surfactant components.

DPPC plays a critical role on surfactant function since this lipid alone can account for the surface tension-lowering proprieties that prevent alveolar collapse and is essential for normal gas exchange to occur in the lung[13].

Surfactant proteins constitute the remaining part (10% approximately) of surfactant, they are designated surfactant protein A (SP-A), SP-B, SP-C, SP-D, and play critical roles in various aspects of surfactant structure, function, and metabolism[14]. They have distinct structures, functions and are expressed at relatively high levels in type II cells.

Both surfactant lipids and proteins are synthesized primarily by type II cells. Surfactant lipids are stored in large, lipid-rich, intracellular organelles, termed lamellar bodies. Lamellar bodies are rich in the surfactant-associated phospholipids, PC and PG, and in two low-molecular-weight, hydrophobic surfactant proteins, SP-B and SP-C, that are co-secreted with the surfactant lipids and interact closely with them[15]. Each type II cell contains 120–180 lamellar bodies (specialized organelles of 0.1–2.4 μm in diameter), which consist of tightly packed concentric membrane lamellae with little intralamellar space, surrounded by the outer membrane[2].

SP-B (MW 8.7 kDa) is required for the formation of tubular myelin from secreted lamellar body material promoting the adsorption of phospholipids from suspended membranes to the air-liquid interface. Surfactant protein B (SP-B) induced lateral stability has been proposed as the mechanism responsible for the functional ability of surfactant to vary surface tension with changing surface area in the stable alveolus[16]. This theory evolved from studies of peptides synthesized according to sequences of SP-B amino acids or mimicking these sequences which showed that SP-B provided cohesiveness to molecules of phospholipids[16,17]. The peptides and SP-B are hydrophobic and are positioned in the acyl side chains of the phospholipid monolayer, with strong electrostatic interactions between the positively charged amino acids and the negatively charged phospholipids. This bonding of SP-B, peptide and phospholipid molecules confers lateral stability on the phospholipid molecules in the monolayer of the alveolus and by virtue of this, the cohesive monolayer is able to prevent collapse of the alveolus[16,18].

SP-C (MW 4.2 kDa) is able to stimulate insertion of phospholipids out of subphase into the air liquid interface in a calcium-dependent manner. SP-B and SP-C alter lipid packing and spreading and enhance the surface tension lowering activity of the lipids, as well as stabilizing the lipid layers during the respiratory cycle.

The hydrophilic surfactant proteins SP-A and SP-D are larger (MW 38kDa and 43 kDa, respectively), relatively abundant, oligomeric proteins that are also synthesized and secreted by type II cells. SP-D and SP-A are structurally related members of the collectin family of C-type mammalian lectins that share distinct collagen-like and globular, carbohydrate-binding domains. SP-A is required for the formation of tubular myelin and plays diverse roles in host-defense functions of the lung[19,20,21]. SP-A binds lipopolysaccharides and various microbial pathogens, enhancing their clearance from the lung. Unlike SP-B and SP-C, SP-A does not play a critical role in surface functions, metabolism, or pulmonary surfactant under normal conditions. SP-D, however, influences the structural forms of pulmonary surfactant and is important in the regulation of alveolar surfactant pool sizes and reuptake[22,23,24].

SP-D is also necessary in the suppression of pulmonary inflammation and in host defense against viral, fungal, and bacterial pathogens.

Taken together, surfactant lipids and proteins play critical roles in reducing surface tension in the alveolus, as required for ventilation, and modulating various aspects of innate host defense of the lung against diverse pulmonary pathogens.

Disaturated phosphatidylcholine

Due to its anphipatic feature DSPC has tension-lowering proprieties that reduce the surface tension at the air-liquid alveolar interface; its adsorption to the monolayer is facilitated by insaturated phosphatilycholines and idrophobic surfactant proteins (Figure 4).

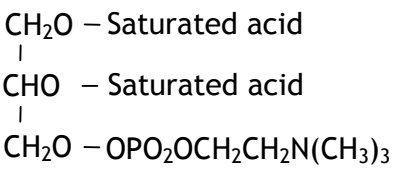


Figure 4. 1,2-saturated-sn-3-phosphocholine phosphatidylcholine (DSPC) formula.

DSPC is synthetized into type II cell endoplasmatic reticulum. In this site there are two different synthesis pathways: the *"de-novo"* synthesis or remodeling synthesis using the free fatty acids derived from the circulating blood. PC is made in all nucleated cells via the choline pathway. Choline can be acquired from diet and via *"de novo"* biosynthesis: choline is produced through the methylation of

phosphatidylethanolamine to PC catalyzed by phosphatidylethanolamine N-methyltransferase. Otherwise, PC can be generated from PC via the action of phospholipases[25]. Unsaturated PC is converted into DSPC by a acylation-deacylation reaction and consequentely phospholipids and hydrophobic proteins are assembled into lamellar bodies[26].

In the fetal lung, DSPC pool increases together with lamellar bodies formation, at approximately the 22° gestational week. In injuried lung, the saturated percentage of PC can decrease through two different mechanisms: unsaturated plasma PC or blood cell derived PC that can damage surfactant; type II cell synthesis and secretion can be decreased[27].

The total surfactant content can be divided into alveolar and an lung (intracellular) pool[28]. However, the total surfactant pool size is not equivalent to the amount of active surfactant. Maintaining adequate surfactant pools within the air space is essential for lung function and is dependent on the dynamic cycle of surfactant metabolism [29]. It is reduced to less than 10 mg/kg surfactant in preterm infants who have respiratory distress syndrome (RDS) compared with term infants who have an estimated pool size of 100 mg/kg surfactant[30]. Exogenous surfactants are given at doses between 10 and 20 times the normal pool sizes during surfactant replacement therapy which approximates the pool size in term infants.

PULMONARY SURFACTANT METABOLISM

Pulmonary surfactant is synthesized, assembled, transported and secreted into the alveolar space where it is degraded. Within the alveolus surfactant is synthesized by the alveolar type II cells, which constitute about 10% of the cells covering the alveolar surface. Surfactant is packaged and stored in special organelles, the lamellar bodies, where the surfactant material is assembled in a series of densely packed bilayers. SP-A, SP-B and SP-C and certain lysosomal proteins are detected in lamellar bodies, but the bulk of SP-A is secreted separately from the lamellar bodies, as must be the case for SP-D because lamellar bodies are devoid of SP-D. After fusion of the lamellar bodies with the plasma membrane, the content of the lamellar bodies is released into the liquid layer covering the epithelial surface, where surfactant is at least partially rearranged into a lattice-like structure, called tubular myelin. It is widely thought (but unproven) that from this tubular myelin, lipids and proteins are inserted into the monolayer, where they can perform their surface-tension reducing function[31]. During a breathing cycle, lipids

are squeezed out of the monolayer. To maintain the primary surface function of the monolayer, i.e., reduction of the surface tension, the loss of lipids from the monolayer has to be compensated by renewed insertion of lipids. Because the *de novo* synthesis of surfactant is insufficient to correct the natural loss (i.e., inactivation of surfactant), the alveolar type II cell not only produces newly synthesized surfactant but also reutilizes inactivated surfactant, derived from the alveolar space.

The massive secretion of surfactant that occurs at birth with the first breath requires prior massive synthesis of surfactant components by type II cells. After birth, considerable proportion of surfactant is continuously recycled, and the demand for *"de novo"* synthesis is much lower. Most of the cholesterol of the surfactant is thought to be derived from serum lipoproteins[32], whereas phospholipids are synthesized by type II cells. Foetal type II cell precursors accumulate considerable amounts of glycogen, which has been suggested to serve as carbon source for lipid synthesis that are typically synthesized in endoplasmatic reticulum and Golgi apparatus[33,34].

Surfactant secretion can be stimulated by a number of mechanisms. Type II cells have beta-adrenergic receptors and respond to beta-agonists with increased surfactant secretion[35]. Purines, such as adenosine triphosphate are potent stimulators of surfactant secretion and may be important for its secretion at birth. Mechanical stretch such as lung distension and hyperventilation, have also been found to be involved in stimulating surfactant secretion. Stretch-mediated enhancement of surfactant secretion during exercise prevents a loss of alveolar surfactant[36]. Hormones also play a role in surfactant secretion. Thyroxine accelerates Type II cell differentiation while acting synergistically with glucocorticoids to enhance the distensibility of the lung and DPPC synthesis. However, glucocorticoids alone are used in clinical practice to induce lung maturity because studies have not shown that the synergistic effect with thyroxine is greater than the effect achieved by glucocorticoids alone.

Surfactant is therefore recycled by type II cells or catabolized by alveolar macrophages in a highly regulated system that maintains precise levels of pulmonary surfactant throughout life. Surfactant turnover is a major pathway for surfactant in the alveolar space.

Type II cells, macrophages and the alveolar lining play a major role in surfactant turnover. Cyclical changes in the alveolar surface appear to promote conversion of newly secreted, apoprotein-rich, active surfactant aggregates into protein-poor,

inactive forms that are ready for clearance[37]. In healthy conditions surfactant components are removed from air spaces through uptake by Type II cells and alveolar macrophages, which contribute equally in surfactant turnover[38]. The phospholipids, organized in multivesicular bodies, are taken up by endocytosis into the Type II cells where they are catabolized by lysosomes or recycled and re-secreted into lamellar bodies; whereas the surfactant proteins are only recycled back into the lamellar bodies for re-secretion with surfactant (Figure 5).

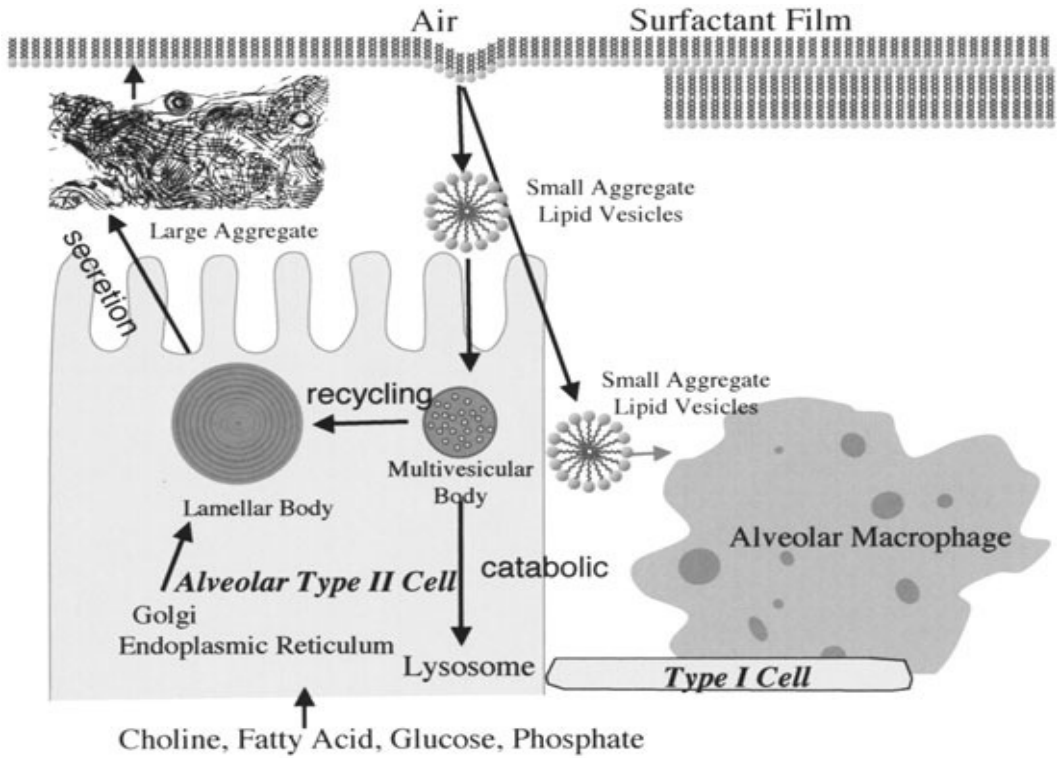


Figure 5. Surfactant metabolism.

Surfactant is also transformed during the cyclic compression and expansion of alveoli from large, highly surface-active aggregates into smaller, less active subtypes[39]. Instead all the surfactant uptaken by macrophages will be catabolized and lost. In total lung macrophages, 20% is represented by alveolar macrophages and 80% is constituted by lung tissue digestive macrophages[40]. In vitro studies estimated that the normal surfactant turnover period is surprisingly short, approximately ranging from 4 to 11 hours[41].

In general, defects in surfactant metabolism occur due to accelerated breakdown of the surfactant complex by oxidation, proteolytic degradation, and inhibition[42,43]. Some inherited surfactant gene defects have also been implicated[8].

PULMONARY SURFACTANT INACTIVATION

Surfactant inhibition, or inactivation, refers to those processes that decrease or abolish the normal surface activity of pulmonary surfactant. The major inhibitory factors include plasma proteins, unsaturated membrane phospholipid, lysophospholipids, free fatty acids, meconium (fetal feces expelled during stress), and high cholesterol levels[44].

Stage of lung maturation is a variable influencing sensitivity of surfactant to inhibition. Sensitivity to inhibition by plasma proteins increases as gestational age decreases[45]. Surfactant inhibition can also arise from degradation of surfactant lipids by phospholipases or of surfactant proteins by proteases. These degradative agents, normally present in the alveolus at very low levels, can be increased during microbial infection and more importantly through secretion by leukocites and type II cells with pulmonary inflammation[11,46]. In addition to above, surfactant can be compromised by reactive oxygen species[47], and by pollutants[48].

The main lytic enzyme implied in PL degradation are the phospholipases 2 (PLA_2) superfamily. Their principle role is to catalyze the hydrolysis of the sn-2 ester bond in a variety of different PLs. The endproducts of these reactions is a PL deriving from position 2 and the remaininig lyso-PL, both of them can serve as lipid mediator precursor: the free PL can be metabolized to form various eicosanoids and related bioactive mediators, while the second can be a platelet activator factors (PAF) and lysophasphatidic acid precursor.

In humans by five main types of PLA_2 have been characterized: secreted PLA_2, cytosolic PLA_2, Ca^{2+} PLA_2, lysosomal PLA_2 and PAF acethylhydrolases. Assignment of enzymes to a certain group is based on the catalytic mechanism (His/Asp, Ser/Asp or Ser/His/Asp hydrolase) as well as functional and structural features on sequence, molecular weight, disulfide bonding patterns, the requirement for Ca^{2+}, etc[49,50,51].

In contrast to lytic enzymes that act chemically to degrade pulmonary surfactant components, cellular lipids and blood-derived proteins typically impair lung surfactant activity through biophysical interactions. Several biophysical mechanisms of action appear to be involved[52,53]. Blood proteins, cell membrane lipids, and FFA all have intrinsic surface activity. These substances adsorb to the air-water interface and form surface films just as do the components of pulmonary surfactant. Competitive adsorption from large proteins like albumin and

haemoglobin has been shown to interfere significantly with the entry of lung surfactant constituents into the interface[52]. FFA also adsorb readily, but unlike large proteins, these small molecules tend to interpenetrate and form mixed films with lung surfactant phospholipids. When the content of fluid unsaturated fatty acids like oleic acid rises to a sufficient level in the surface film, its ability to reach low surface tensions during subsequent dynamic compression is compromised[53]. Cell membrane lipids, including lysophospholipids, have also been shown to impair the surface activity of pulmonary surfactant[54], but detailed mechanistic studies have not been done[55].

PREMATURITY AND RDS IN LUNG

Expected gestational age for normal delivery is at least 37 gestational weeks. If the newborn is delivered earlier it is named pre-term infant. In the last 15 years, improvements done in neonatal care and clinical progress lead to a medical miracle for 24 weeks newborn survival. The premature foetus at 24 weeks is extremely immature in many respects. The tissues defining the potential air-blood barrier are thicker than at term newborn and pulmonary microvascular development has not fully vascularized the saccular mesenchyme. The enzymes and lamellar bodies required for surfactant synthesis and secretion are just appearing as some type II cells begin to mature. A normal foetus at this age is completely surfactant deficient and unable to quickly synthesize or secrete surfactant if delivered; and is not ready to support air breathing. This foetus also has multiple other organ immaturities that only become “abnormalities” if the foetus is delivered (Table 2)[56].

Table 2. Immaturity of organs of normal foetuses at 24 weeks' gestation responsible for morbidity and mortality.

Lungs	About 250,000 saccules, no alveoli Poor microvascular development No surfactant Minimal matrix in tissue
Kidneys	Very low renal blood flow and glomerular filtration Low sodium reabsorption Inability to concentrate
Gut	Low digestive enzymes Poorly developed peristalsis
Skin	Minimal cornification Poor barrier function
Brain	Immature respiratory control
Immune system	Inadequate responses to infection

In term newborns pulmonary surfactant is secreted by type II epithelial cell into the airways of the lung from 24 weeks gestation, although only adequate amounts from 35 weeks gestation. Defective surfactant metabolism leads to both morbidity and mortality in preterm and term neonates. Moreover surfactant metabolic processes are slower in newborns (especially those born prematurely) than in adults or those with lung injury.

The normal foetus will not deliver until term; however recent research into the causes of severe prematurity suggest that there are two main causes of severe prematurity: infection/inflammation and abnormal vascular/placental development (more than 50% of deliveries prior to 32 weeks have an inflammatory association)[57,58]. Indeed many of these preterms do survive with careful neonatal care only because the foetus has responded to the adverse intrauterine environment by improving the maturity of the organ systems.

Neonatal care strategy provides to ventilate and to administer exogenous surfactant to all infant with gestational age less than 28 weeks, and they are diagnosed with respiratory distress syndrome (RDS). RDS is one of the hallmark features of mortality in premature infants with 60% of incidence in newborns delivered prior to 32 gestational weeks. Major pathogenetic causes of this respiratory disorder are pulmonary immaturity and surfactant deficiency. Respiratory failure resulting may require mechanical ventilation, that by itself is a source of inflammation and pulmonary injury. Kramer et al. and coworkers, in *in vivo* studies, demonstrated striking outcome of the inflammatory/injury response

is induced lung maturation. The amounts of surfactant proteins increase in parallel with increases in surfactant lipids in bronchoalveolar lavages; the total effect is a more mature lung structure that contains more surfactant, has an increased compliance an supports a better gas exchange[59]. In addition to surfactant deficit, many other factors are involved in RDS development: among these, genetic factors, such as surfactant specific protein expression, administration of prenatal steroids, the infection and inflammation stage.

BIBLIOGRAPHY

1. Berthiaume Y, Voisin G, Dagenais A. The alveolar type I cells: the new knight of the alveolus? J Physiol 2006;572:609-10.
2. Schmitz G, Muller G. Structure and function of lamellar bodies, lipid-protein complexes involved in storage and secretion of cellular lipids. J Lipid Res 1991;32:1539-70.
3. Homer RJ, Zheng T, Chupp G, et al. Pulmonary type II cell hypertrophy and pulmonary lipoproteinosis are features of chronic IL-13 exposure. Am J Physiol Lung Cell Mol Physiol 2002;283:L52-9.
4. Thorley AJ, Ford PA, Giembycz MA, Goldstraw P, Young A, Tetley TD. Differential regulation of cytokine release and leukocyte migration by lipopolysaccharide-stimulated primary human lung alveolar type II epithelial cells and macrophages. J Immunol 2007;178:463-73.
5. Vanderbilt JN, Mager EM, Allen L, et al. CXC chemokines and their receptors are expressed in type II cells and upregulated following lung injury. Am J Respir Cell Mol Biol 2003;29:661-8.
6. Witherden IR, Vanden Bon EJ, Goldstraw P, Ratcliffe C, Pastorino U, Tetley TD. Primary human alveolar type II epithelial cell chemokine release: effects of cigarette smoke and neutrophil elastase. Am J Respir Cell Mol Biol 2004;30:500-9.
7. Andreeva AV, Kutuzov MA, Voyno-Yasenetskaya TA. Regulation of surfactant secretion in alveolar type II cells. Am J Physiol Lung Cell Mol Physiol 2007;293:L259-71.
8. Nkadi PO, Merritt TA, Pillers DA. An overview of pulmonary surfactant in the neonate: genetics, metabolism, and the role of surfactant in health and disease. Mol Genet Metab 2009;97:95-101.
9. Donn SM, Dalton J. Surfactant replacement therapy in the neonate: beyond respiratory distress syndrome. Respir Care 2009;54:1203-8.
10. Martin JA, Hamilton BE, Ventura SJ, Menacker F, Park MM. Births: final data for 2000. Natl Vital Stat Rep 2002;50:1-101.
11. Gunther A, Ruppert C, Schmidt R, et al. Surfactant alteration and replacement in acute respiratory distress syndrome. Respir Res 2001;2:353-64.
12. Batenburg JJ, Haagsman HP. The lipids of pulmonary surfactant: dynamics and interactions with proteins. Prog Lipid Res 1998;37:235-76.

13. Suwabe A, Mason RJ, Smith D, Firestone JA, Browning MD, Voelker DR. Pulmonary surfactant secretion is regulated by the physical state of extracellular phosphatidylcholine. J Biol Chem 1992;267:19884-90.
14. Perez-Gil J. Structure of pulmonary surfactant membranes and films: the role of proteins and lipid-protein interactions. Biochim Biophys Acta 2008;1778:1676-95.
15. Whitsett JA, Weaver TE. Hydrophobic surfactant proteins in lung function and disease. N Engl J Med 2002;347:2141-8.
16. Cochrane CG. Pulmonary surfactant in allergic inflammation: new insights into the molecular mechanisms of surfactant function. Am J Physiol Lung Cell Mol Physiol 2005;288:L608-9.
17. Cochrane CG, Revak SD. Pulmonary surfactant protein B (SP-B): structure-function relationships. Science 1991;254:566-8.
18. Mazela J, Merritt TA, Gadzinowski J, Sinha S. Evolution of pulmonary surfactants for the treatment of neonatal respiratory distress syndrome and paediatric lung diseases. Acta Paediatr 2006;95:1036-48.
19. Kingma PS, Whitsett JA. In defense of the lung: surfactant protein A and surfactant protein D. Curr Opin Pharmacol 2006;6:277-83.
20. Korfhagen TR, Bruno MD, Ross GF, et al. Altered surfactant function and structure in SP-A gene targeted mice. Proc Natl Acad Sci U S A 1996;93:9594-9.
21. Wright JR. Immunoregulatory functions of surfactant proteins. Nat Rev Immunol 2005;5:58-68.
22. Korfhagen TR, Sheftelyevich V, Burhans MS, et al. Surfactant protein-D regulates surfactant phospholipid homeostasis in vivo. J Biol Chem 1998;273:28438-43.
23. Ikegami M, Whitsett JA, Jobe A, Ross G, Fisher J, Korfhagen T. Surfactant metabolism in SP-D gene-targeted mice. Am J Physiol Lung Cell Mol Physiol 2000;279:L468-76.
24. Ikegami M, Na CL, Korfhagen TR, Whitsett JA. Surfactant protein D influences surfactant ultrastructure and uptake by alveolar type II cells. Am J Physiol Lung Cell Mol Physiol 2005;288:L552-61.
25. Li Z, Vance DE. Phosphatidylcholine and choline homeostasis. J Lipid Res 2008;49:1187-94.
26. Creuwels LA, van Golde LM, Haagsman HP. The pulmonary surfactant system: biochemical and clinical aspects. Lung 1997;175:1-39.

