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Stable isotope tracers to estimate lung surfactant metabolism *in vivo*

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What we call the beginning is often the end.
And to make an end is to make a beginning.
The end is where we start from.
T. S. Eliot

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Abstract

Aim of this thesis was to apply the stable isotopes technique to study pulmonary surfactant kinetics.

Lung surfactant is essential to live, because it prevents the alveoli to collapse during normal breathing. Lung surfactant is composed of lipids and specific proteins, and nowadays it is well known that alterations on the composition and amount of surfactant are involved in acute and chronic lung diseases.

This work presents two studies about lung surfactant kinetics.

The first one is about the synthesis of disaturated phosphatidylcholine, the main lipid in the pulmonary surfactant system, in a murine model of unilateral acid injury.

The second one explains an optimized procedure to evaluate surfactant protein B synthesis and a novel method to study surfactant protein C synthesis in both infants and adults, each with stable isotopes technique.

Riassunto

Obiettivo di questa tesi è stato quello di applicare l'uso degli isotopi stabili come traccianti metabolici allo studio del metabolismo del surfattante polmonare.

Il surfattante polmonare è una sostanza fondamentale per vivere in quanto impedisce agli alveoli di collassare durante l'atto respiratorio. Il surfattante polmonare è composto da lipidi e proteine specifiche, ed è ormai noto che alterazioni nella composizione e nella quantità del surfattante polmonare sono presenti nella malattia respiratoria acuta e cronica.

In questo lavoro vengono presentati due studi sulla cinetica del surfattante polmonare: il primo riguarda la sintesi del lipide maggiormente presente nel surfattante, ovvero la fosfatidilcolina disatura, in una condizione di danno unilaterale da acido in un modello murino.

Il secondo studio riguarda un metodo ottimizzato per misurare la sintesi della proteina B del surfattante e un nuovo metodo per valutare, sempre con l'utilizzo degli isotopi stabili, la sintesi della proteina C del surfattante in bambini e adulti.

Chapter One

Pulmonary surfactant system: as easy as breathing…?

Lungs are the site of gas exchange with blood. To facilitate this gas exchange, the lung has the largest epithelial surface area of the body in contact with the external environment. Together, human lungs contain approximately 2.4 km of airways and 300 to 500 million alveoli, with a total surface area of about 70 square metres in adults. 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. The alveolar surface is formed by two types of epithelial cells, pneumocytes I and pneumocytes II (Figure 1). Type I cells make up 95% of the alveolus, and serve as a thin barrier between blood and air [1]. Type II cells can differentiate in type I cells, and they take part in the immune defence, expressing receptors like Toll- like receptor. Also, they can regulate the transmigration of monocytes across the epithelial layer and participate in T-cell activation. However, the most important role of type II cells is to synthesize and secrete an aqueous fluid covering alveolar epithelial cells called pulmonary surfactant [2].

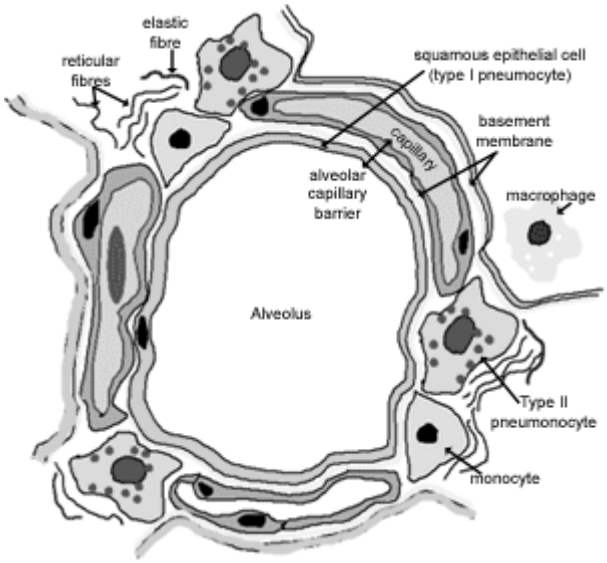

Figure 1: Alveolar structure and its main components.

Pulmonary surfactant is a complex mixture of lipids and proteins that creates a unique interface separating alveolar gas and liquids at the alveolar cell surface, reducing surface tension and preventing atelectasis at the end of expiration. (Table 1)

Increasing evidence also suggest that surfactant is needed in the bronchioli through which air is conducted to the alveoli. A lack of surfactant leads to closure of the small cylindrical airways[3].

Table 1: Functions of pulmonary surfactant
Biophysical functions
Prevents collapse of the alveoli during expiration
Supports inspiratory opening of the lungs
Stabilizes and keeps small airways patent
Improves microciliary transport and removal of particles and cellular debris from the alveoli
Immunological, non biophysical functions
Phospholipids inhibit endotoxin-stimulated cytokine (TNF, IL-1, IL-6 release from macrophages
SP-A and SP-D modulate the phagocytosis, chemotaxis and oxidative burst of macrophages
SP-A and SP-D can opsonise various micro-organisms for easier phagocytosis, and capture bacterial toxins.

The expanding force of gas in an alveolus of radius r is expressed by the law of Laplace:

$$\Delta P = 2\gamma / r$$

where the gas pressure difference (ΔP) needs to keep equilibrium with the collapsing force of surface tension γ. ΔP is approximately equal to the negative pressure around the alveolus. If γ does not change during breathing, ΔP would

increase during expiration as *r* decreases. Alveoli that are surrounded by n excessive negative pressure easily collapse causing pulmonary atelectasis.

During normal breathing γ can be modulated by surfactant phospholipids that are forced to come closer during expiration, leading to a decreased γ value and thus avoiding alveoli collapse [4].

Surfactant system not only protects the lung against alveolar collapse, but, together with alveolar macrophages, constitutes the front line of defence against inhaled pathogens.

These essential functions are strictly linked to the complexity of the lipid-protein system.

Pulmonary surfactant composition

Lung surfactant is composed for the 90% of lipids and for the 10% of proteins (Figure 2).

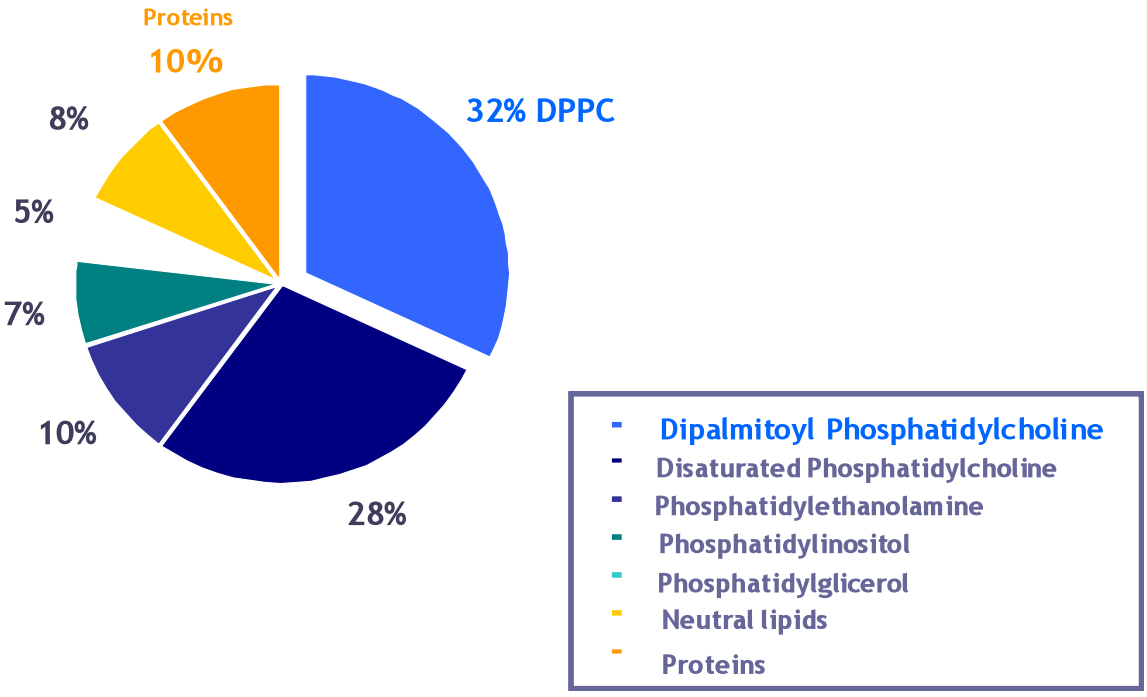

Figure 2: Composition of lung surfactant

Surfactant is synthesized in the endoplasmatic reticulum of type II cells t ype II cells and stored in to specific lysosome-like organelles called lamellar bodies [5-6]. After regulated secretion by exocytosis, lamellar bodies unravel spontaneously in the alveolar fluid to form multilamellar vesicles and highly organised membranes termed tubular myelin [2].