27. Jobe AH. The pathogenesis and treatment of pulmonary edema. Armond Futura Publishing 1998:113-31.
28. Rudiger M, Wendt S, Kothe L, Burkhardt W, Wauer RR, Ochs M. Alterations of alveolar type II cells and intraalveolar surfactant after bronchoalveolar lavage and perfluorocarbon ventilation. An electron microscopical and stereological study in the rat lung. Respir Res 2007;8:40.
29. Forbes A, Pickell M, Foroughian M, Yao LJ, Lewis J, Veldhuizen R. Alveolar macrophage depletion is associated with increased surfactant pool sizes in adult rats. J Appl Physiol 2007;103:637-45.
30. Jobe AH. Why surfactant works for respiratory distress syndrome. Neo Rev 7 2006:e95–e105.
31. ten Brinke A, van Golde LM, Batenburg JJ. Palmitoylation and processing of the lipopeptide surfactant protein C. Biochim Biophys Acta 2002;1583:253-65.
32. Guthmann F, Harrach-Ruprecht B, Looman AC, Stevens PA, Robenek H, Rustow B. Interaction of lipoproteins with type II pneumocytes in vitro: morphological studies, uptake kinetics and secretion rate of cholesterol. Eur J Cell Biol 1997;74:197-207.
33. Bourbon JR, Rieutort M, Engle MJ, Farrell PM. Utilization of glycogen for phospholipid synthesis in fetal rat lung. Biochim Biophys Acta 1982;712:382-9.
34. Carlson KS, Davies P, Smith BT, Post M. Temporal linkage of glycogen and saturated phosphatidylcholine in fetal lung type II cells. Pediatr Res 1987;22:79-82.
35. Mason RJ, Voelker DR. Regulatory mechanisms of surfactant secretion. Biochim Biophys Acta 1998;1408:226-40.
36. Dietl P, Haller T, Mair N, Frick M. Mechanisms of surfactant exocytosis in alveolar type II cells in vitro and in vivo. News Physiol Sci 2001;16:239-43.
37. Jobe AH. Lung Development and Maturation. Neon Perin Med 2002;Mosby, St. Louis.
38. Gurel O, Ikegami M, Chroneos ZC, Jobe AH. Macrophage and type II cell catabolism of SP-A and saturated phosphatidylcholine in mouse lungs. Am J Physiol Lung Cell Mol Physiol 2001;280:L1266-72.
39. Ueda T, Ikegami M, Jobe A. Surfactant subtypes. In vitro conversion, in vivo function, and effects of serum proteins. Am J Respir Crit Care Med 1994;149:1254-9.
40. Ikegami M. Surfactant catabolism. Respirology 2006;11 Suppl:S24-7.

41. Baritussio AG, Magoon MW, Goerke J, Clements JA. Precursor-product relationship between rabbit type II cell lamellar bodies and alveolar surface-active material. Surfactant turnover time. Biochim Biophys Acta 1981;666:382-93.
42. Andersson S, Kheiter A, Merritt TA. Oxidative inactivation of surfactants. Lung 1999;177:179-89.
43. Manalo E, Merritt TA, Kheiter A, Amirkhanian J, Cochrane C. Comparative effects of some serum components and proteolytic products of fibrinogen on surface tension-lowering abilities of beractant and a synthetic peptide containing surfactant KL4. Pediatr Res 1996;39:947-52.
44. Frerking I, Gunther A, Seeger W, Pison U. Pulmonary surfactant: functions, abnormalities and therapeutic options. Intensive Care Med 2001;27:1699-717.
45. Merrill JD, Ballard RA. Pulmonary surfactant for neonatal respiratory disorders. Curr Opin Pediatr 2003;15:149-54.
46. Griese M. Pulmonary surfactant in health and human lung diseases: state of the art. Eur Respir J 1999;13:1455-76.
47. Manzanares D, Rodriguez-Capote K, Liu S, et al. Modification of tryptophan and methionine residues is implicated in the oxidative inactivation of surfactant protein B. Biochemistry 2007;46:5604-15.
48. Bakshi MS, Zhao L, Smith R, Possmayer F, Petersen NO. Metal nanoparticle pollutants interfere with pulmonary surfactant function in vitro. Biophys J 2008;94:855-68.
49. Burke JE, Dennis EA. Phospholipase A2 biochemistry. Cardiovasc Drugs Ther 2009;23:49-59.
50. Burke JE, Dennis EA. Phospholipase A2 structure/function, mechanism, and signaling. J Lipid Res 2009;50 Suppl:S237-42.
51. Schaloske RH, Dennis EA. The phospholipase A2 superfamily and its group numbering system. Biochim Biophys Acta 2006;1761:1246-59.
52. Holm BA, Enhorning G, Notter RH. A biophysical mechanism by which plasma proteins inhibit lung surfactant activity. Chem Phys Lipids 1988;49:49-55.
53. Hall SB, Lu RZ, Venkitaraman AR, Hyde RW, Notter RH. Inhibition of pulmonary surfactant by oleic acid: mechanisms and characteristics. J Appl Physiol 1992;72:1708-16.
54. Holm BA, Notter RH. Effects of hemoglobin and cell membrane lipids on pulmonary surfactant activity. J Appl Physiol 1987;63:1434-42.
55. Holm BA, Wang Z, Notter RH. Multiple mechanisms of lung surfactant inhibition. Pediatr Res 1999;46:85-93.

56. Jobe AH. Lung maturation: the survival miracle of very low birth weight infants. Pediatr Neonatol 2010;51:7-13.
57. McElrath TF, Hecht JL, Dammann O, et al. Pregnancy disorders that lead to delivery before the 28th week of gestation: an epidemiologic approach to classification. Am J Epidemiol 2008;168:980-9.
58. Lahra MM, Jeffery HE. A fetal response to chorioamnionitis is associated with early survival after preterm birth. Am J Obstet Gynecol 2004;190:147-51.
59. Kramer BW, Kallapur S, Newnham J, Jobe AH. Prenatal inflammation and lung development. Semin Fetal Neonatal Med 2009;14:2-7.

Chapter 2

Analytical Methods Using Stable Isotope As Metabolic Tracers

INTRODUCTION

Isotope ratio mass spectrometry (IRMS) and mass spectrometry (MS) are mainly common techniques used to measure stable isotope enrichments since the last century. They mainly differ for sensitivity and precision, with IRMS its possible to measure extremely low enrichments above natural abundance. Stable isotopes can be used as natural or artificial tracer to trace organic molecules metabolism in living organisms and ecosystems.

Nowadays stable isotopes are used in different fields and multiplicity applications, archaeology[1], geochemistry[2], pharmaceutics[3], forensic science[4,5], anti-doping screening[6,7], environmental chemistry[8], and art[9].

Since the last decade, stable isotopes are used in research to determine turnover rates of substrates such as amino acids, glucose and fatty acids as well as energy consumption, distribution volumes of particular metabolites, the elucidation of metabolic pathways and pharmacokinetic studies[10,11,12,13,14]. In clinical diagnosis, stable isotopes are primarily employed for breath tests aiming at the evaluation of hepatic, gastric, small intestine and pancreatic functions, and for the diagnosis of *Helicobacter pylori* infection[15,16,17,18,19,20,21].

STABLE ISOTOPES

All atoms of one element have, by way of definition, the same number of protons in their nucleus, but the number of neutrons may differ if more than one stable combination of the nuclide is possible. The majority of chemical elements consists of a mixture of stable isotopes, which are not subjected to any radioactive disintegration. Due to the identical number of protons, they occupy the same (*isos*) position (*topos*) in the periodic table of elements. Due to difference in mass, stable isotopes of the same element differ in their physical, chemical and biochemical behavior resulting in kinetic and thermodynamic isotope effects. Isotopic distribution of element derives from the origin and the evolution of component they derive from. Therefore two components, with the same formula,

can have different isotopic composition deriving from different origins and/or histories, due to natural fractionation processes. This principle is used for normal abundance measurement in several fields, from agronomy to medicine.

TRACERS LABELED WITH STABLE ISOTOPES

Virtually any metabolic process involving protein, lipid or carbohydrate metabolism can be studied using stable isotopes of carbon (^{13}C), hydrogen (^{2}H, deuterium), nitrogen (^{15}N), or oxygen (^{18}O). In spite of possible small differences in metabolic behavior, molecules labeled with stable isotopes in one or several position are suitable tracers for the study of chemical and biological processes.

For a metabolic study, a tracer in which one or more atoms in the structure is replaced with one of these stable isotopes is administered. It is a misnomer to refer to such a labeled molecule to stable isotope, since the term isotopes refers to elements rather than molecules. Molecules that differ by the number or arrangement of isotopically labeled position are referred to as “isotopemers”. Thus, ^{13}C-leucine is a stable isotopically labeled isotopomer of leucine, not a stable isotope of leucine[22].

The introduction of stable isotopes into biological materials yields tracer substances which can be administered orally or intravenously. With modern analytical techniques, such as mass spectrometry, they can be consecutively be detected unchanged or after conversion into their metabolites.

A large number of different isotopes are used for studies in humans, but most widely used are the stable isotopes of hydrogen, carbon, oxygen and nitrogen.

In this table are summarized mean percent distribution of these elements:

Table 1: Mean percent distribution of principal elements.

Element	Stable istotopes	Natural mean abundance (%)	Standard ratio values	International standard reference
Hydrogen	1H 2H	99.985 0.015	2H/1H = 0.000316	SMOW (Standard Mean Ocean Water)
Carbon	12C 13C	98.892 1.108	13C/12C = 0.0112372	PDB (Pee Dee Belemnite) Fossil calcium carbonate
Nitrogen	14N 15N	99.6337 0.3663	15N/14N = 0.007353	AIR (nitrose from air)
Oxygen	16O 18O	99.7587 0.2039	18O/16O = 0.0039948	SMOW (Standard Mean Ocean Water)
Sulfur	32S 34S	95.02 4.22	34S/32S = 0.0450045	CDT (Canyon Diablo Troilite)

There are several advantages in the use of stable isotopes in medicine. First of all is that they can be used in pediatric and during gestation inasmuch there is no exposition to radiation, as it is with radioactive isotopes.

Indeed more stable isotopes can be administered simultaneously and in repeated doses allowing to study different biological aspects using the same sample.

ISOTOPES' TOXICITY

In animal studies high deuterium doses showed toxic effects, but administered doses were really higher than ones used in human studies. $^{2}H_2O$ amount equal to 10-20 % of body fluid mean an alteration in cellular function, decrease in protein synthesis and enzymatic reaction rate, and if increased to 30-40%, it is fatal[23,24].

In human administered doses are lower and it is established that the maximum doses to which adverse effects appear are 200-400 mg/kg body weight. In clinical studies deuterium is used in a highly lower amount, ranging from 1 to 80 mg/kg body weight[23]. Unlike deuterium, mass difference between ^{12}C and ^{13}C is low and even clinical effects due to ^{13}C administration are unusual. ^{13}C percentage contribution is already high representing 1,1% of the total carbon. In clinical studies, the normal tracer dose administered is 1 mg/kg of ^{13}C (rarely exceeds 25 mg/kg).

STABLE ISOTOPES APPLICATION IN HUMAN DIAGNOSIS AND RESEARCH

A variety of applications for stable isotopes in pediatric research and diagnosis has been described, some example are given in Table 2. For investigative purposes mainly ^{13}C labelled glucose, fatty acid and ^{15}N labelled amino acids and $^{2}H_2O$ or $^{1}H_2{}^{18}O$ are used. Total body water as well as energy expenditure can be determined by measuring the dilution of tracer in the body. Stable isotopes prove to be especially helpful during the examination of unknown metabolic pathways and inborn errors of metabolism. The kinetics of metabolic processes can be elucidated by tracer analyses in precursors, intermediates and endproducts. Direct endproducts such as CO_2, NH_3 and H_2O can be extracted and analysed without or with minor sample processing. More complex metabolites can be studied in tissue or plasma samples after clean up of the analyte. $^{13}CO_2$ breath test are easy to perform and painless for the patient. They are increasingly used particularly in gastro-enterological functional diagnosis. Numerous digestive processes can be evaluated due to the appearance of ^{13}C in breath, indicating the amount of oxidation of specific ^{13}C substrates. A practical example for the use of stable isotope labeled tracer in pediatrics is the examination of gastric emptying in newborn infant using carboxyl^{13}C-acetate.

Table 2: Applications of stable isotopes in pedriatic.

Topic	Tracer	References
Infections		
Helicobacter pylori	^{13}C-urea	17
Gastrointestinal functions		
Gastric emptying	^{13}C-acetate, ^{13}C-ottanoate	25-26
Maldigestion		
Lactase deficiency	^{13}C-lactose	27
Lipase deficiency	^{13}C-trioctanoin, ^{13}C-triolein, ^{13}C-palmitic acid	28
Malabsorption		
Carbohydrates	^{13}C-glucose	12
Fat	Different^{13}C-triglyceride	28
Amino acid	^{13}C-^{15}N- leucine, ^{13}C-leucine	29-31
Body compostiton		
Total body water	$D_2{}^{18}O$	14
Synthetesis procesess		
Gluconeogenesis	2,3-$^{13}C_2$-alanine, 6,6 ^{2}H-glucose	32-33
Albumin	^{15}N glycine, ^{13}C leucine	34-35
Cholesterol	D_2O	36
Fatty acid conversion	^{13}C- linoleic acid, different fatty acid enriched^{13}C(arachidonic, docosaesaenoic)	37-38
Metabolism and metabolic disorder		
Energy expenditure	$D_2{}^{18}O$	39-42
Fructose intolerance	^{13}C-fructose	43
Phenylketonuria	^{2}H-phenilalanine	44

Since 15 years our research group has been investigating on infant nutrition and pulmonary diseases with stable isotope tracers[45,46,47,48,49,50,51,52].

MOLECULAR MASS SPECTROMETRY AND ISOTOPE RATIO MASS SPECTROMETRY

Mass spectrometry (MS) can be defined as the study of ions in gas phase in order to characterize the molecular structure of a sample[53].

Conventional mass spectrometer are utilized in different applications including molecules and biological complex matrices analysis[54].

MOLECULAR MASS SPECTROMETRY

A conventional mass spectrometer is composed by seven parts mainly (Figure 1): introduction system for samples (inlet), ion source, mass analyzer, detector system, vacuum control system, informatics platform for instrument control and data analysis[55].

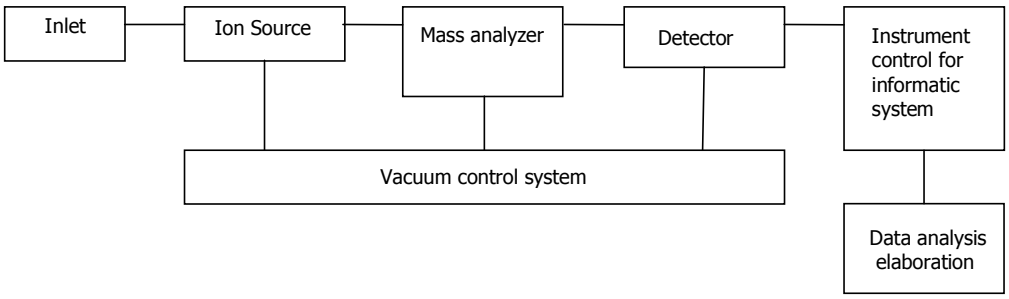


Figure 1. Block diagram of a mass spectrometer instrumentation

Within the ion source takes place the conversion of the sample from liquid to gas phase, and in details the production of positive ions, in gas phase, due to an interaction of an electron beam with sample molecules. Accelerated ions produced in the ion source are focused into the mass analyzer, electromagnetic field, where are separated by their mass (m) to charge (z) ratio (m/z); while into the detector system ions are detected and a ion current is generated, whose intensity is properly amplified and registered.

The ion current intensity produced by a specific m/z ion will be proportional to the amount of this ion into the sample, that depends from ionization proprieties of a specific molecule. Mass analyzer and detector are constantly under high vacuum control. Modern mass spectrometers are fully controlled by sophisticated software and data obtained are automatically recorded by an appropriate management system. The type of inlet, ion source and analyzer characterized a specific instrument and the potentials of a specific system.

The majority of ^{13}C enrichment studies utilize a MS coupled with a gas cromatographer, with an ion source, in an electron impact or chemical ionization mode, an analyzer such as a quadrupole and a dyode for the detection system to enhance the low ion current produced by ionized sample through the analyzer.

The instrument used in this thesis is an Agilent gas cromatographer 6890N interfaced with a 5873 *inert* mass spectrometer (Agilent Technologies, Cernusco sul Naviglio, Milano, Italia) with a ion source operating in electron impact

ionization mode and a single quadrupole analyzer in selected ion monitoring mode in order to measure simultaneously up to four isotopic masses.

ISOTOPE RATIO MASS SPECTROMETRY (IRMS)

The measurement of isotopic differences in natural abundance requires a mass spectrometer with lower sensitivity but higher precision and resolution than conventional mass spectrometer; the instrument utilized is an isotopic ratio mass spectrometer (IRMS). In a IRMS instrumentation there are two different system for sample introduction, the Dual Inlet (DI-IRMS) and Continuos Flow (CF-IRMS). In the first case the sample is prepared for the analysis in off-line mode, transformed into gas before the injection. Despite in CF technique, the sample is injected into an helium flow that, like a carrier, facilitate the introduction of the sample into the ion source. This system can be interfaced with preparative and/or separative techniques, such as elementar analyzer (EA), gas chromatography (GC) and more recently liquid chromatography (LC). The technique used to measure isotopic composition of organic samples is GC, that leads to separate samples previously derivatized in order to transform them in volatile phase (Figure 2).

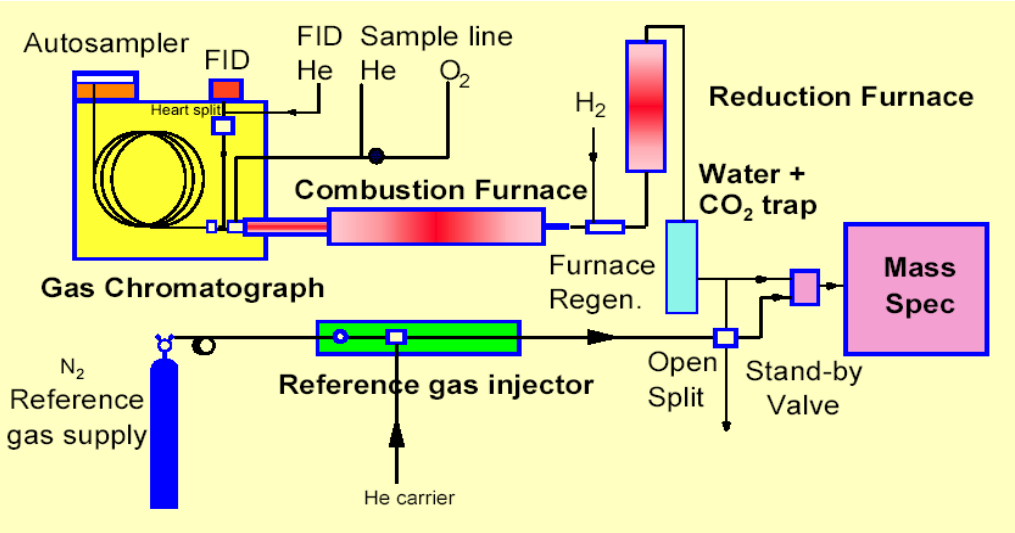


Figure 2. GC-C-IRMS scheme

One splitter at the end of the chromatographic column, leads to the sample to reach the combustion or pyrolisis tube, for nitrogen, carbon, hydrogen and oxygen analysis. For carbon analysis samples pass through oxidation tube made of ceramic and alumina containing a copper, platinum and nichel filaments[56]. The oxidation

into the oven take place for the reaction of copper with pure oxygen at 600-650°C to form CuO (copper oxide). Platinum then react as a catalyzer in the reaction between nichel and oxygen to form NiO (nickel oxide) at 960°C. The reduction oven removes the oxygen excess and reduces the eventually formed species of nitrogen oxide and nitrogen. After the sample combustion to CO_2 and H_2O, water is removed by a nafium trap. The final portion of interface is the open split that leads the helium flow to the magnetic analyzer (Figure 3).

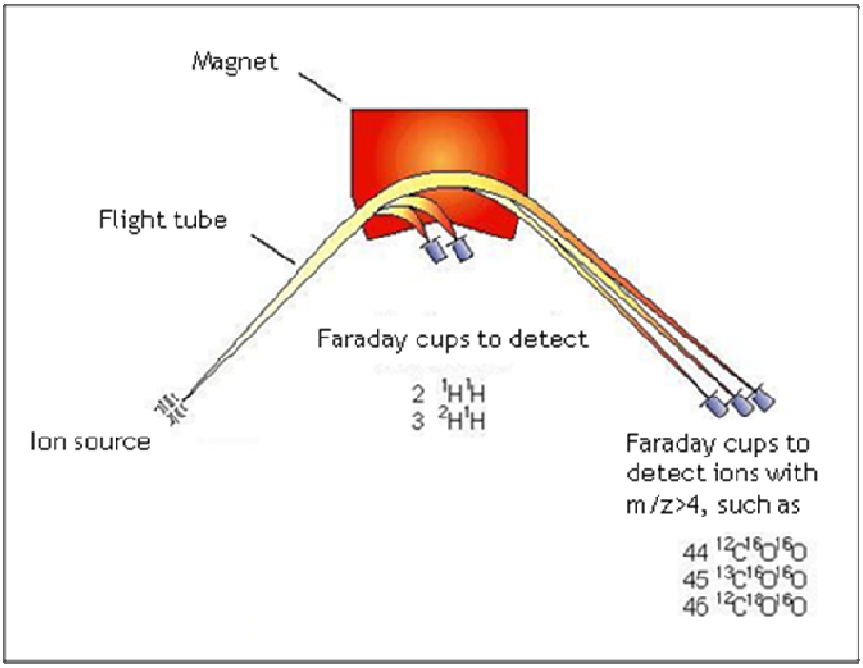


Figure 3. IRMS magnetic analyzer

Isotopic ratio can be calculated from simultaneous measurement of 44 m/z mass and 45 m/z mass, corresponding respectively to $^{12}CO_2$ and $^{13}CO_2$.

To measure hydrogen samples are injected into a pyrolysis reactor at very high temperature (1400°C) that transform quantitatively the molecule in H_2 or CO that consequently are detected by the mass analyzer. Isotopic ratio can be calculated considering 3 m/z corresponding to $^{2}H_2$ and 2 m/z corresponding to $^{1}H_2$.

Isotopic ratios are usually expressed in term of δ (delta) that express the deviations, part per thousand, of a reference standard:

$$\delta X(‰) = \left(\frac{R_{sample} - R_{standard}}{R_{standard}} \right) \text{x } 1000$$

where R= heavy isotope mass/ light isotope mass ratio (i.e. $^{13}C/^{12}C$, $^{2}H/^{1}H$);

δX>0 when the heavy isotope is enriched versus standard in the sample;

δX<0 when the heavy isotope is impoverished or the light isotope is enriched versus standard in the sample.

In order to standardize and compare measurements, δ values are converted in atom percent excess (APE) using a calibration curve obtained by increasing amount of enriched sample. Regarding ^{13}C enrichment, changing of 1 ($^{0}/_{00}$) is approximately equivalent to 0.001 APE shift.

The instrument used in this thesis is a Delta XL Plus (ThermoFinnigan, Rodano, Milano, Italia), interfaced with a combustion system (GC Combustion III) and with a pyrolysis system at high temperature Temperature Convertion (TC), to analyze respectively carbon and hydrogen in organic molecules. To analyze deuterium in urine, IRMS was interfaced with High Temperature Conversion Elemental Analyzer (TC/EA). TC-EA (ThermoFinnigan, Rodano, Milano, Italia) is an elementar analyzer that allow to analyze isotopic composition of oxygen ($^{18}O/^{16}O$) and hydrogen ($^{2}H/^{1}H$) in biological samples.

MASS SPECTROMETRY *versus* ISOTOPE RATIO MASS SPECTROMETERY

In order to measure isotope ratio single quadrupole mass spectrometer must operate in selected ion monitoring (SIM) mode to increase sensitivity. Thus, in this configuration with limited accuracy and precision samples should have a minimum enrichment of 0.05 APE at least[1,57]. Virtually single quadrupole mass spectrometer don't allow accurate and precise measurements of natural abundance or in case with low incorporation of the labeled tracer with an enrichment < 0.05 APE. Simultaneous analysis of two or three isotopomeric species, with GC-IRMS, enable to measure isotopic composition with low enrichment and natural abundances with high accuracy and precision. Low amount of heavy isotopes can be detected with high quantity of light isotope. GC-IRMS sensitivity leads to detect tracer to trace ratio (mol/mol) below 10^{-5} .

BIBLIOGRAPHY

1. Brand WA. High precision isotope ratio monitoring techniques in mass spectrometry. J Mass Spectrom 1996;31:225-35.
2. Craig HR. Isotopic standards for carbon and oxygen correction factors for mass spectrometric analysis of carbon dioxide. Geochim Cosmochim Acta 1957;12:133-49.
3. Jasper JP, Westenberger BJ, Spencer JA, Buhse LF, Nasr M. Stable isotopic characterization of active pharmaceutical ingredients. J Pharm Biomed Anal 2004;35:21-30.
4. Benson S, Lennard C, Maynard P, Roux C. Forensic applications of isotope ratio mass spectrometry--a review. Forensic Sci Int 2006;157:1-22.
5. Fraser I, Meier-Augenstein W. Stable (2)H isotope analysis of modern-day human hair and nails can aid forensic human identification. Rapid Commun Mass Spectrom 2007;21:3279-85.
6. Ayotte C, Levesque JF, Cle roux M, Lajeunesse A, Goudreault D, Fakirian A. Sport nutritional supplements: quality and doping controls. Can J Appl Physiol 2001;26 Suppl:S120-9.
7. Cawley AT, Flenker U. The application of carbon isotope ratio mass spectrometry to doping control. J Mass Spectrom 2008;43:854-64.
8. Schmidt TC, Zwank L, Elsner M, Berg M, Meckenstock RU, Haderlein SB. Compound-specific stable isotope analysis of organic contaminants in natural environments: a critical review of the state of the art, prospects, and future challenges. Anal Bioanal Chem 2004;378:283-300.
9. Fortunato G, Ritter A, Fabian D. Old Masters' lead white pigments: investigations of paintings from the 16th to the 17th century using high precision lead isotope abundance ratios. Analyst 2005;130:898-906.
10. Curtius HC, Vollmin JA, Baerlocher K. Study of metabolic pathways in vivo using stable isotopes. Analyt Chem 1973;45:1107–10.
11. Jones PJ. Stable isotopes in nutrition research: historical perspective and overview. Can J Physiol Pharmacol 1990;68:935-40.
12. Lifschitz CH, Boutton TW, Carrazza F, et al. A carbon-13 breath test to characterize glucose absorption and utilization in children. J Pediatr Gastroenterol Nutr 1988;7:842-7.
13. Pacy PJ, Cheng KN, Thompson GN, Halliday D. Stable isotopes as tracers in clinical research. Ann Nutr Metab 1989;33:65-78.

14. Trowbridge FL, Graham GG, Wong WW, et al. Body water measurements in premature and older infants using H218O isotopic determinations. Pediatr Res 1984;18:524-7.

15. Eggers RH, Kulp A, Tegeler R, et al. A methodological analysis of the 13C-urea breath test for detection of Helicobacter pylori infections: high sensitivity and specifity within 30 min using 75 mg of 13C-urea. Eur J Gastroenterol Hepatol 1990;2:437–44.

16. Hiele M, Ghoos Y, Rutgeerts P, Vantrappen G. Starch digestion in normal subjects and patients with pancreatic disease, using a 13CO2 breath test. Gastroenterology 1989;96:503-9.

17. Koletzko S, Haisch M, Seeboth I, et al. Isotope-selective non-dispersive infrared spectrometry for detection of Helicobacter pylori infection with 13C-urea breath test. Lancet 1995;345:961-2.

18. Shreeve WW, Shoop JD, Ott DG, McInteer BB. Test for alcoholic cirrhosis by conversion of [14C]- or [13C]galactose to expired CO2. Gastroenterology 1976;71:98-101.

19. Solomons NW, Schoeller DA, Wagonfeld JB, Ott D, Rosenberg IH, Klein PD. Application of a stable isotope (13C)-labeled glycocholate breath test to diagnosis of bacterial overgrowth and ileal dysfunction. J Lab Clin Med 1977;90:431-9.

20. Vantrappen GR, Rutgeerts PJ, Ghoos YF, Hiele MI. Mixed triglyceride breath test: a noninvasive test of pancreatic lipase activity in the duodenum. Gastroenterology 1989;96:1126–34.

21. Watkins JR, Klein PD, Schoeller DA, Kirschner BS, Park R, Perman JA. Diagnosis and differentiation of fat malabsorption in children using 13C-labeled lipids: trioctanoin, triolein,and palmitic acid breath tests. Gastroenterology 1982;82:911–7.

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

23. Koletzko B, Demmelmair H, Hartl W, et al. The use of stable isotope techniques for nutritional and metabolic research in paediatrics. Early Hum Dev 1998;53 Suppl:S77-97.

24. Jones PJ, Leatherdale ST. Stable isotopes in clinical research: safety reaffirmed. Clin Sci (Lond) 1991;80:277-80.

25. Pozler O, Neumann D, Vorisek V, Bukac J, Bures J, Kokstein Z. Development of gastric emptying in premature infants. Use of the (13)C-octanoic acid breath test. Nutrition 2003;19:593-6.

26. Barbosa L, Vera H, Moran S, Del Prado M, Lopez-Alarcon M. Reproducibility and reliability of the 13C-acetate breath test to measure gastric emptying of liquid meal in infants. Nutrition 2005;21:289-94.

27. Hiele M, Ghoos Y, Rutgeerts P, Vantrappen G, Carchon H, Eggermont E. 13CO2 breath test using naturally 13C-enriched lactose for detection of lactase deficiency in patients with gastrointestinal symptoms. J Lab Clin Med 1988;112:193-200.

28. Watkins JB, Klein PD, Schoeller DA, Kirschner BS, Park R, Perman JA. Diagnosis and differentiation of fat malabsorption in children using 13C-labeled lipids: trioctanoin, triolein, and palmitic acid breath tests. Gastroenterology 1982;82:911-7.

29. Cortiella J, Matthews DE, Hoerr RA, Bier DM, Young VR. Leucine kinetics at graded intakes in young men: quantitative fate of dietary leucine. Am J Clin Nutr 1988;48:998-1009.

30. de Benoist B, Abdulrazzak Y, Brooke OG, Halliday D, Millward DJ. The measurement of whole body protein turnover in the preterm infant with intragastric infusion of L-[1-13C]leucine and sampling of the urinary leucine pool. Clin Sci (Lond) 1984;66:155-64.

31. Balagopal P, Ford GC, Ebenstein DB, Nadeau DA, Nair KS. Mass spectrometric methods for determination of [13C]Leucine enrichment in human muscle protein. Anal Biochem 1996;239:77-85.

32. Bier DM, Leake RD, Haymond MW, et al. Measurement of "true" glucose production rates in infancy and childhood with 6,6-dideuteroglucose. Diabetes 1977;26:1016-23.

33. Frazer TE, Karl IE, Hillman LS, Bier DM. Direct measurement of gluconeogenesis from [2,3]13C2]alanine in the human neonate. Am J Physiol 1981;240:E615-21.

34. Yudkoff M, Nissim I, McNellis W, Polin R. Albumin synthesis in premature infants: determination of turnover with [15N]glycine. Pediatr Res 1987;21:49-53.

35. Jacobs R, Demmelmair H, Rittler P, et al. Isolation of plasma albumin by ethanol extraction is inappropriate for isotope ratio measurements during the acute phase response. J Chromatogr B Analyt Technol Biomed Life Sci 2005;817:145-51.

36. Biolo G, Agostini F, Simunic B, et al. Positive energy balance is associated with accelerated muscle atrophy and increased erythrocyte glutathione turnover during 5 wk of bed rest. Am J Clin Nutr 2008;88:950-8.

37. Demmelmair H, von Schenck U, Behrendt E, Sauerwald T, Koletzko B. Estimation of arachidonic acid synthesis in full term neonates using natural variation of 13C content. J Pediatr Gastroenterol Nutr 1995;21:31-6.

38. Koletzko B, Demmelmair H, Socha P. Nutritional support of infants and children: supply and metabolism of lipids. Baillieres Clin Gastroenterol 1998;12:671-96.

39. Jones PJ, Winthrop AL, Schoeller DA, et al. Validation of doubly labeled water for assessing energy expenditure in infants. Pediatr Res 1987;21:242-6.

40. Schoeller DA, Hnilicka JM. Reliability of the doubly labeled water method for the measurement of total daily energy expenditure in free-living subjects. J Nutr 1996;126:348S-54S.

41. Roberts SB, Coward WA, Schlingenseipen KH, Nohria V, Lucas A. Comparison of the doubly labeled water (2H2(18)O) method with indirect calorimetry and a nutrient-balance study for simultaneous determination of energy expenditure, water intake, and metabolizable energy intake in preterm infants. Am J Clin Nutr 1986;44:315-22.

42. Lucas A, Ewing G, Roberts SB, Coward WA. How much energy does the breast fed infant consume and expend? Br Med J (Clin Res Ed) 1987;295:75-7.

43. Gopher A, Vaisman N, Mandel H, Lapidot A. Determination of fructose metabolic pathways in normal and fructose-intolerant children: a 13C NMR study using [U-13C]fructose. Proc Natl Acad Sci U S A 1990;87:5449-53.

44. Matalon R, Matthews DE, Michals K, Bier D. The use of deuterated phenylalanine for the in vivo assay of phenylalanine hydroxylase activity in children. J Inherit Metab Dis 1982;5:17-9.

45. Carnielli VP, Sulkers EJ, Moretti C, et al. Conversion of octanoic acid into long-chain saturated fatty acids in premature infants fed a formula containing medium-chain triglycerides. Metabolism 1994;43:1287-92.

46. Cogo PE, Carnielli VP, Bunt JE, et al. Endogenous surfactant metabolism in critically ill infants measured with stable isotope labeled fatty acids. Pediatr Res 1999;45:242-6.

47. Cogo PE, Zimmermann LJ, Rosso F, et al. Surfactant synthesis and kinetics in infants with congenital diaphragmatic hernia. Am J Respir Crit Care Med 2002;166:154-8.

48. Cogo PE, Zimmermann LJ, Meneghini L, et al. Pulmonary surfactant disaturated-phosphatidylcholine (DSPC) turnover and pool size in newborn infants with congenital diaphragmatic hernia (CDH). Pediatr Res 2003;54:653-8.

49. Cogo PE, Zimmermann LJ, Pesavento R, et al. Surfactant kinetics in preterm infants on mechanical ventilation who did and did not develop bronchopulmonary dysplasia. Crit Care Med 2003;31:1532-8.

50. Cogo PE, Zimmermann LJ, Verlato G, et al. A dual stable isotope tracer method for the measurement of surfactant disaturated-phosphatidylcholine net synthesis in infants with congenital diaphragmatic hernia. Pediatr Res 2004;56:184-90.

51. Cogo PE, Toffolo GM, Ori C, et al. Surfactant disaturated-phosphatidylcholine kinetics in acute respiratory distress syndrome by stable isotopes and a two compartment model. Respir Res 2007;8:13.

52. Cogo PE, Simonato M, Mariatoffolo G, et al. Dexamethasone therapy in preterm infants developing bronchopulmonary dysplasia: effect on pulmonary surfactant disaturated-phosphatidylcholine kinetics. Pediatr Res 2008;63:433-7.

53. Liebler DC. Introduction to proteomics: tools for the new biology. Humana Press 2002.

54. Hopfgartner G, Bourgogne E. Quantitative high-throughput analysis of drugs in biological matrices by mass spectrometry. Mass Spectrom Rev 2003;22:195-214.

55. Kinter M. Protein sequencing and identification using tandem mass spectrometry. Wiley-Interscience 2000.

56. Merritt DA, Freeman KH, Ricci MP, Studley SA, Hayes JM. Performance and optimization of a combustion interface for isotope ratio monitoring gas chromatography/mass spectrometry. Anal Chem 1995;67:2461-73.

57. Preston T, Slater C. Mass spectrometric analysis of stable-isotope-labelled amino acid tracers. Proc Nutr Soc 1994;53:363-72.

Chapter 3

Investigation On The Preferential Metabolic Precursors For Surfactant DSPC Biosynthesis In Preterm Newborns With Respiratory Distress Syndrome In First Days Of Life, With Low Energy Intake

INTRODUCTION

Preterm infants have immature lungs and they are inclined to develop RDS, a disease caused by lack of surfactant production and secretion into the alveoli. The primary site of synthesis of pulmonary surfactant are alveolar type II epithelial cell where it is storaged and secreted into alveolar space. While much work has focused on the pathways of pulmonary phospholipid synthesis in animals, little is known on the provision of its synthetic precursors in animals and even more in humans.

Surfactant phospholipids synthesis is characterized by two different pathways: the "*de novo*" fatty acids synthesis and the remodeling synthesis using the free fatty acids deriving from the circulating blood.

a) The precursors recruited in the "*de novo*" fatty acid synthesis are glucose, preferentially, lactate or acetate and this pathway has been shown to occur in the lung or in the liver[1-2]. The "*de novo*" biosynthetic scheme can be considered into two parts: first the synthesis of phospatidic acid from nonlipid precursors, consequently synthesis of DSPC from choline and phosphatidic acid. Phosphatidic acid is the product of acylation of acylglcerol-3-phosphate, which in turn is formed by dihydroxy-acetone phosphate by reduction and than acylation or acylation followed by reduction reactions[3-4]. Glucose, glycerol and free fatty acids are incorporated into phospholipids at this stage of the biosynthetic scheme; glucose and glycerol are metabolized via the glycolitic pathway to dihydroxyacetone phosphate or to acetate and hence fatty acids. Then choline is phosphorilated and transferred to cytidine 5'-diphosphate before reacting with diacyl-glycerol, obtained by phosphatidic acid dephosphorilation, to form DSPC[5]. The interplay among these various

sources of pulmonary fatty acids is poorly understood, but it may be influenced by the concentrations of available substrates, dietary and hormonal factors and altered physiologic states[6-8]. The relative contribution of each of the potential sources of fatty acid (glucose, acetate, lactate and free fatty acid) for incorporation into the lung surfactant DSPC has been studied in animals by radioactive isotopes tracers and more recently by stable isotopes tracers [1, 9-13].

b) In the remodeling biosynthesis mechanism, that has been shown to occur mainly in lung, DSPC is formed by deacylation of the unsaturated species and subsequent reacylation of lyso-PC. Deacylation is catalyzed by PLA_2, a superfamily enzymes present in the lung in different isoforms according to their subcellular district; reacylation can occur by at least two mechanisms, the first being the reacylation of lyso-PC with acyl-CoA, catalyzed by an acyltransferase, the second a transacylation in which two molecules of lyso-PC react to form one molecule of DSPC and a glycerophosphocholine[5].

In the last fifeteen years, our research group focused on the utilization of stable isotope tracer methodology to quantify surfactant DSPC kinetics synthesized from plasma glucose, free palmitate and body water[14-16] in humans. These tracers have not been used simultaneously in previous human studies.

The clinical interest of our research is also focused on the assessment of the dietary contribution of metabolic substrates on surfactant DSPC synthesis it could influence the nutritional management in the first hours and days of life. Intravenous glucose is commonly given as nutritional support in preterm infants during the first hours of life, and intravenous lipids are often withheld for hours or sometimes for days[17]. The effect of early nutritional management in preterm infants on surfactant synthesis is unknown.

The effect of undernutrition on respiratory outcome was studied in animals as much as three decades ago. Brief fasting in rats for up to 72 hours induced a decrease in DPPC content of lung lavage fluid[18], associated with increases in minimal surface tension[19-20] and decreased stability of small liquid bubbles released from lung fragments[21]. Since not all these early reports consistently showed alteration on pressure-volume curves, it was suggested that in some instances there was enough of a surfactant reserve to maintain alveolar stability even if the animals were undernourished[19]. Hyperventilation further worsens lung compliance[22]. Later it was also observed that prenatal starvation decreased

surfactant phospholipids pool and altered lung structure and function in animal models[23-24], whereas overnutrition or increased fatty acid availability to the lungs could be associated with increased surfactant lipid synthesis[25]. No studies have been conducted in animal models of neonatal RDS.

In humans insufficient nutrition is detrimental to the foetus and to the preterm neonate; newborns who are small for dates are at increased risk for adverse respiratory outcome both in the neonatal period and later in infancy[26-27]. No data are available on the effect of dietary manipulation on surfactant production for either adults or infants.

AIM OF THE STUDY

In the first year of my PhD project, we investigated the preferential metabolic precursor for DSPC synthesis. The study combined three stable isotope methods[14-16] to quantify the rate of surfactant DSPC synthesis from body water, plasma glucose and plasma free fatty acid palmitate. We have applied this methodology in preterm infants with RDS, who required exogenous surfactant at birth and prolonged mechanical ventilation, while receiving fat free parenteral nutrition and were receiving no or negligible amounts of enteral feeds.

MATERIAL AND METHODS

Study patients

Surfactant kinetics was studied in 23 preterm infants whose clinical characteristics are reported in Table 1. The Institutional Ethic Committee approved the study and informed consent was obtained from both parents.

Table 1. Mean ± SD clinical characteristics of the three study groups.

	GLUC *vs* PA	GLUC *vs* H_2O	PA *vs* H_2O	p
Patients, (n)	8	8	7	
Birth weight, mean ± SD, (gr)	1155 ± 207[a]	1412 ± 657[b]	898 ± 185[a]	0.08
Gestational age, mean ± SD, (weeks)	28.5 ± 1.2[a]	30.0 ± 2.2[b]	26.9 ± 1.1[a]	<0.01
Prenatal Steroids (%)	63	87	86	0.10
Study age, mean ± SD, (hours)	20 ± 13	30 ± 23	27 ± 35	0.72
Surfactant dose>1 (%)	25 [a]	37 [a]	71 [b]	0.19
Total surfactant dose, mean ± SD, (mg/kg)	200 ± 100	150 ± 75	200 ± 58	0.38
Oxygenation Index, mean ± SD	3.3 ± 1.8	3.5 ± 1.7	3.2 ± 0.9	0.90
Conventional ventilation, mean ± SD, (days)	5.7 ± 4.6	15 ± 14	12 ± 9	0.22
Intake mean ± SD, (Kcal/kg)	31 ± 2	31 ± 5	30 ± 7	0.84
Survival (%)	100	100	100	0.99
BPD at 36 wks (%)	14 [a]	37 [a]	71 [b]	0.10

Values with different superscripts are significantly different with $p<0.05$.

All patients were admitted to the Neonatal Intensive Care Unit of the Department of Paediatrics, University of Padova, Italy. Inclusion criteria were: 1) gestational age between 25 and 30 weeks; 2) respiratory failure requiring endotracheal intubation for an estimated length of time of at least 48 hours; 3) arterial and venous lines placed for clinical monitoring; 4) written informed consent. Exclusion criteria were congenital infections and chromosomal abnormalities. Exogenous surfactant (Curosurf ®, Chiesi Farmaceutici S.p.A, Parma, Italy) was administered endotracheally at a dose of 100 mg/kg, if the mean airway pressure exceeded 7.5 cm of water or if the inspiratory oxygen fraction was higher than 0.40. Infants received a second dose if the same criteria were met after the first dose. We

recorded clinical data and physiological parameters (respiratory rate, heart rate, hemoglobin saturation) hourly during the study. Ventilator parameters and arterial blood gas analysis were recorded before the start of the study and subsequently every 6 hours. The oxygenation index was calculated as $[(MAP*FiO_2)/PaO_2]*100$, where MAP is the mean airway pressure, FiO_2 the fraction of inspired oxygen and PaO_2 the arterial blood partial oxygen tension at each of these time points.

Study design

Patients were divided into 3 groups: 1) Glucose *vs*. Palmitate: newborns received a 24 hours constant intravenous infusion of $U^{13}C$-Glucose (GLUC), and intravenous albumin bound $^2H_3$-Palmitate (PA)[15,28]; 2) Glucose *vs*. Water: patients received a 24 hours intravenous infusion of $U^{13}C$-Glucose and constant primed infusion of ^2H_2O; 3) Palmitate *vs*. Water: patients received a 24 hour intravenous infusion of albumin bound $U^{13}C$-Palmitate and a 48 hour bolus administration of ^2H_2O. $U^{13}C$-Glucose (CIL, Andover, MA) was infused at 0.17 mg/kg/min by the same high precision, calibrated syringe pump. $U^{13}C$-palmitate and $^2H_3$-palmitate (Martek Columbia, MD, USA) were bound to human albumin and infused i.v. at a constant rate of 1.0 $\pm$ 0.26 μmol/kg/h by a high precision, calibrated syringe pump (M22, Harvard Apparatus Co, Inc Natick, MA). ^2H_2O was given as an i.v. bolus of 2 ml/kg at the study start and then 0.125% of fluid intake as ^2H_2O every 12 hours over the next 48 hours to maintain body water deuterium enrichment at plateau[16]. All infants were on total parenteral nutrition without lipids during the first 24 hours of the study.