Secreted surfactant is internalised by type II cells that can be incorporated back to lamellar bodies for recycling and degradation by alveolar macrophages [7].

Lipids and proteins are secreted into the alveolar sub phase to form a tubular myelin reservoir from which multilayers and monolayers form a film.

Lipids

Pulmonary surfactant contains several classes of lipids, such as phospholipids, triglycerides, cholesterol, and fatty acids. Phospholipids composition is highly conserved among mammals [8-9].

Phosphatidylcholine (PC) is the major phospholipid comprising 80% of surfactant lipids.

Surfactant PC contains disaturated PC (DSPC) species, 16:0/16:0-PC.

60% of surfactant PC is present as dipalmitoylphosphatidylcholine (DPPC), and it is the major surface-active component [10]

Mammalian surfactant contains about 10% of 16:0/14:0-PC, 16:0/16:1-PC (30%), and 16:0/18:1-PC and 16:0/18:2-PC species at lower concentrations.

Phosphatidylglicerol (PG) is the second most abundant phospholipid in surfactant and it is present at 7-15%. of the total phospholipid.

It is still not completely understood the role of PG in the pulmonary surfactant system, but it seems to stabilize the alveoli and to take part in the immune response [11-12].

Other minor phospholipids are phosphatidylethanolammine (PE), sphingomyelin (SM), phosphatidylinositol (PI), and phosphatidylserine (PS).

Proteins

Proteins represent about the 10% of the mass of the pulmonary surfactant. There are four specific surfactant protein: SP-A, SP-B, SP-C and SP-D. They are produced by respiratory epithelial cells, each playing a specific role in surfactant homeostasis and host defence.

SP-A and SP-D are hydrophilic, and they are members of the calcium-dependent lectin family of proteins that share collagenous domains (Figure 3). They are able to bind complex carbohydrates, lipids and glycolipids, including those on the surface of the pathogens.

SP-B and SP-C are small, hydrophobic proteins and they play a critical role in enhancing the rate of spreading and stability of surfactant phospholipids. For more details about surfactant protein B and C see chapter 4.

Figure 3: Surfactant proteins: collectins and hydrophobic proteins

Synthesis and regulation of surfactant phospholipids precursors

Surfactant phospholipids derived from a de novo pathway, are dependent on the availability of fatty acids (FA) in type II cells. FA come from the circulation in the form of free FA or triacylglycerols within lipoproteins. FA uptake from the circulation is mediated by membrane FA binding protein.
Alternatively, they can be internalise by alveoli after phospholipid hydrolysis.
Lactate can also be a precursor for de novo synthesized FA in mature type II cells, while in the late fetal period the main precursor is glycogen.
The keratinocyte growth factor (KGF) regulates the type II cell phenotype and stimulates the key enzymes of FA synthesis like acetyl CoA carboxylase, fatty acid synthase (FAS), citrate lyase. These enzymes are up regulated during surfactant lipogenesis in the lung (Figure 4) [13].
Moreover, hormonal and physiologic factors can also stimulate de novo FA synthesis. FAS is activated by glucocorticoids in fetal lung while thyroid hormone and transforming growth factor β1 (TGF-β1) antagonize this effect [14].

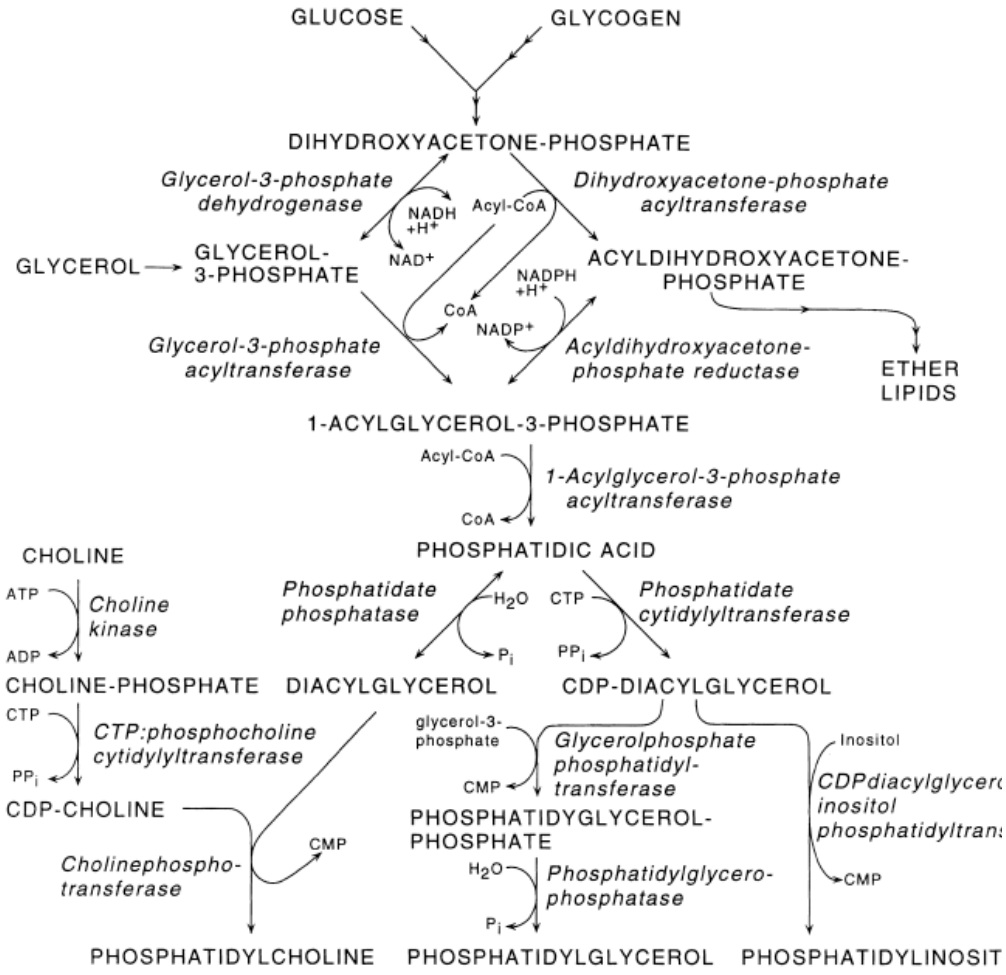

Figure 4: Biosynthesis of phosphatidylcholine, phosphatidylglicerol and phosphatidylinositol.

Surfactant proteins can be synthesized in type II cells but only for surfactant protein C they are the only site of synthesis. Surfactant proteins A, B and D can also be synthesized in Clara cells.

Regulation of surfactant secretion

Surfactant secretion has been studied both *in vivo* in intact animals and *in vitro* with isolated type II cells. Studies have focused primarily on PC or disaturated PC. The phospholipid composition of isolated lamellar bodies is

virtually the same of the lamellar bodies and surfactant phospholipids are secreted together with lamellar bodies [15]. This step occurs through the fusion of the limiting membrane of lamellar bodies with the apical plasma membrane, and then the content of the lamellar bodies is extruded into the alveolar space.

Phospholipids can translocate across the membrane through the ABC transporters; in particular, ABCA3 is in the outer membrane of the lamellar bodies of type II cells.

Lamellar bodies are enriched in SP-B and SP-C and it is likely that these proteins are secreted together with phospholipids by regulated exocytosis of lamellar bodies [15] .

SP-A secretion in isolated type II cells is not stimulated by agonists that stimulate PC secretion.[16]. Lamellar bodies era lacking of SP-D, thus implying that this protein could be secreted independently of the lipids [17-18].

Secretion is an indispensable step in lung surfactant homeostasis. A lack of secretion could lead to a drastic deficiency in surfactant, a condition incompatible with life. To avoid this problem, there are more than one signalling mechanism involved in the regulation of surfactant secretion (Figure 5):

1) The activation of adenylate cyclase (AC), with the subsequent activation of cAMP-dependent protein kinase. This signalling is activated by β-adrenergic and adenosine A_{2B} receptors coupled with a G-protein [19]. Also cholera toxin and forskolin can activate the AC pathway.
2) The second way is mediated by the activation of protein kinase C (PKC), that results in the hydrolysis of phosphatidylinositol bisphosphate and the formation of diacylglicerol (DAG) and inositol triphosphate (IP_3). The consequence of these reactions is the activation of phospholipase D (PLD) that leads to the formation of choline and phosphatidic acid (PA). PA is converted into DAG with the activation of PKC.
3) The third mechanism involves the elevation of intacellular levels of Ca^{++}, [20] that can result from an increase of IP_3 or by ionophores that promote Ca^{++} influx into the cell from the medium. Ca^{++} activates a Ca^{++} -

calmodulin dependent kinase (CaCM-PK) and can activate some PKC isoforms.

In conclusion, PKA, PKC or the mobilization of Ca^{++} can lead to signal protein phosphorilation and so to the surfactant secretion.

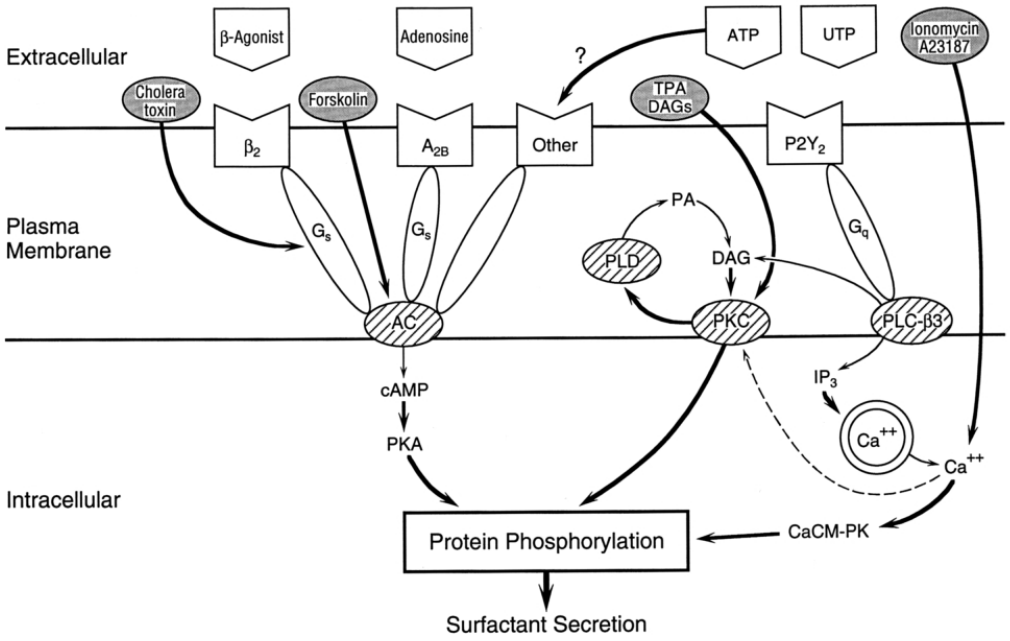

Figure 5: Schematic representation of signal transduction mechanisms mediating surfactant secretion in type II cells. See text for abbreviations.

Surfactant degradation and recycling

Surfactant components can be reutilized by type II cells that internalize alveolar phospholipids to re-incorporate into LB for secretion [21]

Substantial portion of surfactant (25%-95%) are re-internalized in type II cells and this mechanism is promoted by SP-A thanks to its high-affinity receptors present on the cell surface [22]. Mechanisms are not fully defined.

Surfactant components taken up by type II cells are recycled or degraded, while surfactant internalized by macrophages is largely degraded.

Inhibition of surfactant activity

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 phospholipids, lysophospholipids, free fatty acids, meconium (fetal feces expelled during stress), and high cholesterol levels [23].

Surface activity can be inhibited in vitro by phospholipase A2 and C, fatty acids, lyso PC. These molecules are released during lung injury.

SURFACTANT INACTIVATION AND LUNG DISEASES

Inactivation:
- Leakage of plasma proteins
- Lipids: bacterial LPS, PLA2, high cholesterol levels
- oxidative degradation

Lung diseases:
- ARDS, ALI
- Chronic bronchitis
- Cystic Fibrosis
- Asthma

The main lytic enzyme implied in PL degradation are the phospholipases 2 (PLA2) 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 fatty acid (FA) deriving from position 2 and the remaining lyso-PL, both of them can serve as lipid mediator precursor: the free FA 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.

Moreover, cellular lipids and blood proteins can impair lung surfactant activity. Proteins, membrane lipids and free fatty acids (FFA) have intrinsic surface activity, and can compete with the components of surfactant system, and this can seriously interfere with the entry of lung surfactant into the air-liquid interface. 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 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 [24].

Diseases

ARDS

ARDS (acute respiratory distress syndrome) and ALI (acute lung injury) can have a variety of causes, but the most common for adults is sepsis, mechanical trauma, multiple transfusions and gastric aspiration (see Chapter 3 for details). A reduction of synthesis of surfactant can contribute to the decreased surfactant activity in ARDS.

Alterations in the biochemical composition and the biophysical properties of pulmonary surfactant are well documented for patients with acute inflammatory lung diseases such as the acute respiratory distress syndrome. In these patients were noted a reduced relative content of phosphatidylcholine (PC) and phosphatidylglicerol, an increase in minor PL including sphingomyelin, phosphatidylinositol, and phosphatidylethanolamine , a decreased relative content of disaturated PC species, and reduced SP-A, SP-B, and SP-C [25].

Thus, lung injury can reduce both synthesis and function of surfactant lipids and proteins.

Respiratory distress syndrome

Respiratory distress syndrome (RDS) is one of the most common causes of morbidity in preterm neonates. It occurs shortly after birth with apnea, cyanosis, inspiratory stridor, poor feeding and tachypnea. Radiological findings also include reticulogranular “ground glass” appearance.

Preterm neonates with RDS have low amounts of surfactant, and lower quantity of DSPC, PG and surfactant specific proteins than a mature lung.

Nowadays the standard therapy comprises corticosteroids and surfactant replacement therapy.

Surfactant therapy has become the standard of care in management of preterm infants with RDS[26].

Meconium aspiration syndrome

Meconium aspiration syndrome (MAS) is an important cause of morbidity and mortality from respiratory distress in the perinatal period. In the present of fetal distress, gasping can start *in utero* with aspiration of amniotic fluid and its contents, including meconium, leading to acute lung injury.

It has been demonstrated that meconium aspiration can impair surfactant system decreasing its surface adsorption rate [27]

Moreover, MAS is linked with increasing cytokines IL-1β, IL-6 and IL-8 [28].