The isotope tracers infusions were started in all patients within 48 hours from birth and were terminated before 96 hours of life. The start of the study (t = 0) was defined by the start of the tracer administration. In those patients who received exogenous surfactant, the infusion was started at the time of the first surfactant administration. We used a central venous line (umbilical) for tracer infusion, a peripheral line or the second lumen of the central venous line for ^2H_2O administration and an arterial line for blood sampling. Chemical and isotopic purity of all stable isotope tracers were confirmed by gas chromatography and gas chromatography-mass spectrometry.

Blood (0.6 ml) was drawn at time 0, 5.30, 6, 12, 18 and 24 hours from the start of the study to determine the isotopic enrichment of plasma free fatty acid palmitate and plasma glucose. The blood was placed in tubes containing EDTA, immediately centrifuged at 1300 *g* and plasma was stored at -20°C until analysis.

One ml of urine was collected before the start of the study and then every 6 hours for 48 hours and stored at -20°C until analysis.

Tracheal aspirates were obtained before the start of the infusion, at 3 and 6 hours, and every 6 hours thereafter until 72 hours. Afterwards the samples were collected every 12 hours until the extubation or until study day 10. Tracheal aspirates were performed as it follows: 0.5 ml normal saline was injected into the endotracheal tube, and after twenty seconds, suctioning was performed with the tip of the suction catheter beyond the tip of the endotracheal tube. All tracheal aspirates were brought to a final volume of 2 ml. The sample was then gently vortexed and centrifuged at 400 *g* for 10 minutes. The supernatant was stored at -20°C until analysis.

Analytical procedure

a) Plasma lipids

The lipid content was extracted from plasma with chloroform and methanol, according to Folch[29], lipid classes separated by thin layer chromatography (TLC), and their fatty acids derivatized as methyl esters. The separation and identification of free fatty acid methyl esters from the plasma lipid classes were performed by capillary gas chromatography[28,30]. Isotopic enrichments of plasma free fatty acids and of the albumin-bound $U^{13}C$-Palmitate and $^{2}H_3$-Palmitate tracers in the plasma were determined by gas-chromatograph coupled with a quadrupole mass spectrometer (GC-MS) (Voyager, Thermoquest, Rodano, Milano, Italy) operating in negative ionization mode. Selective ion monitoring was carried out at m/z 255, 256, 257 and 271 for $U^{13}C$-palmitate and at m/z 255, 256, 257 and 258 for $^{2}H_3$-Palmitate. Enrichments were expressed as mole percent excess (MPE) that represents the increase in the mole percentage of palmitic acid moiety ($^{2}H_2$ and $U^{13}C$) above the baseline value obtained at time 0 of the study. Each sample was measured in duplicate[31].

b) Plasma glucose

The water fraction of delipidated plasma was passed over anion- and cation-exchange resin[29]. The eluate containing the glucose was derivatized to an aldonitril pentaacetate derivative and enrichment measured by gas-chromatography-combustion interface-isotope ratio mass spectrometry (GC-IRMS) (Finnigan™ DELTA^{plus} XL, Thermo Electron Corporation). Enrichments were expressed as delta ‰. Enrichments were corrected for isotopic dilution contributed by the derivative group[14].

c) Urine water

Urine was filtered with 0.22-μm filter and diluted 1:500 with reference water. Deuterium in the urine was analyzed by GC-IRMS in triplicate and calibrated against Vienna-Standard Mean Ocean Water[32]. Reference water was interspersed every six injections to ensure that memory effect was insignificant[32]. Enrichments were expressed as delta ‰.

d) DSPC isolation and enrichments from tracheal aspirates

Tracheal aspirates' phospholipids were extracted according to Bligh and Dyer[33] and surfactant DSPC was isolated by thin layer chromatography[34] after oxidation with osmium tetroxide[31]. The TLC spot of DSPC was derivatized[35] and the fatty acid methyl esters were extracted with hexane and stored at -20°C. The amount of DSPC in tracheal aspirates was measured from the fatty acids methyl esters by capillary gas chromatography[31]. Tracheal aspirates containing visible blood were not analyzed. Isotopic enrichments were measured by GC-IRMS[14,16]. Each sample was analyzed in duplicate. Enrichments of DSPC-palmitate derived from $U^{13}C$-Glucose and ^2H_2O were measured in delta ‰. Enrichments of DSPC-palmitate derived from $U^{13}C$-Palmitate and $^2H_3$-Palmitate were expressed in MPE by means of calibration curves ranged from 0 to 1% MPE. MPE by GC-IRMS represents the increase in isotopic enrichment above baseline after tracer(s) administration of ^{13}C and ^2H and complete combustion and pyrolisis, respectively. Enrichments were corrected for isotopic dilution contributed by the derivative group.

Calculations

Surfactant kinetics were calculated as previously published[14,16] using the precursor-product approach and assuming isotopic steady state condition. Briefly: 1) Secretion time (ST), defined as the time lag between the start of the tracers infusion and the appearance of the respective labeled fatty acid in surfactant DSPC-palmitate. 2) Fractional Synthesis Rate (FSR) defined as the percentage of the total surfactant DSPC pool synthesized from the respective plasma palmitate, glucose and ^2H_2O per day. It was calculated from the linear portion of the enrichment rise over time relative to the plateau enrichment of the respective precursors. For ^2H_2O the plateau enrichment was corrected for the maximum number (N=22) of deuterium that could be incorporated in the DSPC-palmitate[36]. Total FSR is the sum of the two DSPC-FSR derived either from plasma palmitate, or from glucose and ^2H_2O. 3) Half-life derived from the final, decreasing, mono-exponential portion of the enrichment *versus* time curve. 4) Peak time (PT) as the

time of maximum enrichment of surfactant DSPC-palmitate from the start of the infusion.

Data analysis

Data are presented as individual values and group mean ± SD. We compared groups of patients receiving different tracers and also compared the results of different tracers being simultaneously administered to the same patients. Thus comparisons were made by unpaired and paired t-tests between 2 groups for independent and related samples respectively and by ANOVA among the 3 groups. The level of significance accepted was $p<0.05$. Correlations were assessed by Pearson correlation. Microsoft Excel 2000 (Microsoft Corp, Redmond, WA), Prism 3.0 GraphPad Software and SPSS (15.0 version; SPSS Inc, Chicago, IL) were used for calculations and statistical analysis.

RESULTS

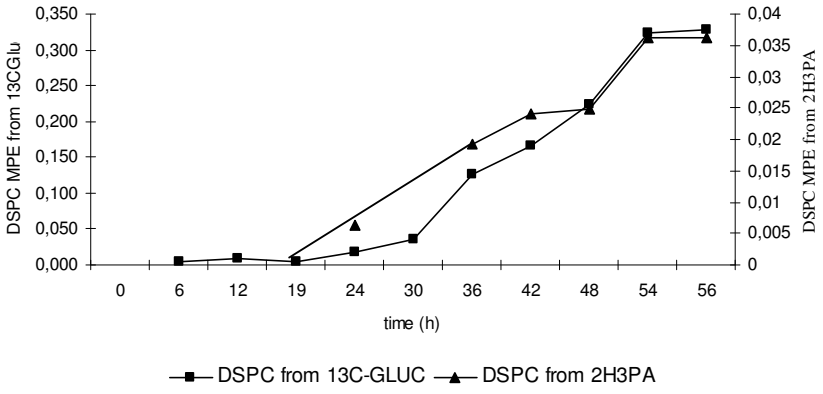
We performed 46 metabolic studies in 23 preterm infants. Eight infants received simultaneously $U^{13}C$-Glucose and ^2H_2O, 8 infants received $^2H_3$-Palmitate and $U^{13}C$-Glucose and 7 received $U^{13}C$-Palmitate and ^2H_2O. Clinical characteristics of the study groups are shown in Table 1 (Material and Methods section). During the first year of my PhD I performed analysis of patients belonging to the group receiving $U^{13}C$-Palmitate and ^2H_2O; while others groups have been previously analyzed in my research group.

All but one infant had mild or severe RDS and required exogenous surfactant. Among the treated infants, 45% required a second dose of exogenous surfactant after 24 ± 12 hours and all showed good clinical response to exogenous surfactant. All infants were on total parenteral nutrition with 7 g/kg/day of carbohydrate and 2 g/kg/day of aminoacids for the first 24 hours of the study. Energy intake in the 3 groups is reported in Table 1. Intravenous lipids and minimal enteral feeding were started after 96 hours of life according with the nutritional policy of the neonatal unit when the study was performed.

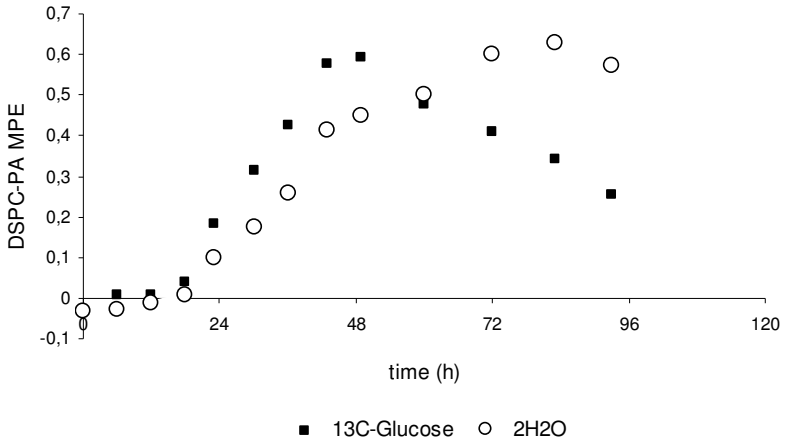
Plasma free fatty acid and plasma glucose concentrations were stable during the study period. The mean plasma glucose level was 2.7 ± 0.9 mmol/L and mean plasma free fatty acids levels were 7.0 ± 4.5 mg/dl. In all infants, the tracers were at steady state in plasma from time 6 to 24 hours for glucose and palmitate, and from 6 to 48 hours for ^2H_2O since the slope of the linear regression of the precursors' (i.e. palmitate, glucose and water) enrichment values vs. time was not significantly different from zero. Mean plasma $U^{13}C$-Glucose enrichment was 670 ± 198 delta ‰ (2.0 ± 0.6 MPE), mean plasma $U^{13}C$-Palmitate MPE was 1.7 ± 0.4, mean plasma $^2H_3$-Palmitate MPE was 0.40 ± 0.15, and mean deuterium enrichment in the urine was 18506 ± 6326 delta ‰ (0.29 ± 0.02 MPE).

A significant incorporation of the i.v. labelled palmitate and of palmitate deriving from *"de novo"* lipogenesis was measurable in the surfactant DSPC-palmitate from all patients. Figure 1 shows the time curves of the ^{13}C and ^2H enrichments of DSPC-palmitate deriving from $U^{13}C$-Glucose and from $^2H_3$-palmitate infusion (Panel A), the time curves of the ^{13}C and ^2H enrichments of DSPC-palmitate deriving from $U^{13}C$-Glucose and from ^2H_2O (Panel B), and the time curves of ^{13}C and ^2H enrichments of DSPC-palmitate deriving from $U^{13}C$-Palmitate and ^2H_2O (Panel C).

Panel A



Panel B



Panel C

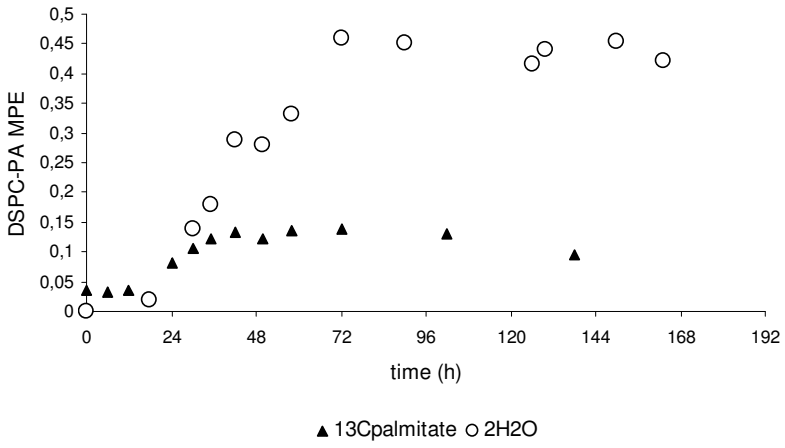


Figure 1. Plots belong to one representative patient. Panel A shows the time curve of the ^{13}C and ^{2}H enrichments of DSPC-palmitate deriving from $U^{13}C$-Glucose (black squares) and from $^{2}H_3$-palmitate (black triangles) infusion, panel B shows the time curves of the ^{13}C and ^{2}H enrichments of DSPC-palmitate deriving from $U^{13}C$-Glucose (black squares) and from $^{2}H_2O$ (white circles), and Panel C shows the time curves of ^{13}C and ^{2}H enrichments of DSPC-palmitate deriving from $U^{13}C$-Palmitate (black triangles) and $^{2}H_2O$ (white circles). All measurements were performed with GC-IRMS.

Kinetics data of all infants studied are shown in Table 2.

Table 2. Mean ± SD kinetics data of the three study groups.

	$U^{13}C$-Glucose	$U^{13}C$ & $^{2}H_3$ Palmitate	$^{2}H_2O$	p
Study, (n)	16	15	15	
Fractional synthesis rate (%/day)	17 ± 11	21 ± 16	15 ± 6	0.36
Secretion Time (hours)	20 ± 6	17 ± 12	24 ± 14	0.32
Peak Time (hours)	58 ± 21	68 ± 21	98 ± 34	<0.01
Half-life* (hours)	78 ± 59	170 ± 128	125 ± 56	0.12

* Half-life could be calculated in only 10 infants receiving IV $U^{13}C$-Glucose, in 8 infants receiving stable isotope palmitate and in 5 infants receiving $^{2}H_2O$, because of early extubation.

Comparisons were made with unpaired t-tests. While DSPC-FSR and ST were not different among the three groups, the slow body water turnover delayed significantly the PT in infants receiving $^{2}H_2O$ compared with infants receiving stable isotope glucose and palmitate. Moreover, since many infants were extubated before complete decay in enrichment, surfactant DSPC-palmitate half-life could be reliably calculated in only 10/16 infants from $U^{13}C$-Glucose, in 8/15 from palmitate and 5/15 from $^{2}H_2O$. Therefore for the purpose of comparing tracers, only the DSPC-FSR was used for the paired statistical analysis. The mean oxygenation index during the study was significantly and negatively correlated with either surfactant DSPC-FSR derived from *"de novo"* lipogenesis (23 infants, R=0.57, p=0.007) and from plasma free fatty acid palmitate (15 infants, R=0.61, p=0.016). This negative correlation was stronger when the oxygenation index was correlated with the total DSPC-FSR (sum of FSR from lipogenesis and that from free fatty acid-palmitate) (15 infants, R=0.72, p=0.002). No significant correlation was found with gestational age and body weight.

Paired analysis of the 8 infants who received simultaneous $U^{13}C$-Glucose and $^{2}H_2O$ gave a mean DSPC-FSR difference of –0.1 ± 3% (p=0.91). FSR from $U^{13}C$-Glucose and $^{2}H_2O$ were highly correlated (R^2=0.93, p=0.001) (Figure 2).

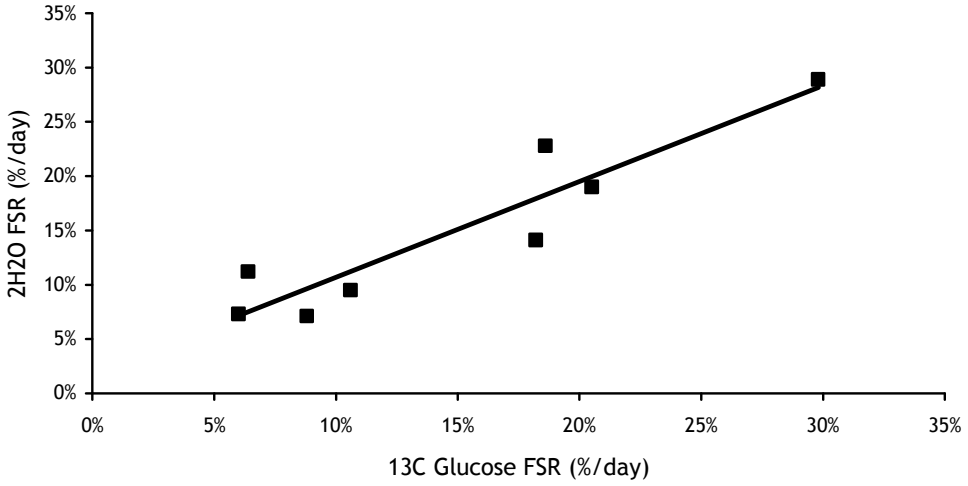


Figure 2. Correlation between fractional synthesis rate of $U^{13}C$-Glucose and ^2H_2O. The two fractional synthesis rates were significantly correlated (R^2=0.93, p=0.001).

Fifteen infants received palmitate and ^2H_2O or $U^{13}C$-Glucose simultaneously. Total DSPC-FSR was 43 ± 30%/day. DSPC-FSR from *"de novo"* lipogenesis significantly correlated with the DSPC-FSR from plasma palmitate (R =0.55, p=0.04). Paired data analysis of FSR showed a mean difference of +4.5 ± 11.8% per day (palmitate vs. glucose) and +4.6 ± 16.3% per day (palmitate vs. ^2H_2O) (p=0.21 by paired t test). The percentage of DSPC-FSR from plasma glucose was 49 ± 20%, and that from plasma free fatty acid-palmitate 51 ± 20%.

DISCUSSION

A better understanding of the metabolic precursors for surfactant synthesis may improve the nutritional management of the patients with respiratory failure with primary or secondary surfactant deficiency. In this study we used the simultaneous administration of stable isotope labeled palmitate, glucose, and water to assess which metabolic precursor is preferentially used for surfactant DSPC synthesis in preterm infants with RDS on parenteral nutrition and low energy intake.

We have previously described a new method to measure pulmonary surfactant DSPC-palmitate synthesis by deuterium incorporation by using low and safe doses of ^2H_2O [16]. As we reported, the method is less invasive and less expensive than the other stable isotope methods and it greatly simplifies the measurement of surfactant kinetics in humans, since ^2H_2O can be given i.v. as well as orally and deuterium enrichment can be measured in plasma or in urine; however in that study we did not compare the DSPC-FSR from body water to that obtained from other lipogenic tracers, such as glucose or acetate[14, 37-38].

Surfactant DSPC production appears to be a carefully timed event in animal experiments with an increased capacity of *"de novo"* fatty acid synthesis and of DSPC content during gestation, followed by a sharp decline of *"de novo"* fatty acids synthesis in the immediate postnatal period, probably related to postnatal diet and increased availability of dietary and thus plasma lipids[1,39]. In the present study we found that the DSPC-palmitate FSR from $U^{13}C$-glucose and from ^2H_2O were highly correlated (Figure 2) and that the mean paired difference was -0.1 ± 3% (p=0.91 by paired t test). This observation confirmed previous findings in animals and also our hypothesis that tracing the *"de novo"* synthesis of palmitate through either $U^{13}C$-glucose or ^2H_2O yields similar results[1].

While the glucose and water trace the contribution of the *"de novo"* FA-DSPC synthesis pathway, plasma free fatty acid-palmitate evaluates the contribution of circulating palmitate to DSPC synthesis. Two of the 3 study groups received simultaneously a tracer for the *"de novo"* fatty acids DSPC synthesis pathway (glucose or water) and a tracer for plasma free fatty acid. DSPC-palmitate kinetics parameters were calculated and comparison considered only for FSR and ST, since fifty percent of DSPC-palmitate peak time and half-life values were missing due to early extubation. No significant differences in DSPC-palmitate FSR and ST were found between infants receiving plasma palmitate and infants receiving glucose or water, suggesting that preterms on low caloric intake and during the first days of

life are able to use indifferently the available metabolic precursors for surfactant DSPC synthesis (Table 2). This was confirmed also by paired analysis (within-groups), where we found that the lipogenic pathway and the incorporation of plasma palmitate contributed almost equally to surfactant synthesis. This observation differs from that of Spence et al. who found that the plasma palmitate pathway contributed twice as much as the *"de novo"* synthetic pathway to surfactant synthesis. The infants in that study, though, were chronologically older (2-3 days, 2 weeks, and 6 weeks), had already received surfactant replacement, were developing chronic lung disease, and were receiving enteral feeds, all of which could have contributed to these differences in results[40].

Moreover it has also been previously reported hypertriglyceridemia and abnormal fat accumulation in the lungs of newborns with bronchopulmonary dysplasia, suggesting an abnormal lipid metabolism in preterm infants developing chronic lung disease[41-42]. An additional methodological explanation can also be considered: the approach of Spence et al. uses ^{13}C-acetate and mass isotopomer distribution analysis to compute the DSPC-palmitate FSR[40], whereas in our study we quantify the DSPC-palmitate FSR derived from body water hydrogen. Thus the two methods measure different aspects of the *"de novo"* palmitate synthesis that may not be theoretically equivalent and may not return identical estimates of DSPC-palmitate FSR. Although the groups of infants in the different tracer protocols were heterogeneous with respect to birth weight and gestational age, and we cannot exclude the possibility that this heterogeneity masked small differences in substrate utilization, all study infants had a similar severity of RDS, which is likely to play a more important role in the kinetics of surfactant metabolism[43-44]. Furthermore, and perhaps more importantly, the majority of our data are obtained comparing different tracers in the same individual and applying paired data statistics. We found a significant inverse correlation between the DSPC-FSR and the oxygenation index suggesting that, in accordance with previous animal studies, lung disease severity affects DSPC synthesis[45-49]. Even when the total DSPC-FSR is enhanced, though, there is no preference for lipogenesis vs. palmitate incorporation in our study infants who received low amounts of lipid intake.

Several methodological issues are relevant to our results: A) surfactant DSPC-palmitate FSR calculated from $^{2}H_2O$ relies on a deuterium isotopic steady state of at least 60 hours, since the body water turnover is a slow process. B) Plasma glucose and palmitate have a much faster turnover possibly leading to a more imprecise determination of DSPC-palmitate FSR in those infants who received a

second surfactant dose during the study. C) Secretion time and peak time are mainly affected by the administration of unlabeled exogenous surfactant, which diluted the endogenous enriched DSPC-palmitate to a variable degree and postpones the point of maximum enrichment. D) DSPC-palmitate half-life has a wide variation and it is artificially prolonged because the slow body water turnover leads to a long residence time of $^{2}H_2O$, which causes a delay of the DSPC isotopic decay over time due to ongoing synthesis. On the other hand the simultaneous infusion of two tracers in 23 infants allowed us to make comparisons between the two tracers fractional incorporation rates within the same subjects, which clearly strengthened the power of the statistical analysis.