References

1. Berthiaume Y, Voisin G, Dagenais A. The alveolar type I cells: the new knight of the alveolus? *J Physiol* 2006: 572(Pt 3): 609-610.
2. Casals C, Canadas O. Role of lipid ordered/disordered phase coexistence in pulmonary surfactant function. *Biochim Biophys Acta* 2012: 1818(11): 2550-2562.
3. Griese M. Pulmonary surfactant in health and human lung diseases: state of the art. *Eur Respir J* 1999: 13(6): 1455-1476.
4. Enhorning G. Surfactant in airway disease. *Chest* 2008: 133(4): 975-980.
5. Haagsman HP, van Golde LM. Synthesis and assembly of lung surfactant. *Annu Rev Physiol* 1991: 53: 441-464.
6. Batenburg JJ. Surfactant phospholipids: synthesis and storage. *Am J Physiol* 1992: 262(4 Pt 1): L367-385.
7. Ridsdale R, Na CL, Xu Y, Greis KD, Weaver T. Comparative proteomic analysis of lung lamellar bodies and lysosome-related organelles. *PLoS One* 2011: 6(1): e16482.
8. Hook GE. Alveolar proteinosis and phospholipidoses of the lungs. *Toxicol Pathol* 1991: 19(4 Pt 1): 482-513.
9. Sullivan LC, Orgeig S, Daniels CB. Control of the development of the pulmonary surfactant system in the saltwater crocodile, Crocodylus porosus. *Am J Physiol Regul Integr Comp Physiol* 2002: 283(5): R1164-1176.
10. Holm BA, Wang Z, Egan EA, Notter RH. Content of dipalmitoyl phosphatidylcholine in lung surfactant: ramifications for surface activity. *Pediatr Res* 1996: 39(5): 805-811.
11. Hallman M, Enhorning G, Possmayer F. Composition and surface activity of normal and phosphatidylglycerol-deficient lung surfactant. *Pediatr Res* 1985: 19(3): 286-292.
12. Numata M, Chu HW, Dakhama A, Voelker DR. Pulmonary surfactant phosphatidylglycerol inhibits respiratory syncytial virus-induced inflammation and infection. *Proc Natl Acad Sci U S A* 2010: 107(1): 320-325.
13. Agassandian M, Mallampalli RK. Surfactant phospholipid metabolism. *Biochim Biophys Acta* 2012.
14. Lu Z, Gu Y, Rooney SA. Transcriptional regulation of the lung fatty acid synthase gene by glucocorticoid, thyroid hormone and transforming growth factor-beta 1. *Biochim Biophys Acta* 2001: 1532(3): 213-222.
15. Rooney SA. Regulation of surfactant secretion. *Comp Biochem Physiol A Mol Integr Physiol* 2001: 129(1): 233-243.
16. Xu X, McCormick-Shannon K, Voelker DR, Mason RJ. KGF increases SP-A and SP-D mRNA levels and secretion in cultured rat alveolar type II cells. *Am J Respir Cell Mol Biol* 1998: 18(2): 168-178.
17. Crouch E, Persson A, Chang D, Parghi D. Surfactant protein D. Increased accumulation in silica-induced pulmonary lipoproteinosis. *Am J Pathol* 1991: 139(4): 765-776.
18. Voorhout WF, Veenendaal T, Kuroki Y, Ogasawara Y, van Golde LM, Geuze HJ. Immunocytochemical localization of surfactant protein D (SP-D) in type II cells, Clara cells, and alveolar macrophages of rat lung. *J Histochem Cytochem* 1992: 40(10): 1589-1597.

19. Gobran LI, Rooney SA. Adenylate cyclase-coupled ATP receptor and surfactant secretion in type II pneumocytes from newborn rats. *Am J Physiol* 1997: 272(2 Pt 1): L187-196.
20. Mason RJ, Voelker DR. Regulatory mechanisms of surfactant secretion. *Biochim Biophys Acta* 1998: 1408(2-3): 226-240.
21. Zimmermann LJ, Janssen DJ, Tibboel D, Hamvas A, Carnielli VP. Surfactant metabolism in the neonate. *Biol Neonate* 2005: 87(4): 296-307.
22. 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(10): 1539-1570.
23. 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(2): 185-191.
24. 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(5): 1708-1716.
25. Schmidt R, Meier U, Yabut-Perez M, Walmrath D, Grimminger F, Seeger W, Gunther A. Alteration of fatty acid profiles in different pulmonary surfactant phospholipids in acute respiratory distress syndrome and severe pneumonia. *Am J Respir Crit Care Med* 2001: 163(1): 95-100.
26. Jobe AH. Mechanisms to explain surfactant responses. *Biol Neonate* 2006: 89(4): 298-302.
27. Bae CW, Takahashi A, Chida S, Sasaki M. Morphology and function of pulmonary surfactant inhibited by meconium. *Pediatr Res* 1998: 44(2): 187-191.
28. Cayabyab RG, Kwong K, Jones C, Minoo P, Durand M. Lung inflammation and pulmonary function in infants with meconium aspiration syndrome. *Pediatr Pulmonol* 2007: 42(10): 898-905.

Chapter Two

Stable isotope tracers

The atoms of one element have the same number of protons but can have a different mass number, that is a different number of neutrons. In fact, the term isotope is formed from the Greek roots isos (ἴσος "equal") and topos (τόπος "place"), meaning that they are at the same place in the periodic table.
Some isotopes, called radionuclides, can undergo a radioactive decay, while some others have never demonstrated a decay, and they are called stable isotopes. The majority of chemical elements consists of a mixture of stable isotopes.

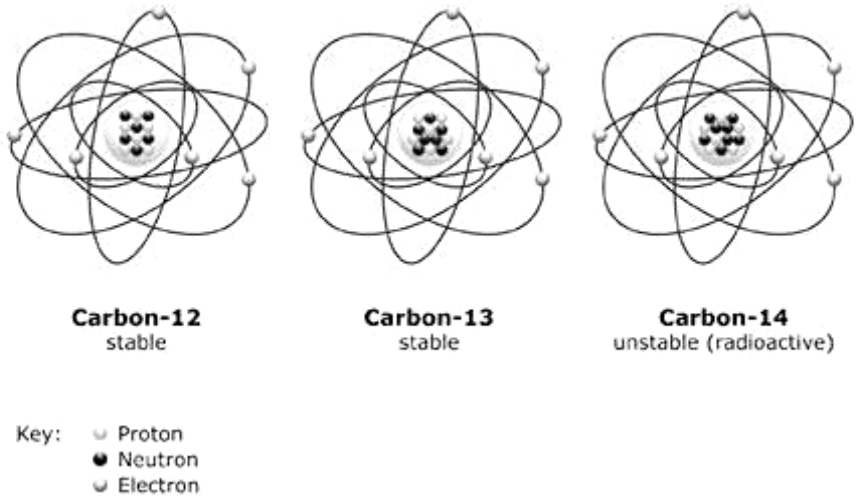

Figure 1: isotopes of carbon

For example carbon can have three main isotopes (figure 1): carbon-12, with 12 neutrons, and carbon-13 and 14, with respectively one or two more neutrons. Carbon-13 is stable, while carbon-14 is not stable but decays at a known rate.
Only hydrogen isotopes have different name: protium is the most abundant form of hydrogen with one proton and no neutrons, deuterium is the stable isotope, with

one proton and one neutron, and tritium is the radionuclide with one proton and two neutrons.

The origin and evolution of the component they derive from, can bring a different isotopic composition. That's why stable isotopes can be used in many different fields, to discriminate different molecules, such as archaeology, geochemistry, pharmaceutics, forensic science, anti-doping screening, environmental chemistry and art.

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[1-5]. 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 [6-12].

Tracers labelled with stable isotopes

Every metabolic process could be studied using stable isotopes. In spite of possible small differences in metabolic behaviour, molecules labelled with stable isotopes in one or several position are suitable tracers for the study of chemical and biological processes. These tracers can be instilled orally or intravenously, and modern analytical techniques allow the measurement of undifferentiated molecules 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. A variety of applications for stable isotopes in research and diagnosis has been described, some example are given in Table 1. 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 end products. Direct end products 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 labelled tracer in paediatrics is the examination of gastric emptying in newborn infant using carboxyl^{13}C-acetate.

TOPIC	**TRACER**	**REF.**
Infections		
Helicobacter pylori	^{13}C-urea	[8]
Gastrointestinal functions		
Gastric emptying	^{13}C-acetate, ^{13}C-ottanoate	[13] - [14]
Maldigestion		
Lactase deficiency	^{13}C-lactose	[15]
Lipase deficiency	^{13}C-trioctanoin, ^{13}C-triolein,^{13}C-palmitic acid	[12]
Malabsorption		
Carbohydrates	^{13}C-glucose	[3]
Fat	Different^{13}C-triglyceride	[12]
Amino acid	^{13}C-^{15}N- leucine, ^{13}C-leucine	[16] - [17]
Body composition		
Total body water	$D_2{}^{18}O$	[5]
Synthesis processes		
Gluconeogenesis	2,3-^{13}C2-alanine, 6,6 ^{2}H-glucose	[18]
Albumin	^{15}N glycine, ^{13}C leucine	[19] - [20]
Cholesterol	D_2O	[21]
Fatty acid conversion	Different fatty acid enriched ^{13}C	[22]
Metabolism and metabolic disorders		
Energy expenditure	$D_2{}^{18}O$	[23-26]
Fructose intolerance	^{13}C-fructose	[27]
Phenylketonuria	^{2}H-phenilalanine	[28]

Table 1: Stable isotopes in clinical practice and research.

Use of stable isotopes: advantages

The application of stable isotopes in medicine has many advantages. First of all, they can be safely administered in children and pregnant women, since they are not radioactive. Moreover, many radioactive isotopes can decay too much faster (e.g. radioactive nitrogen decays in 10 minutes, radioactive oxygen in 123 seconds), precluding long lasting metabolic studies. Also, the employment of ^{14}C or ^{3}H leads to an important discrimination by the metabolic pathway, because of their heavier mass.

Many stable isotopes can be used at the same time, to study simultaneously more than one metabolic pathway in the same subject.

Toxicity of stable isotopes has been studied both in animals and humans.

In animal studies high deuterium doses showed toxic effects, but administered doses were really higher than ones used in human studies. 30-40% of deuterium in total body is fatal [29-30]. 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 [29]. 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.

How to analyze stable isotopes: Mass Spectrometry

Mass Spectrometry is a technique to determine extremely accurate mass of molecules. The three essential functions of a mass spectrometer, and the associated components, are (figure 2):

1. The ions source, where the analyte is converted into ions.
2. The mass analyzer, where ions are separated according to their mass to charge ratio (m/z).
3. The detector, where ions are measured and amplified.

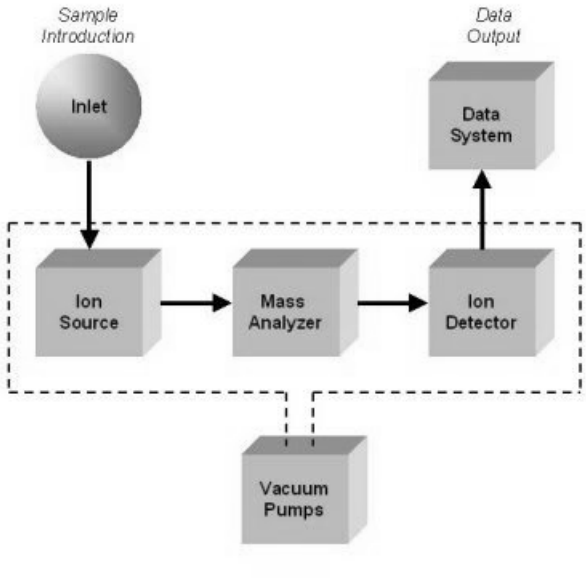

Figure 2: Components of a mass spectrometer.

The role of the ion sources is to turn neutral molecules into charged ions: in this way they can interact with the electromagnetic field of the analyzer, and be separated,

There are two main types of ion sources: Electron Impact Ionizator (EI) and Chemical Ionization (CI).

EI is probably the most common ion source used in mass spectrometry.

In the EI process, the sample of interest is vaporized into the mass spectrometer ion source, where it is impacted by a beam of electrons with sufficient energy to ionize the molecule.

This process can be summarised in this equation:

$$M + e^- \longrightarrow M^+ + 2e^-$$

The ion fragmentation is typical for the molecule of interest.

CI is applied to samples similar to those analyzed by EI and is primarily used to enhance the abundance of the molecular ion. Chemical ionization uses gas phase ion-molecule reactions within the vacuum of the mass spectrometer to produce ions from the sample molecule. The chemical ionization process is initiated with a reagent gas such as methane, isobutane, or ammonia, which is ionized by electron impact. High gas pressure in the ionization source results in ion-molecule reactions between the reagent gas ions and reagent gas neutrals. Some of the products of the ion-molecule reactions can react with the analyte molecules to produce ions.
This kind of ionisation is milder and produces a restrained fragmentation pattern.
Ions produced in the ion source are focused into the mass analyzer, where charged molecules are separated.
The most common analyzer is the “quadrupole” analyzer, that is composed of four metal rods arranged as in figure 3. Each rod pair is connected electrically, and a constant dc current is applied to all the rods, while a radio frequency voltage (RF) is applied between one pair of rods and the other.
Changing RF, the quadrupole can select a specific m/z ratio and thus specific ions. If ions are resonant with the filed, they can pass trough and reach the detector, otherwise they will not (non-resonant ions).

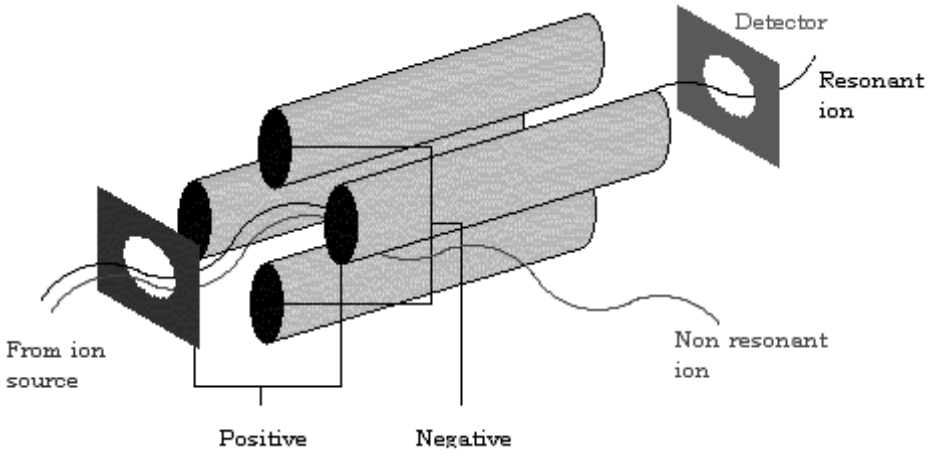

Figure 3: Schematic representation of a quadrupole

Once the ions are separated by the mass analyzer, they reach the ion detector, which generates a current signal from the incident ions. The most commonly used detector is the electron multiplier, which transfers the kinetic energy of incident ions to a surface that in turn generates secondary electrons.

Isotope Ratio Mass Spectrometry

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).

The organic sample is pyrolised to its fundamental components, like CO, CO_2,, H_2.

Water is removed and the sample is ionised in a EI source. As analyzer, it is used a magnetic analyzer because it permits a very precise measurement of isotopes ratio. Each cup of the analyzer can monitor a single m/z ratio. Ratios are usually expressed as *delta*, that represents the variation of the isotopic ratio of the sample versus the isotopic ratio of the sample:

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

where R= heavy isotope mass/ light isotope mass ratio (i.e. $^{13}C/^{12}C$, $^{2}H/^{1}H$); $\delta X>0$ when the heavy isotope is enriched versus standard in the sample; $\delta X<0$ when the heavy isotope is impoverished or the light isotope is enriched versus standard in the sample.

Isotopic enrichment has to be compared to a standard, to provide comparable data. Standard are shown in table 2.

Element	**Stable isotopes**	**Natural mean abundance (%)**	**Standard ratio values**	**International standard reference**
Hydrogen	^{1}H ^{2}H	99.985 0.015	$^{2}H/^{1}H = 0.000316$	VSMOW (Vienna Standard Mean Ocean Water)
Carbon	^{12}C ^{13}C	98.892 1.108	$^{13}C/^{12}C =$ 0.0112372	PDB (Pee Dee Belemnite)
Oxygen	^{16}O ^{18}O	99.7587 0.2039	$^{18}O/^{16}O =$ 0.0039948	VSMOW (Vienna Standard Mean Ocean Water)

Table 2: International standard references for the stable isotopes of hydrogen, carbon and oxygen.