From a nutritional point of view, study infants received fat free parenteral nutrition with 5.2 ± 1.5 mg/kg/min of glucose, 2 g/kg/day of amino acids, and energy intake of 28 to 40 Kcal/kg/day. Although this energy intake reflects what is often prescribed to preterm infants in the first days of life[17], it provides an energy intake well below energy expenditure. We do not know if the relative contributions of *"de novo"* lipogenesis and circulating fatty acids to the DSPC-FSR could be increased by higher energy intake or by adding fat emulsion to the parenteral nutrition. Higher glucose intake could increase the *"de novo"* lipogenesis and decrease the contribution of fat by shutting down lipolysis and reducing plasma free fatty acid. Alternatively increasing plasma triglycerides and free fatty acid either from increased lipolysis of body stores or by administering i.v. lipid emulsions could increase the availability of free fatty acid precursors. Studies in preterm infants addressing both the issues are in progress.

In vitro data suggested that the alveolar type II cells are capable of active *"de novo"* fatty acid synthesis, which is inhibited when exogenous palmitate is available[1,9]. In vivo ^{3}H-acetate and ^{14}C-palmitate have been used by many researchers to investigate the incorporation of fatty acids from plasma and *"de novo"* synthesis into surfactant[10,45,50]. The major limitation of these studies is that the fatty acid contribution to surfactant phosphatidylcholine incorporation was based only on product enrichment without taking into account the precursor enrichment. Therefore it is difficult to compare these data directly with the values obtained in our study. More recently Martini et al. used a simultaneous infusion of uniformly labeled ^{13}C acetate and palmitate in adult pigs to quantify surfactant DSPC kinetics during low-dose glucose infusion[11,15,51,52]. They showed that the phosphatidylcholine-bound palmitate incorporation rate from plasma palmitate was 10 times higher than that derived from *"de novo"* synthesis. During

high rate glucose infusion, the contribution of *"de novo"* synthesis of palmitate to phosphatidylcholine synthesis significantly increased and the contribution from plasma preformed palmitate significantly decreased[12]. It is conceivable that these large and fasted animals had significant body fat stores and a high rate of lipolysis, and therefore a large amount of plasma free fatty acid were available for DSPC synthesis. Clearly this does not reflect the metabolic condition of the preterm infants in this study.

The FSR values from plasma palmitate and from glucose found in our study were higher than those found by us and by others in preterm infants with RDS; these values are closer to the values found in term infants with no lung disease[14,15,53-56]. However the purpose of this study was to compare two metabolic tracers in the same infants and thus, patient selection criteria, degree and stage of respiratory failure and treatment modality could account for the difference in measurements between this and our previous studies.

In summary this study provides new data on the contribution of the *"de novo"* synthesis and preformed pathways to the DSPC-palmitate synthesis in preterm infants on low energy intake. Further studies on the quality and amount of energy intake are needed to assess the role of nutrition on surfactant metabolism in preterm newborns.

BIBLIOGRAPHY

1. Maniscalco WM, Finkelstein JN, Parkhurst AB. De novo fatty acid synthesis in developing rat lung. Biochim Biophys Acta 1982;711:49-58.
2. Wang MC, Meng HC. Synthesis of phospholipids and phospholipid fatty acids by isolated perfused rat lung. Lipids 1974;9:63-7.
3. Fisher AB, Huber GA, Furia L, Bassett D, Rabinowitz JL. Evidence for lipid synthesis by the dihydroxyacetone phosphate pathway in rabbit lung subcellular fractions. J Lab Clin Med 1976;87:1033-40.
4. Wykle RL, Malone B, Snyder F. Biosynthesis of dipalmitoyl-sn-glycero-3-phosphocholine by adenoma alveolar type II cells. Arch Biochem Biophys 1977;181:249-56.
5. Rooney SA. Lung surfactant. Environ Health Perspect 1984;55:205-26.
6. Buechler KF, Rhoades RA. Fatty acid synthesis in the perfused rat lung. Biochim Biophys Acta 1980;619:186-95.
7. Rhoades RA, Shaw ME, Eskew ML. Influence of altered O2 tension on substrate metabolism in perfused rat lung. Am J Physiol 1975;229:1476-9.
8. Bassett DJ, Fisher AB, Rabinowitz JL. Effect of hypoxia on incorporation of glucose carbons into lipids by isolated rat lung. Am J Physiol 1974;227:1103-8.
9. Maniscalco WM, Stremmel W, Heeney-Campbell M. Uptake of palmitic acid by rabbit alveolar type II cells. Am J Physiol 1990;259:L206-12.
10. Jobe A. An in vivo comparison of acetate and palmitate as precursors of surfactant phosphatidylcholine. Biochim Biophys Acta 1979;572:404-12.
11. Martini WZ, Chinkes DL, Barrow RE, Murphey ED, Wolfe RR. Lung surfactant kinetics in conscious pigs. Am J Physiol 1999;277:E187-95.
12. Martini WZ, Irtun O, Chinkes DL, Barrow RE, Wolfe RR. Glucose effects on lung surfactant kinetics in conscious pigs. Am J Physiol Endocrinol Metab 2000;279:E920-6.
13. Martini WZ, Irtun O, Chinkes DL, Barrow RE, Wolfe RR. Surfactant phosphatidylcholine in thermally injured pigs. Crit Care Med 2001;29:1417-22.
14. Bunt JE, Zimmermann LJ, Wattimena JL, van Beek RH, Sauer PJ, Carnielli VP. Endogenous surfactant turnover in preterm infants measured with stable isotopes. Am J Respir Crit Care Med 1998;157:810-4.

15. Cogo PE, Carnielli VP, Bunt JE, et al. Endogenous surfactant metabolism in critically ill infants measured with stable isotope labeled fatty acids. Pediatr Res 1999;45:242-6.
16. Cogo PE, Gucciardi A, Traldi U, Hilkert AW, Verlato G, Carnielli V. Measurement of pulmonary surfactant disaturated-phosphatidylcholine synthesis in human infants using deuterium incorporation from body water. J Mass Spectrom 2005;40:876-81.
17. Grover A, Khashu M, Mukherjee A, Kairamkonda V. Iatrogenic malnutrition in neonatal intensive care units: urgent need to modify practice. JPEN J Parenter Enteral Nutr 2008;32:140-4.
18. Gail DB, Massaro GD, Massaro D. Influence of fasting on the lung. J Appl Physiol 1977;42:88-92.
19. Faridy EE. Effect of food and water deprivation on surface activity of lungs of rats. J Appl Physiol 1970;29:493-8.
20. Weiss HS, Jurrus E. Starvation of compliance and surfactant of the rat lung. Respir Physiol 1971;12:123-9.
21. Garbagni R, Coppo F, Grassini G, Cardellino G. Effects of lipide loading and fasting on pulmonary surfactant. Respiration 1968;25:458-64.
22. Thet LA, Alvarez H. Effect of hyperventilation and starvation on rat lung mechanics and surfactant. Am Rev Respir Dis 1982;126:286-90.
23. Lechner AJ, Winston DC, Bauman JE. Lung mechanics, cellularity, and surfactant after prenatal starvation in guinea pigs. J Appl Physiol 1986;60:1610-4.
24. Harding R, Tester ML, Moss TJ, et al. Effects of intra-uterine growth restriction on the control of breathing and lung development after birth. Clin Exp Pharmacol Physiol 2000;27:114-9.
25. Chander A, Fisher AB. Choline-phosphate cytidyltransferase activity and phosphatidylcholine synthesis in rat granular pneumocytes are increased with exogenous fatty acids. Biochim Biophys Acta 1988;958:343-51.
26. Regev RH, Lusky A, Dolfin T, Litmanovitz I, Arnon S, Reichman B. Excess mortality and morbidity among small-for-gestational-age premature infants: a population-based study. J Pediatr 2003;143:186-91.
27. Lum SS, Fletcher WS, O'Dorisio MS, Nance RW, Pommier RF, Caprara M. Distribution and functional significance of somatostatin receptors in malignant melanoma. World J Surg 2001;25:407-12.

28. Cogo PE, Giordano G, Badon T, et al. Simultaneous measurement of the rates of appearance of palmitic and linoleic acid in critically ill infants. Pediatr Res 1997;41:178-82.
29. Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497-509.
30. Carnielli VP, Pederzini F, Vittorangeli R, et al. Plasma and red blood cell fatty acid of very low birth weight infants fed exclusively with expressed preterm human milk. Pediatr Res 1996;39:671-9.
31. Torresin M, Zimmermann LJ, Cogo PE, et al. Exogenous surfactant kinetics in infant respiratory distress syndrome: A novel method with stable isotopes. Am J Respir Crit Care Med 2000;161:1584-9.
32. Gucciardi A, Cogo PE, Traldi U, et al. Simplified method for microlitre deuterium measurements in water and urine by gas chromatography-high-temperature conversion-isotope ratio mass spectrometry. Rapid Commun Mass Spectrom 2008;22:2097-103.
33. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911-7.
34. Touchstone JC, Levitt RE, Soloway RD, Levin SS. Separation of conjugated dihydroxy bile acids by thin-layer chromatography. J Chromatogr 1979;178:566-70.
35. Christie WW, Brechany EY, Shukla VK. Analysis of seed oils containing cyclopentenyl fatty acids by combined chromatographic procedures. Lipids 1989;24:116-20.
36. Diraison F, Pachiaudi C, Beylot M. In vivo measurement of plasma cholesterol and fatty acid synthesis with deuterated water: determination of the average number of deuterium atoms incorporated. Metabolism 1996;45:817-21.
37. Merchak A, Patterson BW, Yarasheski KE, Hamvas A. Use of stable isotope labeling technique and mass isotopomer distribution analysis of [(13)C]palmitate isolated from surfactant disaturated phospholipids to study surfactant in vivo kinetics in a premature infant. J Mass Spectrom 2000;35:734-8.
38. Bohlin K, Patterson BW, Spence KL, et al. Metabolic kinetics of pulmonary surfactant in newborn infants using endogenous stable isotope techniques. J Lipid Res 2005;46:1257-65.
39. Ridsdale R, Post M. Surfactant lipid synthesis and lamellar body formation in glycogen-laden type II cells. Am J Physiol Lung Cell Mol Physiol 2004;287:L743-51.

40. Spence KL, Zozobrado JC, Patterson BW, Hamvas A. Substrate utilization and kinetics of surfactant metabolism in evolving bronchopulmonary dysplasia. J Pediatr 2005;147:480-5.

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

42. Wareham JA, Ferlauto JJ, Wells DH, Newell RW. Hypertriglyceridemia in infants with bronchopulmonary dysplasia. J Pediatr 1989;114:458-60.

43. Cogo PE, Zimmermann LJ, Pesavento R, et al. Surfactant kinetics in preterm infants on mechanical ventilation who did and did not develop bronchopulmonary dysplasia. Crit Care Med 2003;31:1532-8.

44. Bohlin K, Merchak A, Spence K, Patterson BW, Hamvas A. Endogenous surfactant metabolism in newborn infants with and without respiratory failure. Pediatr Res 2003;54:185-91.

45. Guice KS, Oldham KT, Wolfe RR, Simon RH. Lung injury in acute pancreatitis: primary inhibition of pulmonary phospholipid synthesis. Am J Surg 1987;153:54-61.

46. Oldham KT, Guice KS, Stetson PS, Wolfe RR. Bacteremia-induced suppression of alveolar surfactant production. J Surg Res 1989;47:397-402.

47. Gross I, Ilic I, Wilson CM, Rooney SA. The influence of postnatal nutritional deprivation on the phospholipid content of developing rat lung. Biochim Biophys Acta 1976;441:412-22.

48. Rooney SA, Gobran L, Gross I, Wai-lee TS, Nardone LL, Motoyama EK. Studies on pulmonary surfactant. Effects of cortisol administration to fetal rabbits on lung phospholipid content, composition and biosynthesis. Biochim Biophys Acta 1976;450:121-30.

49. Rubin JW, Clowes GH, Jr., Macnicol MF, Gavin JW. Impaired pulmonary surfactant synthesis in starvation and severe nonthoracic sepsis. Am J Surg 1972;123:461-7.

50. Jobe A, Ikegami M, Sarton-Miller I. The in vivo labeling with acetate and palmitate of lung phospholipids from developing and adult rabbits. Biochim Biophys Acta 1980;617:65-75.

51. Cavicchioli P, Zimmermann LJ, Cogo PE, et al. Endogenous surfactant turnover in preterm infants with respiratory distress syndrome studied with stable isotope lipids. Am J Respir Crit Care Med 2001;163:55-60.

52. Hellerstein MK, Neese RA. Mass isotopomer distribution analysis: a technique for measuring biosynthesis and turnover of polymers. Am J Physiol 1992;263:E988-1001.

53. Cogo PE, Zimmermann LJ, Rosso F, et al. Surfactant synthesis and kinetics in infants with congenital diaphragmatic hernia. Am J Respir Crit Care Med 2002;166:154-8.

54. Cogo PE, Zimmermann LJ, Verlato G, et al. A dual stable isotope tracer method for the measurement of surfactant disaturated-phosphatidylcholine net synthesis in infants with congenital diaphragmatic hernia. Pediatr Res 2004;56:184-90.

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

56. Bunt JE, Carnielli VP, Janssen DJ, et al. Treatment with exogenous surfactant stimulates endogenous surfactant synthesis in premature infants with respiratory distress syndrome. Crit Care Med 2000;28:3383-8.

Chapter 4

Evaluation Of $^{2}H_{3}$-U^{13}C- Dipalmitoyl-Phosphatidylcholine Metabolism As Newly Surfactant Tracer In Newborns With Lung Diseases

INTRODUCTION

1. SURFACTANT THERAPY REPLACEMENT

Respiratory failure secondary to surfactant deficiency is a major cause of morbidity and mortality in preterm infants. Surfactant therapy is a life-saving treatment that substantially reduces mortality and respiratory morbidity for this population. Surfactant replacement was established as an effective and safe therapy for immaturity-related surfactant deficiency by early 1990's. Randomized studies and controlled clinical trials confirmed that surfactant replacement reduces initial inspired oxygen, ventilation requirements, thoracic air leaks, as well as the incidence of RDS, death, pneumothorax, and pulmonary interstitial emphysema[1]. Surfactant therapy has revolutionized neonatal care and is used routinely for preterm infants with RDS becoming a standard practice in the developed world.

The rationale of surfactant replacement in acute RDS of immature preterm infants is obvious: substitution of primary surfactant deficiency and counterbalancing surfactant inactivation or inhibition by increasing the pool size of the surfactant[2]. The majority of immature infants has a rapid and sustained response after one dose of surfactant as reflected by an improvement in oxygenation and gas exchange, but a subgroup of infants has a suboptimal response or an early relapse. Newborns that don't show any improvement after a single dose, namely "poor responders" and babies with early relapse after surfactant administration could have an underlying disease process affecting the alveolar-capillary integrity and inducing surfactant inactivation and dysfunction[3]. There are a variety of commercially available surfactant preparations, including both synthetic and naturally derived surfactants. Exosurf Neonatal® (Colfosceril Palmitate HSE, Glaxo Wellcome, Middlesex, United Kingdom) is a synthetic mixture of the primary surface-active phospholipid DPPC, together with agents that facilitate dispersion

and surface adsorption. Exosurf Neonatal® does not, therefore, contain surfactant proteins. Survanta® (beractant, Ross Laboratories, Columbus, OH) and Curosurf® (poractant alpha, Dey, Napa, CA) are natural surfactants derived by organic solvent extraction of lipids from lung tissue, that contain SP-B and SP-C. Supplementation with additional lipids (Survanta®) or further extraction of neutral lipids (Curosurf®) improves surface properties. For example Curosurf is unique in that, apart from being produced from pig lungs rather than cow lungs, it goes through an additional preparation step of liquid gel chromatography, leaving only polar lipids and SP-B and SP-C with a phospholipid concentration of 80 mg/ml[4]. Natural animal surfactants may also be derived from organic solvent extraction of alveolar lavage fluid, such as Infasurf® (Forest Laboratories, St. Louis, MO, for Ony, Inc., Amherst, NY), and do not require further supplementation. Surfactant protein B concentrations are as much as 20 times higher with Infasurf® than other natural surfactants such as Survanta®[3].

A meta-analysis of six clinical study comparisons[5,6,7] using neonatal mortality as an outcome shows a reduction favoring Curosurf than Survanta. Indeed babies treated with Curosurf are more likely to need only one dose of surfactant especially when the initial dose is 200 mg kg^{-1}, as recently published by our research group[8]. Recent investigation has further elucidated the function of surfactant-associated proteins and their contribution toward surfactant and lung immune defense functions. As the field of neonatology moves away from intubation and mechanical ventilation of preterm infants at birth toward more aggressive use of nasal continuous positive airway pressure, the optimal timing of exogenous surfactant therapy remains unclear.

2. RESPIRATORY DISORDERS IN INFANTS: PNEUMONIA, BRONCHOPULMONARY DYSPLASIA, MECONIUM ASPIRATION SYNDROME AND CHORIOAMNIONITIS

Surfactant treatment in preterm infants and term newborns with RDS-like severe respiratory failure has become part of an individualized treatment strategy in many intensive care units around the world. These babies constitute heterogeneous groups of gestational ages, lung maturity, as well as of the underlying disease processes and postnatal interventions. The pathophysiology of respiratory failure in preterm infants is characterized by a combination of primary surfactant deficiency and surfactant inactivation as a result of plasma proteins

leaking into the airways from areas of epithelial disruption and injury. Various pre- and postnatal factors, such as chorioamnionitis, pneumonia, sepsis and asphyxia, induce an injurious inflammatory response in the lungs of preterm infants, which may subsequently affect surfactant function, synthesis and alveolar stability. Surfactant inactivation and dysfunction is also a hallmark in newborns with meconium aspiration syndrome, bronchopulmoary dysplasia, pneumonia and other disorders affecting the pulmonary function[2]. Term infants can also present with the clinical picture of RDS as a result of surfactant inactivation, secondary to conditions such as meconium aspiration or congenital pneumonia[9].

Pneumonia

Viruses, atypical, and typical bacteria cause the vast majority of childhood pneumonia[10-11]. The distribution of pathogens varies with age and clinical setting. Identifying the etiologic agent(s) responsible for pneumonia remains a challenge, primarily because of difficulty in obtaining adequate samples for culture and in differentiating infection from colonization and lack of reliable diagnostic methods[12,13]. The surfactant system is impaired in viral and bacterial pneumonia. In viral pneumonia there is reduction in surfactant activity due to injury to the type II epithelial cells. In bacterial pneumonia increased surface tension associated with reduced levels of SP-A, lipids, phosphatydilcholine/sphingomyelin ratio and phosphatidylglycerol has been demonstrated[14]. Herting et al., in animal study, demonstrate that surfactant improved lung function and mitigated bacterial growth in immature rabbits with experimentally induced neonatal GBS pneumonia[15].

Meconium aspiration syndrome

The pathophysiology of meconium aspiration syndrome is complex and multifactorial and is characterized by acute airway obstruction, chemical pneumonitis, and secondary infection. It occurs in 1% to 5% of all term and near-term liveborn deliveries[3]. Meconium itself is a mixture of gastrointestinal secretions, sloughed intestinal epithelial cells, and potentially any substances capable of appearing in amniotic fluid, which is swallowed by the fetus. Many of these, especially bile salts, can inactivate surfactant. There is also a direct toxic effect in the lung, causing chemical pneumonitis. Meconium in fact, can activate alveolar macrophages and induce inflammation and vascular leakage.

Inflammatory mediators, such as cytokines and eicosanoids, can also inhibit surfactant, as can the protein that leaks into the alveolar spaces[16]. Several studies have shown inactivation of surfactant by the components of meconium, including cholesterol, free fatty acids, and bilirubin, as well as plasma proteins from increased capillary permeability[17,18]. There may be alteration in pulmonary vasoreactivity, leading to pulmonary vasoconstriction and secondary persistent pulmonary hypertension of the newborn. If intrauterine hypoxemia is present, there may be vascular remodeling with pulmonary hypertension. Reduced pulmonary blood flow may cause pulmonary ischemia, with damage to the type II cells and reduced surfactant production. Airway obstruction may cause increased resistance, and surfactant deficiency and parenchymal lung changes may require the use of high ventilatory support and substantial supplemental oxygen, contributing to lung injury. Thus, breaking this vicious cycle by using surfactant-replacement therapy is an attractive hypothesis. Two approaches have been attempted: surfactant replacement and surfactant lavage. It appears that surfactant-replacement therapy for meconium aspiration syndrome consistently improves gas exchange and short-term outcomes, especially avoidance of air leaks and the need for extracorporeal membrane oxygenation[19]. Sun et al. demonstrated amelioration of surfactant inhibition, with improved compliance and oxygenation, by large dose surfactant replacement therapy in an animal model of meconium aspiration syndrome[3, 18].

Bronchopulmonary Dysplasia

Bronchopulmonary dysplasia is a term used to describe the chronic lung changes that accompany mechanical ventilation, particularly in preterm infants. It was first coined in a population of relatively late preterm babies subjected to moderate ventilatory support[20]. Today, affected infants tend to be more preterm and subjected to less ventilatory support, and are often described as having the "new BPD"[18]. The etiology of BPD is multifactorial and includes ventilator-induced lung injury, inflammation, inadequate antioxidant systems, infection, exposure to steroids, and inadequate nutrition, all superimposed upon a still developing immature lung in terms of underdeveloped parenchyma, altered alveolarization, and deficient surfactant production[21,22]. Ultimately, this results in a decrease in alveolarization and can impair growth and development. It is estimated that 30–40% of infants <1,500 g at birth are affected to some extent[23], and it is thus a considerable burden to the health-care system. Considerable investigation into

ways to prevent or ameliorate BPD is underway. One of these is late surfactant-replacement therapy. The introduction of early surfactant-replacement therapy has not altered the incidence of BPD, although this may be a demographic quirk. In other words, very preterm infants who might not have survived without surfactant replacement therapy are now surviving, only to subsequently develop BPD.

Chorioamnionitis

An important cause of preterm labor is chorioamnionitis, which affects up to 60% of extremely preterm infants. Chorioamnionitis is an antenatal inflammatory state associated with bacterial invasion of the uterine environment and subsequent neutrophil invasion of the placenta[24], that has been associated with an adverse perinatal outcome such as chronic lung disease. Antenatal inflammation enhances lung maturation and increases surfactant production in preterm animal models[25-26]. Correspondingly, chorioamnionitis is associated with decreased incidence of RDS in many human studies[27]. Despite accelerating lung maturation, antenatal inflammation is associated with adverse lung development[28]. Ikegami et al.[29], in animal study, showed that a considerable evidence links inflammation to decreased surfactant function, due to a reduction of exogenous surfactant efficacy after antenatal inflammation[30]. Histologic results of the examination of placenta are usually not available within the first days of life, and prenatal diagnosis of chorioamnionitis remains difficult[31].

3. INFLAMMATORY MEDIATORS: IL-8 AND LTB_4

Although the lung is primarily involved in gas exchange, it has a unique relationship with the environment and must protect itself from infection. An extensive alveolar-capillary membrane containing immune and non-immune cells is constantly exposed to microbial challenges, and consequently is expected to generate a brisk innate host response to both inhaled and hematogenous pathogens to clear the offending microorganism and preserve gas exchange. While a variety of factors are involved in the innate response in the lung, cytokines constitutes the largest and most pleiotropic group of mediators. They are involved in the recognition of microbes, the recruitment of leukocytes, and the removal of the invading microorganism[32]. While toll-like receptors mediate pathogen

recognition by the innate immune system in response to unique molecular patterns presented by the microorganism, it is increasingly clear that induction of cytokines is necessary for the full development of the innate host defense. The extravasation of leukocytes in the lung is a salient feature of the innate response and the production of chemokines plays a critical role in this process of recruitment and maintenance of leukocyte sequestration during infection. Among these potent chemoattractant factors IL-8 is the predominant in activating leukocytes to mount an immune response and initiate wound healing. IL-8 derives primarily from mononuclear phagocytes, endothelial, and epithelial cells but also from T cells, eosinophils, neutrophils. During inflammation response, IL-8 appears relatively late in comparison with other chemottractants and later it persists for at least 24 hours[33].

Leukotrienes are inflammatory mediators generated from arachidonic acid by the 5-lypo-oxygenase pathway[34-35]. Leukotriene B_4 (LTB_4) has an important role among the mediators involved in the inflammatory response to lung infections, it is released by neutrophils and macrophages and, in turn, it stimulates neutrophil chemotaxis, enhances neutrophil-endotelial interactions and stimulates neutrophil activation, leading to degranulation and release of mediators and enzymes[36]. Neutrophils release LTB_4 in response to various activating stimuli, and also have a high density of cell surface LTB_4 receptors[37].

4. USE OF DSPC TRACERS IN *IN VITRO* AND *IN VIVO* STUDIES

To study surfactant metabolism labeled substrates can be administered endovenously or intratracheally. Endovenous infusion of labeled precursors allows to measure surfactant synthesis: labeled precursors are incorporated by type II cell and used to synthesized surfactant components, that is secreted in alveolar space and can be collected by sequential tracheal aspirates[38,39,40]. Endotracheal instillation of labeled phospholipids leads to trace directly pulmonary surfactant obtaining pool size evaluation and half life estimation that represents a global measurement of surfactant turnover[41].

The first approach of tracing lung surfactant metabolism dating 1980, when Okano et al.[42] investigated ^{14}C palmitate and ^{3}H choline incorporation and metabolism in surfactant phospholipids in "*in vitro*" studies. They showed that these tracers had different labeling profiles: ^{3}H choline had high reactivity with PG, while ^{14}C palmitate was preferentially incorporated by PC. In a recent study by our research

group, we found that during the first days of life infants are able to use indifferently the available metabolic precursors, namely stable isotope palmitate, glucose and acetate for surfactant DSPC synthesis[43-45]. Jacobs and coworkers, with radioactive isotopes, injected intraperitoneally a trace dose of ^{3}H choline and simultaneously ^{14}C DPPC intratracheally to 3 days old rabbits, and demonstrated that the synthetic DPPC had the same turnover as that obtained from the incorporation of ^{3}H choline in the natural surfactant DSPC[46]. On the basis of these studies our research group administered endotracheally $U^{13}C$ –PA DPPC to study surfactant metabolism *in vivo* in human newborns[40,41, 47-50]. and we reported for the first time data on surfactant DSPC pharmacokinetics in humans[8, 51-52].

In this thesis we used 1-$^{2}H_{3}$,2-U ^{13}C dipalmitoyl-phosphatidylcholine, a newly and doubly labeled synthesized surfactant tracer, instilled into trachea together with exogenous surfactant to study surfactant catabolic rate *in vivo* in newborns with various lung diseases.

AIM OF THE STUDY

In the last two years of my PhD project we evaluated surfactant kinetics with the use of a newly synthesized stable isotope tracer: 1^2H_3,2-$U^{13}C$ dipalmitoyl-phosphatidylcholine. Newborns with different lung diseases received either a tracer dose or 1^2H_3,2-$U^{13}C$ dipalmitoyl-phosphatidylcholine mixed with 100 or 200 mg/kg of exogenous surfactant, to measured pulmonary surfactant catabolism by analyzing the isotopic enrichment of disaturated phosphatidylcholine isolated from tracheal aspirates. From the enrichment decay curves, we derived half-life of administered exogenous surfactant and the surfactant pool size at the time of dosing.

MATERIAL AND METHODS

Study patients

We studied 64 newborn infants who were admitted to the Neonatal Intensive Care Unit of the Department of Pediatrics, University of Padua, or the Division of Neonatology of the Polytechnic University of Marche (Ancona, Italy). Infants were recruited if they required synchronized intermittent mandatory ventilation for an estimated length of time of at least 24 hours. The ethics committees of both institutions approved study protocol, and written informed consent was obtained from both parents. Newborns with congenital malformations, severe asphyxia with resulting neurological problems, renal or liver failure were excluded from the study.

Patients were classified in groups accordingly to their lung disease:

- RDS: diagnosis was based on clinical data and on chest radiograms[53], after exclusion of infections.
- Chorioamnionitis: 1) clinical: diagnosed as maternal fever and two or more of these criteria: uterine tenderness, malodorous vaginals discharge, foetal tachycardia, leukocytosis especially neutrophils, positive blood colture and bacteria and/or inflammatory cells in aminotic fluid, and membrane rupture. 2) Histological: polymorphonuclear leukocyte infiltration (≥ 5 scattered neutrophils pre high power field) of the chorionic plate or of the extraplacental foetal membranes, presence of inflammatory mediators by istological infections concerning chorionic plate or of the extraplacental foetal membranes[54].
- Pneumonia: diagnosis was based on the following criteria: respiratory distress, radiographic findings, and at least one of the following laboratory signs of infections: (a) presence of blood cultrure, (b) increase C reactive protein plasma levels (CRP), (c) leukocytosis (d) immature to total ratio of neutrophilic granulocytes (I:T ratio) > 0.2[55].
- BPD: persistent respiratory symptoms, hypoxemia with oxygen dependence and typical radiological signs at 36 gestational weeks.

According to the attending neonatologist, exogenous surfactant (Curosurf ®, Chiesi Farmaceutici S.p.A, Parma, Italy) was administered endotracheally at a dose of 100 or 200 mg/kg, if the mean airway pressure (MAP) exceeded 7.5 cm of water or if the inspiratory oxygen fraction (FiO_2) was higher than 0.40. Infants

received a second dose if the same criteria were met after the first dose. Clinical data and physiological parameters (respiratory rate, heart rate, hemoglobin saturation) were recorded hourly during the study. Ventilator parameters and arterial blood gas analysis were recorded before the start of the study and subsequently every 6 hours. The oxygenation index (OI) was calculated as $[(MAP*FiO_2)/PaO_2]*100$, where PaO_2 is the arterial blood partial oxygen tension at each of these time points. $AaDO_2$ is the alveolar-arterial oxygen difference, calculated as $[FiO_2*(713)-pCO_2/0.8]-pO_2$, where pO_2 and pCO_2 are the partial oxygen tension and carbon dioxide tension measured by hemogasanalysis at each of these time points.

Ethical aspects

During the last 15 years, our research group has been studied surfactant kinetics in more than two hundreds patients, both adults and infants, by using stable isotopes as metabolic tracers with no evidence of any complication. Isotope tracers have been safely used in humans in the last forty years according to numerous researchers[56]. The Institutional Ethic Committee approved the study and informed consent was obtained from both parents.

Study design

Patients were divided into 2 groups: 1) Surfactant: newborns received simultaneously an endotracheal dose of 100 or 200 mg/kg of exogenous surfactant mixed with 2 mg/ml/kg dose of 1^2H_3,2-$U^{13}C$ dipalmitoyl-phosphatidylcholine (1^2H_3,2-$U^{13}C$-PA DPPC) (Avanti Polar Lipids, AL) as tracer; 2) Tracer: newborns received an endotracheal administration of the tracer dose at 2 mg/ml/kg of 1^2H_3,2-$U^{13}C$-PA DPPC mixed with a small amount of exogenous surfactant as a vehicle.

For these investigations we referred to a method previously published by our research group[57]. In all patients the start of the study (t = 0) was defined by the start of the tracer administration. A central venous line (umbilical), a peripheral line or an arterial line has been used for blood sampling. Chemical and isotopic purity of the 1^2H_3,2-$U^{13}C$-PA DPPC stable isotope tracer was confirmed by gas chromatography and gas chromatography-mass spectrometry. Tracheal aspirates were obtained before the start of the administration and every 6 hours thereafter until 72 hours or till the extubation. Tracheal aspirates were performed as it follows: 0.5 ml normal saline was injected into the endotracheal tube, and after

twenty seconds, suctioning was performed with the tip of the suction catheter beyond the tip of the endotracheal tube. All tracheal aspirates were brought to a final volume of 2 ml with normal saline. The sample was then gently vortexed and centrifuged at 400 *g* for 10 minutes. The supernatant was divided as 400 ul aliquots and stored at -80 °C until analysis. Fifty ul of each labelled exogenous surfactant dosing, or tracing dose were stored -80°C in order to determine the $^{2}H_3$-PA and U^{13}C-PA enrichment of DPPC.

Analytical procedure

DSPC isolation and enrichments from tracheal aspirates

Tracheal aspirates' phospholipids were extracted according to Bligh and Dyer[58] and surfactant DSPC was isolated by thin layer chromatography using as mobile phase a mixture of cholorform/methanol/ potassium chloride/ triethylammine (40:12:33:8:24) after oxidation with osmium tetroxide[59]. The TLC spot containing DSPC fatty acids was methylated adding 3 ml of methanolic HCl 3N, samples were incubated at 100°C for 60 minutes and after cooling, pH neutralization was made with 2.5 ml of 10% K_2CO_3[60]. The fatty acid methyl esters were extracted with 500 ul of hexane and stored at -20° C. The amount of DSPC in tracheal aspirates was measured from the fatty acids methyl esters by capillary gas chromatography[61]. Tracheal aspirates containing visible blood were not analyzed. The enrichment of two different moieties U^{13}C-PA and $^{2}H_3$-PA of the tracer in TA were determined by gas chromatograph coupled with a quadrupole mass spectrometer (GC-MS) (6890N, 5873 *inert* Agilent Technologies, Cernusco sul Naviglio, Milano, Italy) with a ion source operating in electron impact ionization mode. Selective ion monitoring was carried out at m/z 270, 271 and 273 for $^{2}H_3$-PA and at m/z 270, 271 and 286 for U^{13}C-PA. Enrichments were expressed as mole percent excess (MPE) that represents the increase in the mole percentage of each palmitic acid moieties ($^{2}H_3$ PA and U^{13}C PA) above the baseline value obtained at time 0 of the study. Each sample was measured in duplicate.

Cytokine Assays

IL-8 and LTB_4 were measured in tracheal aspirates using commercially available ELISA kits (IL-8 AviBion, Human ELISA, Orgenium; LTB4 ELISA Cayman Chemical Ann Arbor, MI). The assays were performed according to manufacturer's instructions. The volume of samples analyzed was 50 ul. All samples were analysed in duplicate, and values from duplicates were nearly identical. The lower limits of detection were 3.9 pg/ml for both kits. Concentrations were measured at study

start for IL-8 and LTB_4, and after 12 hours of the tracer dose administration for IL-8.

Calculations

Data were analyzed under the following assumptions: (1) exogenous surfactant DSPC is distributed in the alveolar pool and subsequently internalized and recycled by the type II cells; (2) endogenous DSPC is synthesized by lung parenchyma, secreted in the alveoli, and recycled before degradation; (3) exogenous surfactant DSPC is distributed homogeneously in the lungs, and the system is at steady state; and (4) DSPC kinetics are linear. Under these assumptions, DSPC kinetics can be modeled conveniently on the basis of the tracer/tracee ratio (TTR), that is, the ratio of exogenous (tracer) to endogenous (tracee) DSPC[62-63]. This variable is different from enrichment, which measures the ratio of the labeled component of exogenous surfactant to unlabeled DSPC, originating from both exogenous and endogenous sources. Therefore, enrichment (E) measured at time t was converted to TTR by using the following formula: $TTR(t) = E(t)[(E_I + 1)/[E_I - E(t)]]$, where E_I is the percentage of [U-^{13}C and $^{2}H_3$palmitic acid] DPPC with respect to unlabeled DSPC in the administered surfactant dose. TTR data were fitted to either a monoexponential or biexponential decay model, and DSPC kinetic parameters were calculated as follows. In the monoexponential model, that is, $TTR(t) = Ae^{-Kt}$, the DSPC half-life (in hours) is $\ln(2)/k$, the DSPC pool size (in milligrams per kilogram) is dose/A. In the biexponential model, that is, $TTR(t) = A_{1e}{}^{-k_{1t}} + A_{2e}{}^{-k_{2t}}$, the DSPC half-life (in hours) is $\ln(2)/k_2$, the DSPC pool size (in milligrams per kilogram) is dose/$(A_1 + A_2)$. Dose (in milligrams per kilogram) is the administered surfactant DSPC dose, t is time (in hours), and, for the biexponential model, k_2 indicates the rate constant of the slower component, responsible for the late portion of TTR decay[8].

Data analysis

Data are presented as mean ± SD or median (IQR range) according to the variable distribution. Groups were compared by paired t-tests (Wilcoxon and Mann-Whitney Test) by oneway ANOVA, with LDS as post Hoc Test, and Kruskal-Wallis test. The level of significance accepted was $p<0.05$. Microsoft Excel 2000 (Microsoft Corp, Redmond, WA), Prism 3.0 GraphPad Software and SPSS (15.0 version; SPSS Inc, Chicago, IL) were used for calculations and statistical analysis.

RESULTS

In the last two years of my PhD project we prospectively enrolled 64 infants, 34 received exogenous surfactant therapy at the start of the study while 30 received only the tracer dose. All of them received a 1^2H_3,2-$U^{13}C$-PA DPPC dose in order to measure surfactant kinetics.

Infants were retrospectively divided into Surfactant group and Tracer group. Infants belonging to each group were classified according to their specific lung disease.

1. SURFACTANT GROUP: DSPC KINETICS

In Surfactant group 19 infants had RDS, 7 had RDS with chorioamnionitis and 8 had pneumonia. Gestational age, neonatal weight and total amount of surfactant doses were lower in RDS with chorioamnionitis infants with respect to the other groups; while 5°minute Apgar score, prenatal steroids, patent ductus arteriosus, number of surfactant doses, age at ventilator intubation, BPD at 36 week of gestation and mortality, were similar among all groups. Clinical characteristics of all patients studied, classified in subgroups, are reported in Table 1.

Table 1. Surfactant group: clinical characteristics of infants studied.

	RDS	RDS with Chorioamnionitis	Pneumonia	P
Prenatal and neonatal data				
Patients, (n)	19	7	8	/
Gender, (male/female)	12/7	2/5	6/2	0.371
Gestational age, mean ± SD, (weeks)	29 ± 4	26 ± 2 [a]	32 ± 6 [b]	0.030
Neonatal weight, mean ± SD, (gr)	1203 ± 714	835 ± 249 [a]	1681 ± 1104 [b]	0.113
5° minutes Apgar score, [median (IQR)]	8 (6-9)	7 (7-8)	8 (7-8)	0.893
Prenatal steroids, (%)	90	100	63	0.066
Patent ductus arteriosus, (%)	69	57	63	0.957
N° surfactant doses, n=1/n=2/n=3	11/5/3	5/2/0	1/7/0	0.090
Total surfactant dose, mean ± SD, (mg/kg)	224 ± 86	180 ± 73 [b]	268 ± 53 [a]	0.106
Intubation age, mean ± SD, (hours)	4.6 ± 9.5	0	8.8 ± 12.1	0.204
Conventional ventilation, mean ± SD, (days)	7.0 ± 8.2	5.9 ± 7.5	5.3 ± 2.4	0.834
N° of 100 vs 200 doses	21/9	7/2	10/5	0.839
Outcome				
BPD at 36 weeks, (%)	42	57	37	0.742
Mortality, (%)	10	14	0	0.576

Values with different superscripts are significantly different with $p<0.05$.

Clinical severity and ventilator parameters during all the studies performed are reported in Table 2.

Table 2. Surfactant group: clinical severity of all studied performed.

	RDS	RDS with Chorioamnionitis	Pneumonia	P
Study, (n)	26	7	10	/
>1 study, (n)	6/26	0/7	2/10	/
CRP > 20 mg/L at study start, (%)	4 [a]	57 [b]	50 [b]	0.002
CRP > 20 mg/L during study, (%)	11 [a]	57 [b]	70 [b]	0.003
Age at study start, mean ± SD, (hours)	19 ± 22	34 ± 67	26 ± 31	0.552
Weight at study start, mean ± SD, (gr)	1104 ± 635	828 ± 260	1512 ± 1001	0.131
Study duration, mean ± SD, (hours)	44 ± 23	54 ± 17	56 ± 20	0.279
Interval between 1° and 2° dose, mean ± SD, (hours)	27 ± 14[a]	97 ± 129[b]	28 ± 19[a]	0.019
Interval between 2° and 3° dose, mean ± SD, (hours)	114 ± 129	/	/	/
Oxigenation Index before study start, mean ± SD	3.3 ± 5.1	6.5 ± 5.0	12.4 ± 10.2	0.201
Oxigenation Index during study, mean ± SD	5.7 ± 6.6	3.9 ± 1.9	4.0 ± 2.0	0.589
$AaDO_2$ before study start, mean ± SD	189 ± 133	190 ± 204	242 ± 180	0.699
$AaDO_2$ during study, mean ± SD	104 ± 67	93 ± 42	94 ± 34	0.840
PaO_2/FiO_2 before study start, mean ± SD	128 ± 71	172 ± 107	139 ± 85	0.505
PaO_2/FiO_2 during study, mean ± SD	207 ± 65	212 ± 56	208 ± 35	0.978

Values with different superscripts are significantly different with $p<0.05$.

We measured kinetics after 43 surfactant administrations traced with 1^2H_3,2-$U^{13}C$-PA DPPC: 26 doses were given for RDS, 7 for RDS associated with chorioamnionitis

and 10 for pneumonia. After the first dose of exogenous surfactant, 8 infants (6 RDS and 2 pneumonia) required a second surfactant dose which has been traced another time with 1^2H_3,2-$U^{13}C$-PA DPPC and considered as a new DSPC kinetics study. Before the study start and during the study we found that C-reactive protein (CRP) levels >20 mg/L were significantly more frequent in infants with chorioamnionitis and pneumonia compared with RDS infants (p=0.002 and p=0.003 respectively). Ages, weight, duration of study were similar among groups. Infants with RDS and RDS with chorioamnionitis required the second surfactant dose at a significant delayed time than infant with RDS (p=0.019). Indexes of respiratory disease severity didn't differ among groups neither before surfactant administration nor after surfactant dose.

Figure 1 shows the enrichment decay curves of $^2H_3$-PA and $U^{13}C$-PA obtained from a representative studied infant.

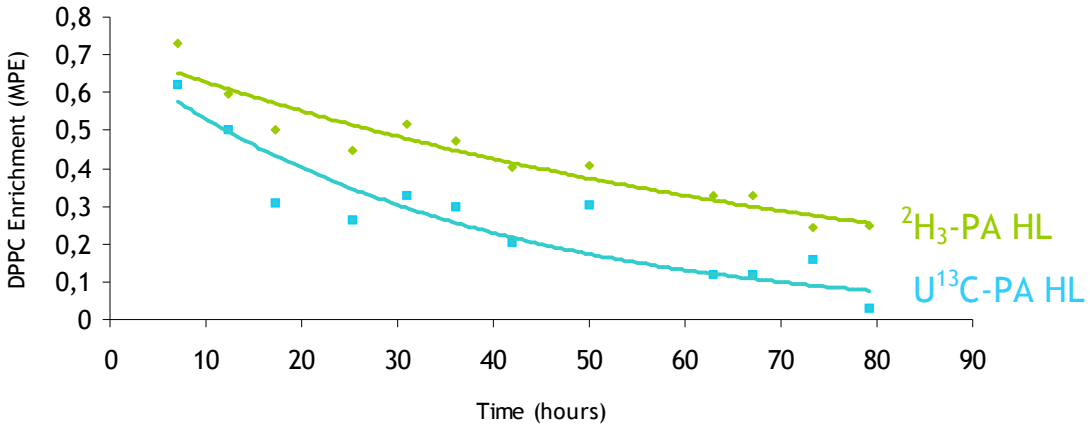
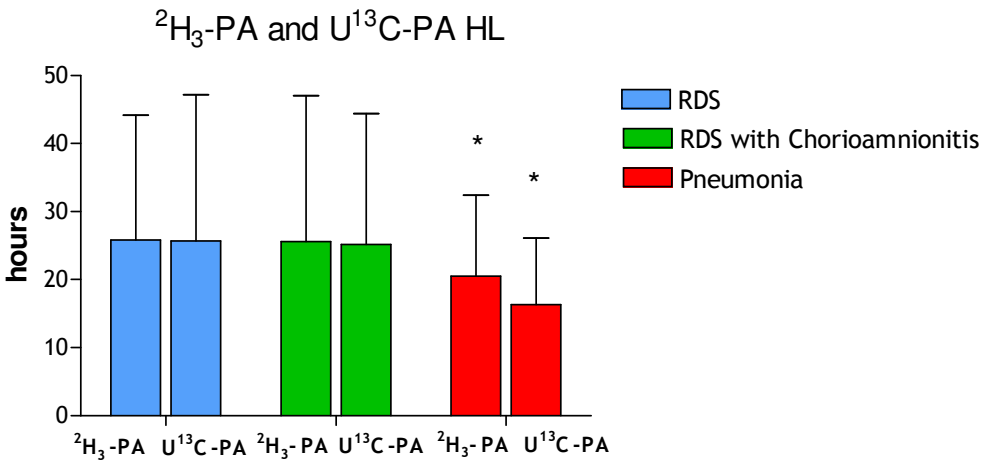


Figure 1. DSPC enrichment decay curve over time in a study patient.

Mean DSPC $^2H_3$-PA half life (HL) was 25.8 ± 18.4 hours in RDS, 25.6 ± 21.4 hours in RDS with chorioamnionitis and 20.5 ± 11.9 hours in pneumonia. Mean DSPC $U^{13}C$-PA HL was 25.7 ± 21.5 hours in RDS, 25.2 ± 19.2 hours in RDS with chorioamnionitis and 16.3 ± 9.8 hours in pneumonia. Non-parametric paired test showed a significant shorter DSPC $U^{13}C$-PA HL compared with DSPC $^2H_3$-PA HL in pneumonia patients (p=0.050). No differences between DSPC $^2H_3$-PA HL and DSPC $U^{13}C$-PA HL were found either in RDS or RDS with chorioamnionitis (Figure 2).