References

1. Curtius HC, Vollmin JA, Baerlocher K. Study of metabolic pathways in vivo using stable isotopes. *Anal Chem* 1973: 46(7): 1107-1110.
2. Jones PJ. Stable isotopes in nutrition research: historical perspective and overview. *Can J Physiol Pharmacol* 1990: 68(7): 935-940.
3. Lifschitz CH, Boutton TW, Carrazza F, Beyreiss K, Schmitz J, Ricour C, Shulman R, Nichols BL. A carbon-13 breath test to characterize glucose absorption and utilization in children. *J Pediatr Gastroenterol Nutr* 1988: 7(6): 842-847.
4. Pacy PJ, Cheng KN, Thompson GN, Halliday D. Stable isotopes as tracers in clinical research. *Ann Nutr Metab* 1989: 33(2): 65-78.
5. Trowbridge FL, Graham GG, Wong WW, Mellits ED, Rabold JD, Lee LS, Cabrera MP, Klein PD. Body water measurements in premature and older infants using H218O isotopic determinations. *Pediatr Res* 1984: 18(6): 524-527.
6. Atherton JC, Spiller RC. The urea breath test for Helicobacter pylori. *Gut* 1994: 35(6): 723-725.
7. 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(2 Pt 1): 503-509.
8. Koletzko S, Haisch M, Seeboth I, Braden B, Hengels K, Koletzko B, Hering P. Isotope-selective non-dispersive infrared spectrometry for detection of Helicobacter pylori infection with 13C-urea breath test. *Lancet* 1995: 345(8955): 961-962.
9. 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(1): 98-101.
10. 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(3): 431-439.
11. 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(4): 1126-1134.
12. Watkins P. Testing for mitotic crossing over and induced aneuploidy using Aspergillus nidulans as part of the UKEMS test programme. *Mutat Res* 1982: 100(1-4): 133-138.
13. 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(7-8): 593-596.
14. 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(3): 289-294.
15. 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(2): 193-200.

16. 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(4): 998-1009.
17. 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(1): 77-85.
18. Bier DM, Leake RD, Haymond MW, Arnold KJ, Gruenke LD, Sperling MA, Kipnis DM. Measurement of "true" glucose production rates in infancy and childhood with 6,6-dideuteroglucose. *Diabetes* 1977: 26(11): 1016-1023.
19. Yudkoff M, Nissim I, McNellis W, Polin R. Albumin synthesis in premature infants: determination of turnover with [15N]glycine. *Pediatr Res* 1987: 21(1): 49-53.
20. Jacobs R, Demmelmair H, Rittler P, Kellermann J, Koletzko B, Krick M, Jauch KW, Hartl WH. 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(2): 145-151.
21. Biolo G, Agostini F, Simunic B, Sturma M, Torelli L, Preiser JC, Deby-Dupont G, Magni P, Strollo F, di Prampero P, Guarnieri G, Mekjavic IB, Pisot R, Narici MV. 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(4): 950-958.
22. 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(1): 31-36.
23. Jones PJ, Winthrop AL, Schoeller DA, Swyer PR, Smith J, Filler RM, Heim T. Validation of doubly labeled water for assessing energy expenditure in infants. *Pediatr Res* 1987: 21(3): 242-246.
24. 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(1): 348S-354S.
25. 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(3): 315-322.
26. 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(6590): 75-77.
27. 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(14): 5449-5453.
28. 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(1): 17-19.
29. Koletzko B, Demmelmair H, Hartl W, Kindermann A, Koletzko S, Sauerwald T, Szitanyi P. The use of stable isotope techniques for nutritional and metabolic research in paediatrics. *Early Hum Dev* 1998: 53 Suppl: S77-97.

30. Jones PJ, Leatherdale ST. Stable isotopes in clinical research: safety reaffirmed. *Clin Sci (Lond)* 1991: 80(4): 277-280.

Chapter Three

Aspiration pneumonia: effect of unilateral lung injury on surfactant disaturated phosphatidylcholine synthesis in mice

Aspiration pneumonia is the result of the abnormal entry of endogenous secretion or exogenous substances into the lower airways. In aspiration pneumonia there is a breakdown of the defences protecting the tracheobronchial tree as well as pulmonary complications.

There are three categories of pulmonary aspiration:

1- Chemical pneumonitis is caused by fluids like acid, kerosene, gasoline, milk, mineral oil, alcohol. This is independent from bacterial infection.
2- Oropharyngeal secretion aspiration results in bacterial infection of the lower airways by commensals of the oropharynx
3- Aspiration of inert fluids, like saline, water, barium that aren't toxic to the lung but that can cause airway obstruction

Table 1. Risk factors for aspiration-induced lung injury

Depressed level of consciousness
Sedation
Alcohol intoxication
Traumatic brain injury
Encephalopathy
Seizure disorder
Impaired/depressed gag reflex
Presence of nasogastric or endotracheal intubation
Bulbar paralysis
Gastrointestinal disorders
Esophageal motility disorders
Gastroesophageal reflux
Gastroparesis
Bowel obstruction/ileus
Drugs
Anticholinergics
Adrenergic agents
Nitrates
Phosphodiesterase inhibitors
Calcium channel blockers
Others
Obesity
Labor

There are some conditions that can predispose to the development of pulmonary aspiration, like altered consciousness, anatomic disorders, physiologic disorders, neurologic disorders, mechanical disruption of the normal defence barriers and accidental ingestion of substances (Table 1).

Aspiration of gastric contents is a severe complication seen in intensive care patients [1]. Acid

aspiration can contribute to acute respiratory distress syndrome (ARDS) or acute lung injury (ALI) and is associated with high morbidity/mortality rates.
Aspiration-associated ALI/ARDS carries a 30% mortality and accounts for up to 20% of all deaths attributable to anaesthesia [2]. Also, it can lead to bacterial pneumonia.
ALI/ARDS can lead to pulmonary inflammation, alveolar-capillary permeability injury, hypoxemia and protinaceous oedema with loss of lung compliance [3-4].
The main steps of AP-induced lung damage are an early marked alveoli's capillary injury due to the caustic effect of the acid. In fact, acid aspiration induces lung injury in a biphasic pattern. There is initially an increase in lung permeability without marked evidence of inflammation. The second and more important response is the presence of neutrophils in the lung and alveolar spaces [5]. In animal models it has been demonstrated that the adhesion and activation of neutrophils mediated by eicosanoids [6] and tumor necrosis factor (TNF).
TNF-alpha increases expression of endothelial cells adhesion molecules (e.g. intercellular adhesion molecule-1, E-selectin) that mediate the anchoring of neutrophils to endothelial cells.
After that, neutrophils migrate into the lung in response to a putative chemotactic gradient to release oxidants and proteases that mediate lung damage[7].
Alveolar-endothelial barrier damage and neutrophils infiltration cause alterations of surfactant homeostasis and, during the late inflammation stage, a delayed fibrotic lung tissue remodelling resulting in a lack or impaired functionality of the involved lung section.
In particular, disaturated phosphatidylcholine (DSPC) is the key component of the phospholipid film covering the alveoli for its uncommon characteristic molecular conformation that comprise two saturated residues (mainly palmitate residues) (Figure 1).

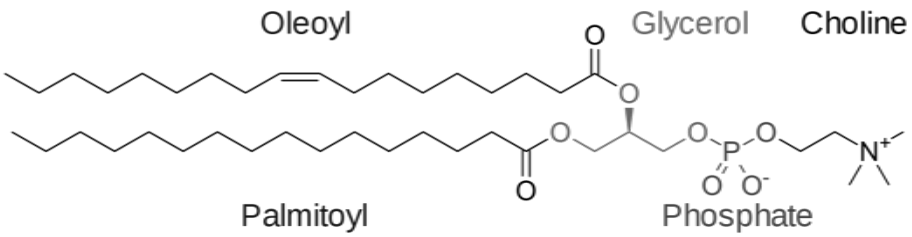

Figure 1. *Palmitoyl-oleyl-PC.* An example of a PC species. Saturated species (DSPC) contains two saturated residues. The most common saturated residue is the palmitate one.

This packed configuration avoids alveolar collapse during expiration and permits alveolar expansion during inspiration [8]. Type II cells are the only cells involved in the modulation of surfactant synthesis, secretion, uptake, and catabolism. Alveolar macrophages contribute to surfactant homeostasis mainly by catabolising surfactant components [9].

Alterations of the pulmonary surfactant system, in particular of the PL profile, have been confirmed in the course of ARDS, or less specifically, in lung diseases with pronounced alveolar inflammation (Figure 2)[10].