* = p<0.05

Figure 2. DSPC mean ± SD $^2H_3$-PA HL and ^{13}C-PA-HL in Surfactant group.

Mean DSPC pool sizes (PS) were not different between $^2H_3$-PA and U^{13}C-PA in each group of infants. Estimation of PS showed that RDS infants tended to have lower alveolar pool size than other two groups, although the difference was not statistically significant (Figure 3).

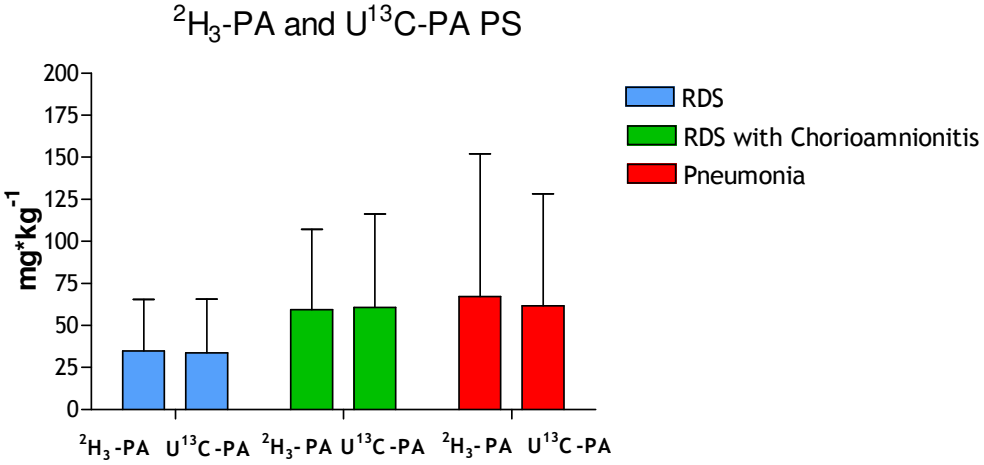


Figure 3. DSPC mean ± SD $^2H_3$-PA PS and U^{13}C-PA PS in Surfactant group.

2. TRACER GROUP: DSPC KINETICS

In Tracer group 8 infants had pneumonia, 11 had BPD and 11 infants had no lung disease but required mechanical ventilation for major abdominal surgery or

neurological failure. Clinical characteristics of these infants are not comparable among all groups as expected by study design and they are reported in details in Table 3. Infants with BPD had more steroids therapy, patent ductus arteriosus, more doses of surfactant and they needed mechanical ventilation earlier than other two groups.

Table 3. Tracer group: clinical characteristics of infants studied.

	Controls	Pneumonia	BPD	P
Prenatal and neonatal data				
Patients, (n)	11	8	11	/
Gender, (male/female)	8/3	6/2	4/7	0.132
Gestational age, mean ± SD, (weeks)	34 ± 5 [a]	39 ± 2 [b]	25 ± 1 [c]	0.000
Neonatal weight, mean ± SD, (gr)	1955 ± 813 [a]	3128 ± 671 [b]	718 ± 202 [c]	0.000
5° min Apgar score, [median (IQR)]	7 (5-9) [a]	10 (10-10) [b]	6 (6-8) [a]	0.016
Prenatal steroids, (%)	36 [a]	/	92 [b]	0.000
Patent ductus arteriosus, (%)	27 [a]	13 [a]	83 [b]	0.004
N° surfactant doses n=0/n=1/n=2/n=3/n=4	8/2/1/0/0 [a]	5/3/0/0/0 [a]	1/4/7/0/1 [b]	0.003
Total surfactant dose, mean ± SD, (mg/kg)	165 ± 125	167 ± 58	242 ± 106	0.364
Intubation age, mean ± SD, (hours)	16 ± 32	28 ± 39 [a]	1 ± 3 [b]	0.110
Conventional ventilation, mean ± SD, (days)	13.4 ± 10.9[a]	4.8 ± 1.5[a]	61.7 ± 78.8[b]	0.059
Number of 100 vs. 200 doses	2/2	1/2	17/5	0.206
Outcome				
BPD at 36 weeks, (%)	0 [a]	0 [a]	100 [b]	0.000
Mortality, (%)	9	12	33	0.485

Values with different superscripts are significantly different with $p<0.05$.

Clinical severity and ventilator parameters during the study period are reported in Table 4.

Table 4. Tracer group: clinical severity of all studied performed.

	Controls	Pneumonia	BPD	P
Study, (n)	11	8	18	
>1 study, (n)	0/11	0/8	5/18	
CRP > 20 mg/L at study start, (%)	0 [b]	50 [a]	6 [b]	0.013
CRP > 20 mg/L during study, (%)	9 [b]	87 [a]	6 [b]	0.001
Age at study start, mean ± SD, (hours)	360 ± 384 [a]	72 ± 72 [a]	869 ± 538 [b]	0.000
Weight at study start, mean ± SD, (gr)	2100 ± 751 [c]	3125 ± 671 [b]	1065 ± 282 [a]	0.000
Study duration, mean ± SD, (hours)	60.7 ± 13.8	69.5 ± 13.7 [a]	49.8 ± 21.2 [b]	0.037
Interval between 1° and 2° dose, mean ± SD, (hours)	50.2 (n=1)	/	20.9 ± 46.4	0.551
Interval between 2° and 3° dose, mean ± SD, (hours)	/	/	18.96 (n=1)	/
$AaDO_2$ before study start, mean ± SD	76 ± 64 [a]	268 ± 154 [b]	126 ± 69 [a]	0.004
$AaDO_2$ during study, mean ± SD	82 ± 63	123 ± 69	149 ± 91	0.209

We measured kinetics after 37 1^2H_3,2-$U^{13}C$-PA DPPC administrations: 11 tracer doses were given to controls, 8 to pneumonia patients and 18 to infants with BPD. After the first tracer dose, 5 newborns (BPD) received a second dose of surfactant tracer.

C-reactive protein levels were significantly higher either before the study start or during the study in pneumonia group, as expected by study design. Age at the study start was higher for BPD infants, as expected according with the inclusion criteria. Duration of study and interval between repeated surfactant doses were

similar among all infants. $AaDo_2$ at the study start was lower in controls and BPD groups with respect to pneumonia group as expected by the study design.
Mean DSPC HL tended to be lower in pneumonia group, although not significantly being 18.2 ± 7.11 hours in controls, 13.4 ± 5.3 hours in pneumonia and 15.7 ± 10.2 in BPD (p=0.494), (Figure 4).

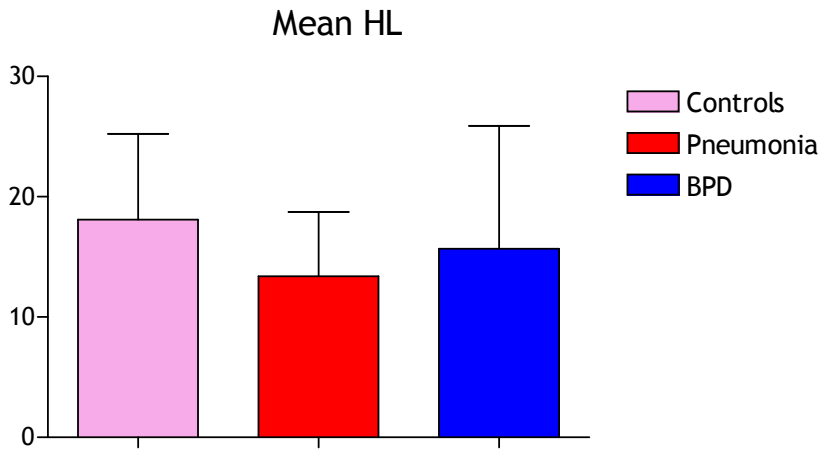


Figure 4. Mean DSPC HL in Tracer groups.

Mean DSPC $^{2}H_3$-PA HL was 16.4 ± 6.7 hours in controls, 14.9 ± 5.1 hours in pneumonia and 13.4 ± 8.6 in BPD. Mean DSPC $U^{13}C$-PA HL was 19.9 ± 8.3 hours in controls, 11.9 ± 6.5 hours pneumonia and 17.2 ± 13.3 hours in BPD (Figure 5 and 6). DSPC $U^{13}C$-PA HL was significantly different between controls and pneumonia group (Mann-Whitney Test p=0.048) (Figure 5).

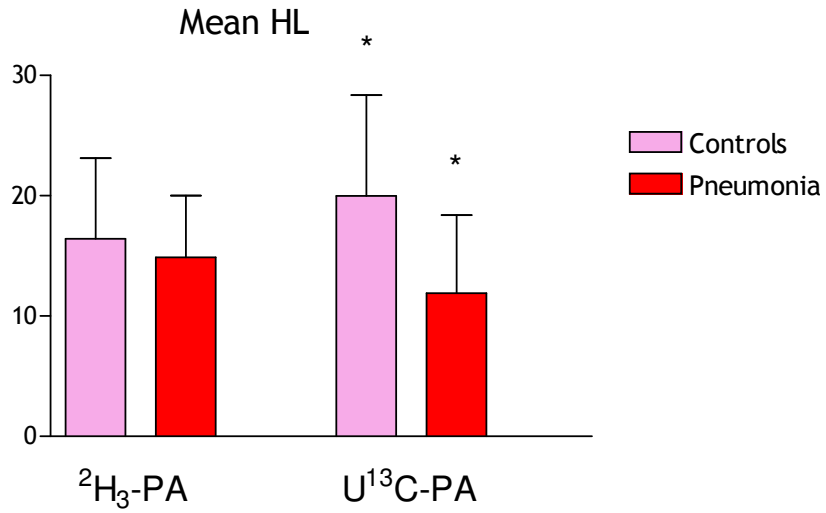


Figure 5. DSPC $^{2}H_3PA$ and $U^{13}C$-PA HL between controls and Pneumonia.

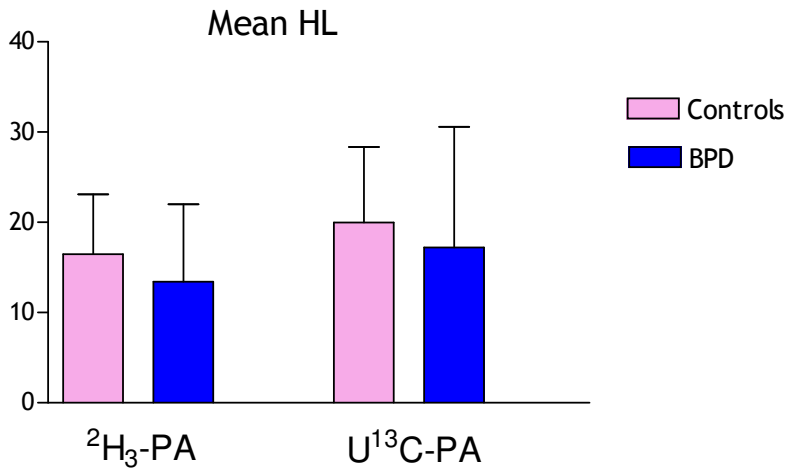


Figure 6. DPPC $^{2}H_3PA$ and $U^{13}C$-PA HL between controls and BPD.

Mean DSPC pool sizes (PS) were not different among the three groups (p=0.646) and they are reported in Figure 7.

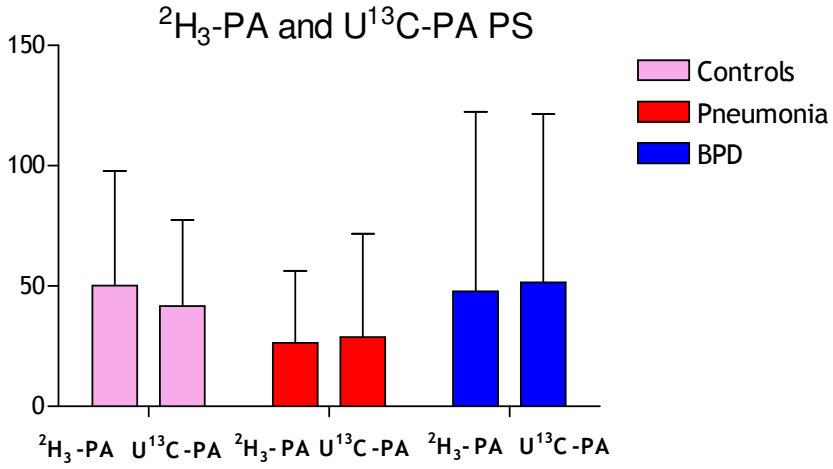
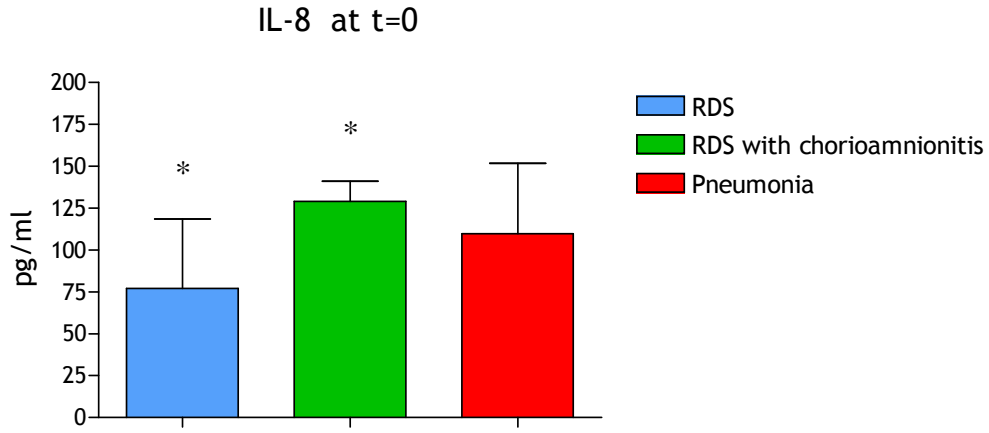


Figure 7. DSPC $^{2}H_3$-PA PS and $U^{13}C$-PA PS, mean ± SD in Tracer groups.

3. INFLAMMATORY MEDIATORS

ELISA IL-8 and LTB_4 mean concentrations in tracheal aspirates are depicted in Figure 8 and 9. At the time 0 of the study IL-8 was 77.0 ± 41.5 pg/ml in RDS, 129.1 ± 11.9 pg/ml in chorioaminionitis and 109.8 ± 42.0 pg/ml in pneumonia group. The difference was statistically different for the RDS with chorioamnionitis group

compared to the RDS group (Anova p=0.085, post hoc test p=0.040). After at least 12 hours from surfactant administration, IL-8 values were 122.3 ± 51.2 pg/ml, 113.4 ± 75.8 pg/ml and 115.9 ± 38.8 pg/ml in RDS, chorioamnionitis and pneumonia group respectively (p=0.952).



* = p<0.05

Figure 8 a. IL-8 mean ± SD at t=0 and concentration in Surfactant subgroups.

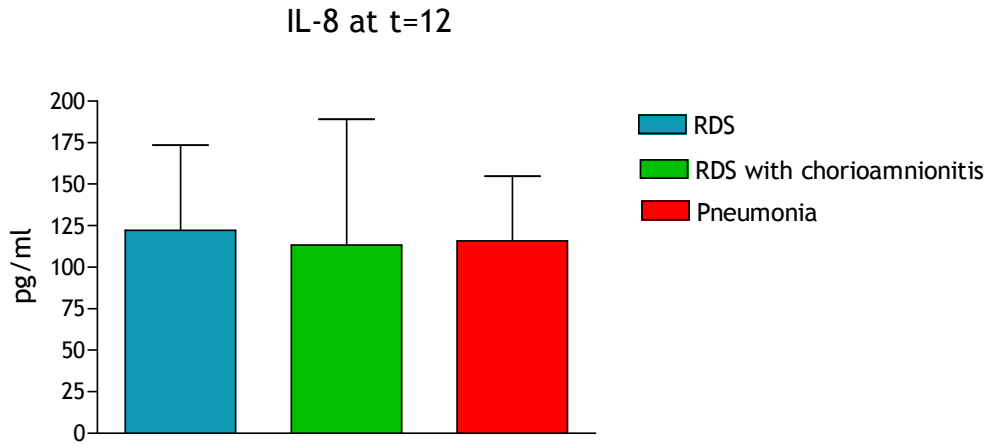


Figure 8 b. IL-8 mean± SD at t=12 concentration in Surfactant subgroups.

We analyzed LTB_4 values at time zero of the study in tracheal aspirates in 34 Surfactant group: 10 pneumonia, 6 chorioamnionitis, 18 RDS. In 3 samples of chorioamnionitis group, LTB_4 concentrations were too high and they need to be re-analyzed. For Tracer group available data are not enough to be represented.
Figure 9 represents LTB_4 results from Surfactant group.

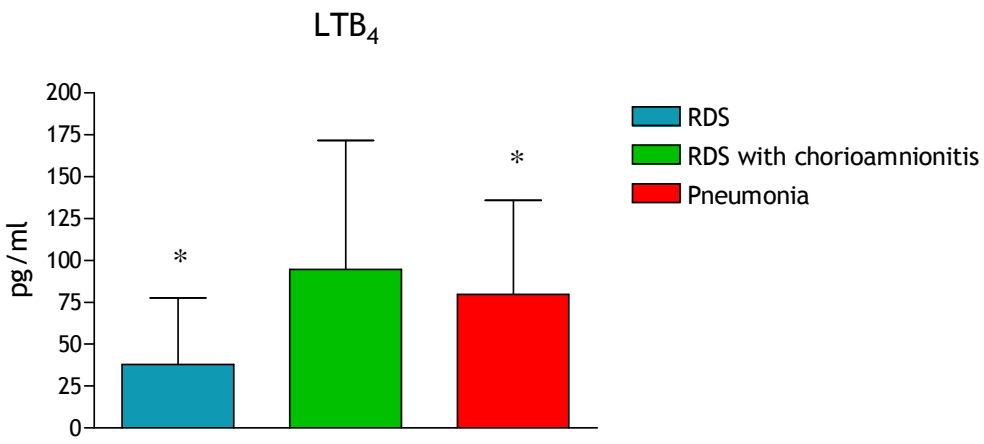


Figure 9. LTB4 mean± SD concentrations in Surfactant subgroups.

LTB_4 mean values were 37.9 ± 39.7 pg/ml in RDS, 94.6 ± 77.0 pg/ml in chorioamnionitis and 79.8 ± 56.1 pg/ml in pneumonia group (Oneway Anova p=0.05). LSD post hoc test between RDS and pneumonia was significantly different (p=0.038).

DISCUSSION

In this prospective study we described a novel and safe method that is applicable to investigate surfactant kinetics in human infants who required endotracheal intubation due to different lung diseases. We used a DSPC tracer with the PA moieties differently labeled with stable isotopes: in sn-1 with $^{2}H_{3}$-PA, in sn-2 with U^{13}C-PA to trace exogenous surfactant, and we reported for the first time on the direct measurement of the kinetics of the two surfactant DSPC-palmitate moieties in newborns. We previously reported, on endogenous surfactant metabolism by infusing surfactant precursors, such as glucose and palmitate (^{2}H and ^{13}C labeled), to measure DSPC enrichment in serial tracheal aspirates[43,64-65]. Subsequently stable isotopes were also used to trace *in vivo* exogenous surfactant pharmacokinetics. In infants with RDS exogenous surfactant was administered endotracheally mixed with U^{13}C-DPPC (with both PA moieties uniformly labeled with ^{13}C) leading to trace DSPC directly; DSPC enrichment was measured by GC/MS and pulmonary DSPC pool size and half life were estimated in premature infants with different lung diseases[41,51]. Exogenous surfactant therapy, labeled with U^{13}C-DPPC, led to investigate *in vivo* DSPC kinetics in order to understand different factors that could affect DSPC half life and therefore surfactant treatment efficacy. In a recent study, by our research group, DSPC half life estimation was assessed in premature infants who received exogenous surfactant therapy at 100 or 200 mg/kg doses. Exogenous surfactant given to preterm infants with RDS at a dose of 200 mg/kg resulted in a longer DSPC half life, fewer retreatments and better oxygenation index values[8]. In another study, with the same analytical and clinical method, we tried to find a correlation among clinical and respiratory parameters and surfactant kinetics in order to evaluate which factors were associated with surfactant redosing. Infants requiring surfactant retreatment had a significantly shorter DSPC half life. Clinical predictors factors for surfactant retreatment were: lower birth weight, worse RDS radiological score, lower first surfactant dosing (100 mg/kg vs. 200 mg/kg), poorer response to the first surfactant dose, ventilation using conventional modalities[52].

In this thesis we administered a newly synthesized DSPC tracer, with two palmitate moieties differently labeled (1-$^{2}H_{3}$,2-U^{13}C PA-DPPC), in order to investigate surfactant metabolism accordingly to different lung diseases such as RDS, RDS with chorioamnionitis, pneumonia and BPD. In infants with RDS and RDS with chorioamnionitis comparison between DSPC $^{2}H_{3}$ PA and U^{13}C PA half lives

showed no differences in the catabolic rate of disappearance, while in infants with pneumonia DSPC $U^{13}C$ PA half life was shorter than DSPC $^{2}H_3$ PA; suggesting that catabolic mechanism is altered during pneumonia. In healthy lung, type II cells and alveolar macrophages contribute equally in surfactant turnover[66]. The phospholipids, organized in multivesicular body, are taken up by endocytosis into the type II cells where they are catabolized by lysosome or recycled and re-secreted into lamellar body; conversely all the surfactant uptaken by macrophages will be catabolized. Both of these cells activate PLA_2 superfamilies, lytic enzyme implied in phospholipids degradation, that are responsible of lung DSPC catabolism. They hydrolyze phospholipid in the sn-2 position leading to a lyso-phospholipid and a free fatty acid. Lysosomal PLA_2 and secreted PLA_2 are classes mainly produced by type II cells and alveolar macrophages, respectively. During inflammation, when macrophages and neuthrophils recruitment is increased even secreted PLA_2 activity is higher, as shown by De Luca and coworkers[67]. Supporting data by Malloy et al. gave evidence that in an animal model of LPS induced acute pulmonary inflammation, *in vivo* clearance of surfactant lipids by type II cells is unaltered and that the increased clearance of surfactant lipids is primarily due to either macrophages or neutrophils that are recruited to the alveolar spaces[68]. This altered turnover was also confirmed in our infants with pneumonia, belonging to tracer group, in which a faster tracer breakdown, presumably due to the increased catabolism of surfactant by $sPLA_2$ lead to a shorter DSPC $U^{13}C$-PA half life found in pneumonia compared with controls group ($p=0.048$). These data corroborate findings of our previous studies in which we administered uniformly labeled ^{13}C PA-DPPC in term and preterm infants with RDS and pneumonia[51].