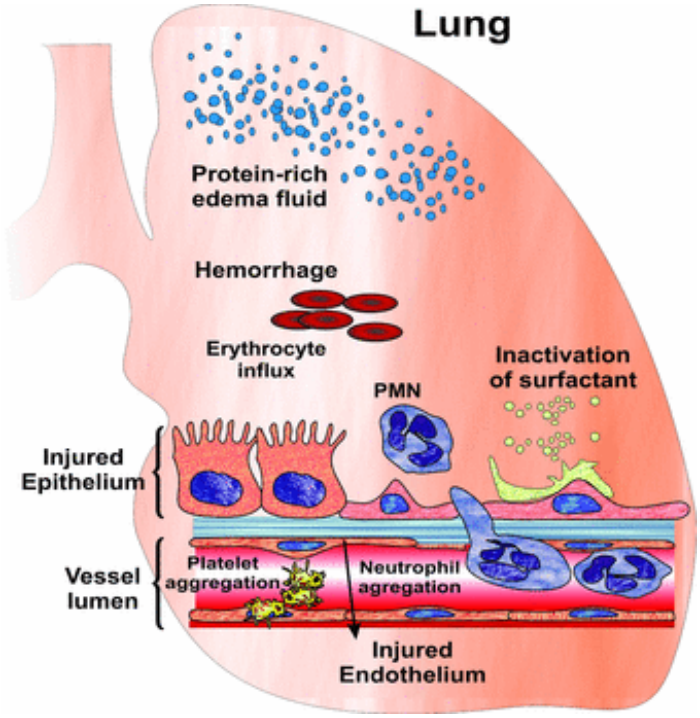

Figure 2: Mechanisms of lung injury in the early stage of ARDS.

To clarify the mechanisms of lung surfactant damage/recovery in the late phase of aspiration pneumonitis we used a formerly characterized murine model of AP [11], that includes an acid load instilled only in the right bronchus, and we applied the sensitivity of gas-chromatography isotope ratio mass spectrometry (GC-IRMS) to measure DSPC synthesis and secretion by means of stable isotopes (deuterated water as precursor of DSPC-palmitate biosynthesis) [12-14]. Lung inflammation was assessed by inflammation markers in both injured and non-injured lungs. We demonstrated that acid aspiration injury in a single lung triggers the contralateral non-injured lung to increase DSPC synthesis and pools to overcome a respiratory impairment occurring in the injured lung.

Materials and methods

Animal Protocol

CD-1 mice (male, 32 ± 2.2 g, Charles River Laboratories, Lecco, Italy) were maintained under standard laboratory conditions. Procedures involving animals and their care were in accordance with NIH's Guide for the Care and Use of Laboratory Animals 8th Edition. Local Ethic Committee approved study's protocol.

Animals with lung injury (n=20) were anaesthetized prior to any procedure with a 400 mg/kg intraperitoneal injection of 2.5% Tribromoethanol (Avertin®, Sigma-Aldrich, Milan, Italy). Lung injury was induced by instillation of 1.5 ml/kg HCl 0.1 M with a PE10 tube into the right bronchus through a small tracheal incision. The bronchial catheter was then removed and the tracheal incision sutured. During instillation and for the next 10 minutes, mice were mechanically ventilated (Inspira asv, Harvard Apparatus, Holliston, Massachusetts, USA with the following parameters: V_T 8-10 ml/kg; RR 130, PEEP 2 cmH_2O, FiO_2 1) and kept in a reverse Trendelenburg position (45°), tilted to the right side (45°) to confine the instilled fluid to the right lung. Treated animals were then placed in an oxygenated chamber (FiO_2 = 0.5) until full awake [11]. Eight healthy mice were

used as “controls”, so not treated but ventilated as treated mice. Therefore, the study included 4 groups of lungs: (1) Injured (I) right lung; (2) contralateral non-injured (NI) left lung; (3) control right lung; (4) control left lung.

Isotope Infusion Protocol

Previous unilateral injury studies from our group showed that inflammation in both lungs reached its maximum peak within 12 to 24 h from acid instillation [11, 15] , therefore the administration of deuterated water was performed after 18 h from the lung injury.

All study mice (n=28) received an intraperitoneal dose of 1 ml/kg 10% (v/v) of deuterated water (Cambridge Isotope Laboratories, Andover, MA) to assess DSPC-palmitate synthesis and secretion. Animals were sacrificed at 4, 8, 10, 12, 20 and 24 h after isotopes administration that corresponded to 22, 26, 28, 30, 38 and 42 h from HCl injury (Figure 3). Four animals were used for each time point. The age matched control animals did not receive any surgical manipulation but only the intraperitoneal dose of deuterated water and thereafter sacrificed at 4, 8, 10, 12, 20 and 24 hours after isotopes administration.

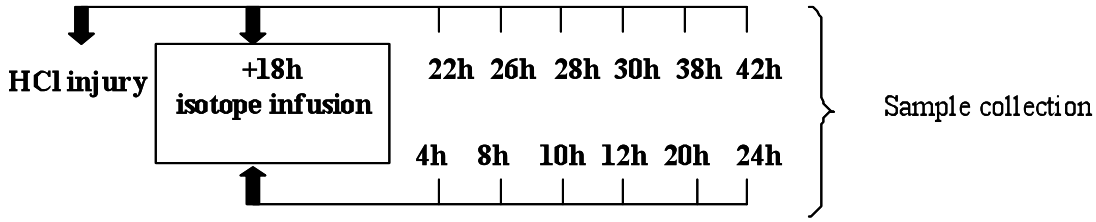

Figure 3. *Study Design.* Mice were anesthetized and an unilateral (right) HCl injury was performed. After 18 h, at the point of maximum inflammation, mice were injected with deuterated water as precursor of DSPC biosynthesis. Lung tissue and BALF were collected at subsequent time points.

Samples collection

After opening the chest and “proper injury” assessment (see Results Section for details), right and left lung were subjected to selective bronchoalveolar lavage (BAL) performed as follows: 0.6 ml of 0.9% NaCl was instilled in the right

main bronchus 3 times, the withdrawal fluid was recovered and pooled in a single aliquot; 0.4 ml of 0.9% NaCl was instilled 3 times in the left main bronchus to obtain the left bronchoalveolar lavage fluid (BALF). During the instillation procedure each lung was isolated with a clamp onto the opposite main bronchus to confine a lung during the lavage of the contralateral one. BALF was centrifuged 10 min at 1,500 x g to eliminate cells and cell debris. Supernatants were stored at –80°C until analysis. Lungs were dissected, weighed and separately homogenized in 0.9% NaCl with protease inhibitors (Complete Mini, Roche, Mannheim, Germany). The homogenate was then sonicated 3 times for 10 s at full speed (Labsonic 1510, B.Braun, Melsungen, Germany), centrifuged 10 min at 20,000 x g and the supernatant stored at –80°C until analysis.

Urine of each mouse was collected to assess body water deuterium enrichment (see below).

Total proteins and MPO assay

BALF and lung tissue homogenates total proteins were quantified by a spectrophotometric assay according to Lowry [16]. Myeloperoxydase (MPO) activity, as marker of neutrophil infiltration, was assayed in BALF and homogenates according to Bradley [17] modified as follows: BALF or homogenates were centrifuged for 40 min at 20,000 x g, supernatants were added to 2.9 ml of revealing buffer (50 mM KH_2PO_4, H_2O_2 30% and 0.168 mg/ml *o*-dianisidine hydrochloride). Absorbance was recorded for 4 min at 460 nm. Each sample was determined in duplicate [18].

DSPC isolation

DSPC was isolated from BALF and lung homogenates with a modification of the procedure described by Mason et al. [19] Three-hundred µl of sample were added with 10 µg of pentadecanoyl phosphatidylcholine 1 mg/ml (Sigma Aldrich, Milano, Italy) and lipids were extracted as described before [20]. The extracted lipid fraction was dried under nitrogen stream, combined with 500 µl of 10 mg/ml

osmium tetroxyde in carbon tetrachloride, and left for 8 hours at room temperature in the dark. Samples were then dried under nitrogen, dissolved in 100 µl of chloroform/methanol (2:1), spotted onto 20 x 20 cm silica gel plates (Silicagel 60, Merck KGaA, Darmstadt, Germany) and fractionated with chloroform/methanol/isopropanol/triethylamine/potassium chloride 0.5% in water (40/12/33.3/24/8).

Fatty acids methyl ester preparation

To perform GC analysis, DSPC was hydrolyzed and volatilized by methylation under acidic conditions[21-22] . After neutralization with potassium carbonate 10%, 200 µl of n-hexane were added to the neutralized solution, mixed for 10 min and centrifuged at 400 x g for 5 min. The upper n-hexane phase, containing esterified fatty acids, was transferred to a glass vial-insert and analysed.