Usual therapy for neonatal pneumonia includes mechanical ventilation and antibiotics (at the beginning with wide spectrum, later focused therapy is used if a specific pathogen is identified). Exogenous surfactant therapy is a wide used strategy in RDS treatment due to well known efficacy and benefits, while in pneumonia the surfactant therapy is still controversial. In animal studies, with induced bacterial pneumonia, growing of bacteria colony was decreased and pulmonary activity was improved after surfactant administration[15, 69]. In human studies few data are available concerning surfactant therapy in "not-RDS" infants[70]. In two studiesinfant with pneumonia were studied and after surfactant administration ventilation and oxygenation parameters significantly improved[71,72].

Chemical pneumonia due to meconium inhalation is characterized by surfactant deficiency caused by bilirubin, cholesterol, biliar salts, enzymes and free fatty

acids contained in meconium. All of these molecules can inactivate and alter surfactant functionality. In newborn with MAS exogenous surfactant therapy improved gas exchange and clinical outcome[23, 73] even though a recent strategy implying alveolar lavages with diluted surfactant had more efficacy[74,75,19].

Pool size represents the tracer dilution in infant's lung endogenous surfactant. The DSPC pool estimated with our model is likely to be associated with the actual metabolically active surfactant, where the tracer is distributed[57, 76]. Pool size calculated in all infants studied showed no statistical differences, therefore this tracers allow an estimation of surfactant pool size regardless the position of the labeled molecules. Moreover RDS pool size was lower compared with other infants who received exogenous surfactant therapy, as already found in other studies[41, 77,8]. Conversely BPD group showed a greater surfactant pool compared with controls as found in animal[78] and in human studies[57, 76]. This finding can be explained either by repeated surfactant doses required by infants developing BPD, that increase endogenous surfactant pool, or by delayed lung development leading to impaired surfactant recycling and increased surfactant synthesis as shown in animal study[29].

Our findings highlight a faster DSPC catabolism in infants with pneumonia, probably due to increased $sPLA_2$ activity, suggesting a role of exogenous surfactant therapy in neonatal pneumonia.

In this study we measured IL-8 and LTB_4 as inflammatory markers in order to estimate lung inflammation among infants belonging to surfactant group. Evaluation of pulmonary inflammatory markers is a newsworthy neonatal area of study. Recently it has been shown a correlation between pro-inflammatory cytokines in blood and bronchoalveolar aspirates in preterm with severe lung injury. In a recent study by Ambavalan et al.[79], several blood cytokines were measured, in very preterm infants with evolving BPD or died due to pulmonary injury; they had higher concentrations of interleukin (IL) 1ß, 6, 8 and 10 and interferon γ and lower concentration of interleukin 17, regulated on activation, normal T cell expressed and secreted (RANTES), and tumor necrosis factor ß. Cytokines levels were evaluated from 0 to 3 days of life (named early cytokines) and from 14 to 21 days (named late cytokines) and showed different concentrations depending on dosing time. Early cytokines were IL-8, IL-10 and INF-γ responsible of neuthrophil recruitment leading to pulmonary edema; late cytokine was IL-6 that was higher in infants developing BPD or in infants who died. In another study, in preterm infants with BPD on days 1 and 3 IL-8 levels were

elevated and neuthrophil counts were low, while on days 3 and 7 IL-6 was higher and neuthrophil counts increased in BPD with respect to RDS. Thus IL-8 and IL-6 temporally precede the influx of neutrophils in tracheal aspirates from mechanically ventilated preterm infants with RDS[80]. Paananen et al.[81] describes a relationship between chorioamnionitis and plasmatic cytokines: IL-6, IL-8, IL-10, and granulocyte colony stimulating-factor (G-CSF). On days 1 and 7 cytokine levels were measured from cord blood and plasma. In infants with chorioamnionitis high concentration in cord blood decreased during the first postantal day and were associated with the severity of chorioamnionitis. At day 1 IL-8 concentration predicted the risk of BPD. De Dooy et al.[31] identified inflammatory variables either in tracheal aspirates and in blood that were associated with histological chorioamnionitis (diagnosed by light microscopy). On day 1 they found significantly higher concentration of tracheal aspirates cytokines IL-1ß, IL-6, IL-8, IL-10 and TNFα in patient with chorioamnionitis; after multivariate analysis IL-8 in tracheal aspirates remains the only independent and single strongest factor associated with chorioamnoinitis. Contradictory evidences in literature correlate chorioamnionitis with acute and chronic lung injury and use of different therapies. Wattenberg et al.[82] described that preterm exposed to histologically confirmed chorioamnionitis in uterus, showed low incidence of developing RDS but higher incidence for developing BPD; these data were confirmed by De Dooy with higher cytokine levels on day 1 demonstrating that inflammation was originated in uterus. On the contrary Van Marter et al.[83] considered ventilated and not-ventilated preterm infants and speculated that chorioamnionitis determined lower incidence of BPD.

LTB_4 has an important role among the mediators involved in the inflammatory response to lung infections, it is release by macrophages and neutrophils and, in turn, it stimulates neutrophil chemotaxis and activation, leading to degranulation and release of inflammatory markers and enzymes. Many authors report LTB_4 evaluations in different lung diseases, but most of them measured this mediators in exhaled breath condensate (EBC)[84-86]. Piotrowski et al.[87] comparing eicosanoids concentrations between EBC and bronchoalveolar fluids concluded that LTB_4 can be equally measured in both matrices. Carraro et al.[36] measured LTB_4 in EBC in children with community acquired pneumonia, they found that LTB_4 was higher in this group compared with healthy controls and decreased after 1 week.

In this thesis two inflammatory mediators, IL-8 and LTB_4, were measured in tracheal aspirates of surfactant group of infants studied. IL-8 at study start (t=0)

was significantly higher in infants with RDS with chorioamnionitis compared to RDS; in the following 12 hours comparable concentrations were found in RDS and pneumonia. LTB_4, measured at study start, were significantly higher in pneumonia compared with controls and BPD. Our data are in agreement with other researchers that concluded that IL-8 is an early cytokine involved in inflammatory cascade who reaches peak concentration later (on day 1 values are elevated, first peak concentration is on days 3-4 and a second peak is on days 14-21)[79]; indeed in our study IL-8 level at t=0 was higher in chorioamnionitis due to an early activation in uterus during gestation, while in RDS and pneumonia group values were lower because they didn't already reach maximum concentration. After 12 hours IL-8 reached higher concentration even in RDS and pneumonia and IL-8 levels among all groups equalize.

LTB_4 that rapidly increases during inflammatory activation, was higher in pneumonia group, while in RDS and RDS with chorioamnionitis showed lower values probably because it was already in the decreasing part of concentration curve.

In conclusion in this study we utilized a newly synthesized tracer, doubly labeled, in order to trace *in vivo* surfactant metabolism. A shorter DSPC half life was found in sn-2 in infants with pneumonia, either with or without exogenous surfactant therapy, in comparison with other infant groups; this mechanism can be explained by a altered catabolic rate that occur in diffuse inflammation and support conventional therapies with exogenous surfactant replacement in term and preterm infants affected by pneumonia. Considerable investigation into ways to ameliorate pneumonia are underway. To better understand phatophysiological mechanisms that characterize different lung disorders, surfactant kinetics studies should be considered in order to define appropriate therapeutic strategies.

Study limitations and further analysis to perform

A larger number of infants are needed to confirm the higher DSPC breakdown rate in preterm and term newborns with pneumonia. We will also measure LTB_4 values at t=12, as already performed for IL-8; we will estimate either LTB_4 or IL-8 at different time of study, also for infants belonging to Tracer group.

BIBLIOGRAPHY

1. Engle WA. Surfactant-replacement therapy for respiratory distress in the preterm and term neonate. Pediatrics 2008;121:419-32.
2. Wirbelauer J, Speer CP. The role of surfactant treatment in preterm infants and term newborns with acute respiratory distress syndrome. J Perinatol 2009;29 Suppl 2:S18-22.
3. Merrill JD, Ballard RA. Pulmonary surfactant for neonatal respiratory disorders. Curr Opin Pediatr 2003;15:149-54.
4. Robertson B. Structural and functional characterization of porcine surfacctant isolated by liquid gel chromatography. Prog Resp Res 1990;25:237-46.
5. Ramanathan R, Rasmussen MR, Gerstmann DR, Finer N, Sekar K. A randomized, multicenter masked comparison trial of poractant alfa (Curosurf) versus beractant (Survanta) in the treatment of respiratory distress syndrome in preterm infants. Am J Perinatol 2004;21:109-19.
6. Bloom BT, Kattwinkel J, Hall RT, et al. Comparison of Infasurf (calf lung surfactant extract) to Survanta (Beractant) in the treatment and prevention of respiratory distress syndrome. Pediatrics 1997;100:31-8.
7. Bloom BT, Clark RH. Comparison of Infasurf (calfactant) and Survanta (beractant) in the prevention and treatment of respiratory distress syndrome. Pediatrics 2005;116:392-9.
8. Cogo PE, Facco M, Simonato M, et al. Dosing of porcine surfactant: effect on kinetics and gas exchange in respiratory distress syndrome. Pediatrics 2009;124:e950-7.
9. McFetridge L, McMorrow A, Morrison PJ, Shields MD. Surfactant Metabolism Dysfunction and Childhood Interstitial Lung Disease (chILD). Ulster Med J 2009;78:7-9.
10. McIntosh K. Community-acquired pneumonia in children. N Engl J Med 2002;346:429-37.
11. Michelow IC, Olsen K, Lozano J, et al. Epidemiology and clinical characteristics of community-acquired pneumonia in hospitalized children. Pediatrics 2004;113:701-7.
12. Isaacs D. Problems in determining the etiology of community-acquired childhood pneumonia. Pediatr Infect Dis J 1989;8:143-8.
13. Kayser FH. Changes in the spectrum of organisms causing respiratory tract infections: a review. Postgrad Med J 1992;68 Suppl 3:S17-23.

14. Stinson SF, Ryan DP, Hertweck S, Hardy JD, Hwang-Kow SY, Loosli CG. Epithelial and surfactant changes in influenzal pulmonary lesions. Arch Pathol Lab Med 1976;100:147-53.

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

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

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

18. Sun B, Curstedt T, Song GW, Robertson B. Surfactant improves lung function and morphology in newborn rabbits with meconium aspiration. Biol Neonate 1993;63:96-104.

19. Donn SM, Dalton J. Surfactant replacement therapy in the neonate: beyond respiratory distress syndrome. Respir Care 2009;54:1203-8.

20. Sun B, Curstedt T, Robertson B. Surfactant inhibition in experimental meconium aspiration. Acta Paediatr 1993;82:182-9.

21. Auten RL, Notter RH, Kendig JW, Davis JM, Shapiro DL. Surfactant treatment of full-term newborns with respiratory failure. Pediatrics 1991;87:101-7.

22. Laughon M, Bose C, Moya F, et al. A pilot randomized, controlled trial of later treatment with a peptide-containing, synthetic surfactant for the prevention of bronchopulmonary dysplasia. Pediatrics 2009;123:89-96.

23. Khammash H, Perlman M, Wojtulewicz J, Dunn M. Surfactant therapy in full-term neonates with severe respiratory failure. Pediatrics 1993;92:135-9.

24. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. N Engl J Med 2000;342:1500-7.

25. Kramer BW, Kallapur S, Newnham J, Jobe AH. Prenatal inflammation and lung development. Semin Fetal Neonatal Med 2009;14:2-7.

26. Kramer BW, Ladenburger A, Kunzmann S, et al. Intravenous lipopolysaccharide-induced pulmonary maturation and structural changes in fetal sheep. Am J Obstet Gynecol 2009;200:195 e1-10.

27. Been JV, Rours IG, Kornelisse RF, Jonkers F, de Krijger RR, Zimmermann LJ. Chorioamnionitis alters the response to surfactant in preterm infants. J Pediatr 2010;156:10-5 e1.

28. Kramer BW. Antenatal inflammation and lung injury: prenatal origin of neonatal disease. J Perinatol 2008;28 Suppl 1:S21-7.
29. Ikegami M, Jobe A, Yamada T, et al. Surfactant metabolism in surfactant-treated preterm ventilated lambs. J Appl Physiol 1989;67:429-37.
30. Meyer KC, Zimmerman JJ. Inflammation and surfactant. Paediatr Respir Rev 2002;3:308-14.
31. De Dooy J, Colpaert C, Schuerwegh A, et al. Relationship between histologic chorioamnionitis and early inflammatory variables in blood, tracheal aspirates, and endotracheal colonization in preterm infants. Pediatr Res 2003;54:113-9.
32. Strieter RM, Belperio JA, Keane MP. Host innate defenses in the lung: the role of cytokines. Curr Opin Infect Dis 2003;16:193-8.
33. Borish LC, Steinke JW. 2. Cytokines and chemokines. J Allergy Clin Immunol 2003;111:S460-75.
34. Busse WW. Leukotrienes and inflammation. Am J Respir Crit Care Med 1998;157:S210-3; discussion S47-8.
35. Da Dalt L, Callegaro S, Carraro S, Andreola B, Corradi M, Baraldi E. Nasal lavage leukotrienes in infants with RSV bronchiolitis. Pediatr Allergy Immunol 2007;18:100-4.
36. Carraro S, Andreola B, Alinovi R, et al. Exhaled leukotriene B4 in children with community acquired pneumonia. Pediatr Pulmonol 2008;43:982-6.
37. Csoma Z, Kharitonov SA, Balint B, Bush A, Wilson NM, Barnes PJ. Increased leukotrienes in exhaled breath condensate in childhood asthma. Am J Respir Crit Care Med 2002;166:1345-9.
38. Cavicchioli P, Zimmermann LJ, Cogo PE, et al. Endogenous surfactant turnover in preterm infants with respiratory distress syndrome studied with stable isotope lipids. Am J Respir Crit Care Med 2001;163:55-60.
39. Bunt JE, Zimmermann LJ, Wattimena JL, van Beek RH, Sauer PJ, Carnielli VP. Endogenous surfactant turnover in preterm infants measured with stable isotopes. Am J Respir Crit Care Med 1998;157:810-4.
40. Cogo PE, Carnielli VP, Bunt JE, et al. Endogenous surfactant metabolism in critically ill infants measured with stable isotope labeled fatty acids. Pediatr Res 1999;45:242-6.
41. Torresin M, Zimmermann LJ, Cogo PE, et al. Exogenous surfactant kinetics in infant respiratory distress syndrome: A novel method with stable isotopes. Am J Respir Crit Care Med 2000;161:1584-9.

42. Okano G, Sato T, Akino T. Biosynthetic routes of molecular species of lung phosphatidylglycerol. Tohoku J Exp Med 1981;135:265-73.
43. Cogo PE, Ori C, Simonato M, et al. Metabolic precursors of surfactant disaturated-phosphatidylcholine in preterms with respiratory distress. J Lipid Res 2009;50:2324-31.
44. Bohlin K, Patterson BW, Spence KL, et al. Metabolic kinetics of pulmonary surfactant in newborn infants using endogenous stable isotope techniques. J Lipid Res 2005;46:1257-65.
45. Spence KL, Zozobrado JC, Patterson BW, Hamvas A. Substrate utilization and kinetics of surfactant metabolism in evolving bronchopulmonary dysplasia. J Pediatr 2005;147:480-5.
46. Jacobs H, Jobe A, Ikegami M, Conaway D. The significance of reutilization of surfactant phosphatidylcholine. J Biol Chem 1983;258:4159-65.
47. Cogo PE, Toffolo GM, Ori C, et al. Surfactant disaturated-phosphatidylcholine kinetics in acute respiratory distress syndrome by stable isotopes and a two compartment model. Respir Res 2007;8:13.
48. Cogo PE, Zimmermann LJ, Meneghini L, et al. Pulmonary surfactant disaturated-phosphatidylcholine (DSPC) turnover and pool size in newborn infants with congenital diaphragmatic hernia (CDH). Pediatr Res 2003;54:653-8.
49. Cogo PE, Zimmermann LJ, Rosso F, et al. Surfactant synthesis and kinetics in infants with congenital diaphragmatic hernia. Am J Respir Crit Care Med 2002;166:154-8.
50. Cogo PE, Zimmermann LJ, Verlato G, et al. A dual stable isotope tracer method for the measurement of surfactant disaturated-phosphatidylcholine net synthesis in infants with congenital diaphragmatic hernia. Pediatr Res 2004;56:184-90.
51. Verlato G. Surfactant kinetics in newborn infants with pneumonia and respiratory dystress syndrome. Ital J Pediatr 2003;29:414-9.
52. Cogo PE, Facco M, Simonato M, et al. Pharmacokinetics and clinical predictors of surfactant redosing in respiratory distress syndrome. Intensive Care Med 2010.
53. Thome U, Topfer A, Schaller P, Pohlandt F. Comparison of lung volume measurements by antero-posterior chest X-ray and the SF6 washout technique in mechanically ventilated infants. Pediatr Pulmonol 1998;26:265-72.

54. Smulian JC, Bhandari V, Vintzileos AM, et al. Intrapartum fever at term: serum and histologic markers of inflammation. Am J Obstet Gynecol 2003;188:269-74.
55. Rudiger M, Friedrich W, Rustow B, Schmalisch G, Wauer R. Disturbed surface properties in preterm infants with pneumonia. Biol Neonate 2001;79:73-8.
56. Jones PJ, Leatherdale ST. Stable isotopes in clinical research: safety reaffirmed. Clin Sci (Lond) 1991;80:277-80.
57. Cogo PE, Zimmermann LJ, Pesavento R, et al. Surfactant kinetics in preterm infants on mechanical ventilation who did and did not develop bronchopulmonary dysplasia. Crit Care Med 2003;31:1532-8.
58. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911-7.
59. Mason RJ, Nellenbogen J, Clements JA. Isolation of disaturated phosphatidylcholine with osmium tetroxide. J Lipid Res 1976;17:281-4.
60. Touchstone JC, Levitt RE, Soloway RD, Levin SS. Separation of conjugated dihydroxy bile acids by thin-layer chromatography. J Chromatogr 1979;178:566-70.
61. Christie WW, Brechany EY, Shukla VK. Analysis of seed oils containing cyclopentenyl fatty acids by combined chromatographic procedures. Lipids 1989;24:116-20.
62. Cobelli C, Toffolo G. Constant specific activity input allows reconstruction of endogenous glucose concentration in non-steady state. Am J Physiol 1990;258:E1037-40.
63. Cobelli C, Toffolo G, Foster DM. Tracer-to-tracee ratio for analysis of stable isotope tracer data: link with radioactive kinetic formalism. Am J Physiol 1992;262:E968-75.
64. Hallman M, Merritt TA, Pohjavuori M, Gluck L. Effect of surfactant substitution on lung effluent phospholipids in respiratory distress syndrome: evaluation of surfactant phospholipid turnover, pool size, and the relationship to severity of respiratory failure. Pediatr Res 1986;20:1228-35.
65. Griese M, Dietrich P, Reinhardt D. Pharmacokinetics of bovine surfactant in neonatal respiratory distress syndrome. Am J Respir Crit Care Med 1995;152:1050-4.
66. Ikegami M. Surfactant catabolism. Respirology 2006;11 Suppl:S24-7.
67. De Luca D, Baroni S, Vento G, et al. Secretory phospholipase A2 and neonatal respiratory distress: pilot study on broncho-alveolar lavage. Intensive Care Med 2008;34:1858-64.

68. Malloy JL, Wright JR. In vivo clearance of surfactant lipids during acute pulmonary inflammation. Respir Res 2004;5:8.

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

70. Greenough A. Expanded use of surfactant replacement therapy. Eur J Pediatr 2000;159:635-40.

71. Lotze A, Mitchell BR, Bulas DI, Zola EM, Shalwitz RA, Gunkel JH. Multicenter study of surfactant (beractant) use in the treatment of term infants with severe respiratory failure. Survanta in Term Infants Study Group. J Pediatr 1998;132:40-7.

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

73. Halliday HL, Speer CP, Robertson B. Treatment of severe meconium aspiration syndrome with porcine surfactant. Collaborative Surfactant Study Group. Eur J Pediatr 1996;155:1047-51.

74. Lam BC, Yeung CY. Surfactant lavage for meconium aspiration syndrome: a pilot study. Pediatrics 1999;103:1014-8.

75. Wiswell TE, Knight GR, Finer NN, et al. A multicenter, randomized, controlled trial comparing Surfaxin (Lucinactant) lavage with standard care for treatment of meconium aspiration syndrome. Pediatrics 2002;109:1081-7.

76. Cogo PE, Toffolo GM, Gucciardi A, Benetazzo A, Cobelli C, Carnielli VP. Surfactant disaturated phosphatidylcholine kinetics in infants with bronchopulmonary dysplasia measured with stable isotopes and a two-compartment model. J Appl Physiol 2005;99:323-9.

77. Jobe A. Metabolism of endogenous surfactant and exogenous surfactants for replacement therapy. Semin Perinatol 1988;12:231-44.

78. Janssen DJ, Carnielli VP, Cogo PE, et al. Surfactant phosphatidylcholine half-life and pool size measurements in premature baboons developing bronchopulmonary dysplasia. Pediatr Res 2002;52:724-9.

79. Ambalavanan N, Carlo WA, D'Angio CT, et al. Cytokines associated with bronchopulmonary dysplasia or death in extremely low birth weight infants. Pediatrics 2009;123:1132-41.

80. Munshi UK, Niu JO, Siddiq MM, Parton LA. Elevation of interleukin-8 and interleukin-6 precedes the influx of neutrophils in tracheal aspirates from preterm infants who develop bronchopulmonary dysplasia. Pediatr Pulmonol 1997;24:331-6.

81. Paananen R. Blood cytokines during the perinatal period in very preterm infants: relationship of inflammatory response and bronchopulmonary dysplasia. J Pediatr 2000;154:39-43.

82. Watterberg KL, Demers LM, Scott SM, Murphy S. Chorioamnionitis and early lung inflammation in infants in whom bronchopulmonary dysplasia develops. Pediatrics 1996;97:210-5.

83. Van Marter LJ, Dammann O, Allred EN, et al. Chorioamnionitis, mechanical ventilation, and postnatal sepsis as modulators of chronic lung disease in preterm infants. J Pediatr 2002;140:171-6.

84. Ko FW, Leung TF, Wong GW, et al. Measurement of tumor necrosis factor-alpha, leukotriene B4, and interleukin 8 in the exhaled breath condensate in patients with acute exacerbations of chronic obstructive pulmonary disease. Int J Chron Obstruct Pulmon Dis 2009;4:79-86.

85. Bayley DL, Abusriwil H, Ahmad A, Stockley RA. Validation of assays for inflammatory mediators in exhaled breath condensate. Eur Respir J 2008;31:943-8.

86. Biernacki WA, Kharitonov SA, Barnes PJ. Increased leukotriene B4 and 8-isoprostane in exhaled breath condensate of patients with exacerbations of COPD. Thorax 2003;58:294-8.

87. Piotrowski WJ, Antczak A, Marczak J, Nawrocka A, Kurmanowska Z, Gorski P. Eicosanoids in exhaled breath condensate and BAL fluid of patients with sarcoidosis. Chest 2007;132:589-96.