Quantification of DSPC pools

DSPC amount was assessed as reported previously[22]. Briefly, fatty acid methyl esters were injected into a GC-FID system (HP 5890, Palo Alto, CA, USA) and their amounts were determined from the fatty acid peak-areas using the previously added internal standard. DSPC lung homogenate and BALF DSPC pools were expressed as umol/g wet lung weight. In addition, BALF pools were corrected for the estimated BALF recovery obtained as described below.

Estimate of BALF recovery

In order to estimate the recovery of DSPC from the airways, right and left lungs of 4 healthy mice that were not part of the study, were sequentially washed 3 times. Right and left BALFs were collected and the three lavages of the right and left lungs were analysed separately. The incremental recoveries of DSPC were plotted using the sample equation $y=A*\exp(Bx) + C$ and the asymptote of

the resulting curve was automatically calculated (LabView Software, National Instruments Corporation) to estimate DSPC recovery.

Measurement of deuterium enrichment

DSPC-palmitate methyl ester deuterium enrichments were measured by GC-IRMS (DELTA plus XL, Thermo Fisher Scientific, Rodano, Italy). The DSPC fatty acids were separated [23] and the $^{2}H/^{1}H$ ratios of the hydrogen gas obtained by quantitative pyrolysis were determined by the simultaneous integration of the m/z 2 ($^{1}H^{1}H$) and m/z 3 ($^{2}H^{1}H$) ion beams over time. The $^{2}H/^{1}H$ ratios were automatically corrected by the ISODAT 2.0 software for the H_3^+ contribution to m/z 3. Each sample was analysed in triplicate. A blank run with pure n-hexane was interspersed every six injections to insure absence of palmitate ghost peaks and to avoid memory effects [24].

Urine deuterium enrichment

Urinary deuterium enrichment, representative of the body water enrichment, was measured by a thermal conversion/elemental analysis (TC/EA, Thermo Fisher Scientific) device coupled with an IRMS [25]. Briefly 100 µl of urine were deproteinized with sulphosalicylic acid, cooled in an ice bath for 10 min, and centrifuged at 2,300 x g for 10 min. The supernatant was isolated, diluted 1:20 with distilled water of known isotopic enrichment and injected into the TC/EA device .

Kinetic calculations

Results were expressed as ð (delta) $^{2}H[‰]$ = (1000 ð x ($^{2}H/^{1}H$sample - $^{2}H/^{1}H$standard)/$^{2}H/^{1}H$standard). The δ ^{2}H represents the increase in isotopic enrichment above baseline of ^{2}H. Urine enrichments were normalized against Vienna Standard Mean Ocean Water (VSMOW, International Atomic Energy Agency, Vienna, Austria), which defines 0‰ as δ ^{2}H. Delta ^{2}H values of fatty

acids vs. VSMOW were obtained using a standard mixture of fatty acids with standard δ ^{2}H ("Mixture F8", kindly provided by Prof. Arndt Schimmelmann, Dept. of Geological Sciences, University of Indiana, Bloomington, IN, USA) [23]. The DSPC fractional synthesis rate (FSR) was obtained by dividing the slope of the linear enrichment increase of DSPC-palmitate by the urine enrichments multiplied by 64.8% under steady state conditions [26]. FSR represents the fraction of DSPC pool synthesized in one day from the isotope precursors. FSR was standardized per gram of the respective wet lung. The DSPC absolute synthetic rate (ASR) is the product of DSPC's FSR multiplied by the respective DSPC pool (lung homogenate or BALF) and it represents the amount of DSPC synthesized in one day in that pool, expressed as umol/g wet weight/day. Since DSPC is synthesized in the lung tissue and secreted into the alveolar space, for the purpose of this study we considered as indicator of DSPC's synthesis the ASR calculated in the lung homogenate, and as indicator of DSPC's secretion the ASR value calculated in the BALF fluid.

Statistical Analysis

Data were expressed as mean ± SD. Comparison between two groups were performed by Student t-test. Two-way analysis of variance (ANOVA) was used to evaluate the effects of HCl injury, time, and their interaction on the continuous variables. One-way ANOVA with the Dunnett's multiple comparison test was used to compare I and NI lungs with the respective control lungs at each time point. P values of less than 0.05 were considered statistically significant. Analysis was performed by Prism 4.0 (GraphPad Software Inc. La Jolla, CA, USA).

Results

Animal Protocol

Selectivity of the injury was assessed by lung macroscopic examination during the autopsy. Animals with bilateral or left injury were excluded. We previously confirmed the macroscopic view showed that the majority of the

histopathologic changes described affected the right caudal pulmonary lobe [11]. In order to verify the selectivity of acid instillation and to exclude a relevant contamination of the left lung, we performed preliminary experiments (unpublished data, under review) in which mice were treated with a bolus of 1.5 ml/kg of methylene blue administered in the same way used for HCl instillation, and mechanically ventilated reverse in a Trendelenburg position (45°), tilted to the right side. We macroscopically confirmed a confinement of the instilled fluid in the right lung (principally, in the caudal lobe) while neither the parenchyma, the hilum or the main right bronchus had signs of the blue dye (data not shown).

Estimate of BALF recovery from the lungs

Three sequential BALF's from 4 different mice were recovered and analysed separately for both the right and the left lung. The asymptote of the resulting curve was calculated with the sample equation y=A*exp (Bx) + C (Figure 4). We estimated a 100% recovery in 0.233 mg of DSPC from the right lungs and of 0.220 mg of DSPC mg from the left lungs. The mean DSPC amount in our (pooled) right BALF was 0.189 ± 0.05 mg, corresponding to 81% recovery of total DSPC after 3 lavages. The mean DSPC pool size for the left lung was 0.176 ± 0.04 mg, corresponding to a 80% recovery.

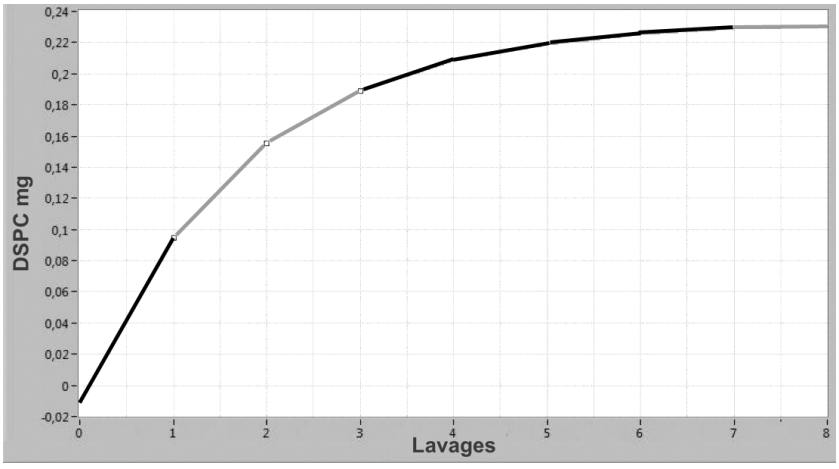

Figure 4. *Asymptotic calculation of BALF lavages DSPC recovery.* Three serial BALF were collected from the right and left lungs of 4 control mice and analyzed separately. DSPC amounts were plotted against the BALF serial number and the total amount of DSPC extrapolated from an exponential curve fitting. The figure shows the representative plot of the right lungs experiment.

Total Proteins and Myeloperoxidase activity

Total protein content in the I and NI lung is represented in figure 3. Total BALF proteins were significantly increased by HCl injury ($p=0.002$) and by the time from the study start ($p=0.002$) (both lungs, two ways ANOVA). (Figure 5a). In the lung homogenate the total protein content was affected only by HCl injury ($p<0.0001$) (Figure 5b). Moreover, mean BALF protein content of all pooled time points increased 3 times in I lungs ($p=0.004$) and 2 times in the NI lungs ($p=0.02$), compared with control lungs by Student's t-test (Figure 5c).

BALF MPO activity was significantly increased in both I and the NI lungs over time ($p=0.0004$, two ways ANOVA) and tended to be more pronounced in the I lung compared with the NI lungs (I = 45.61 ± 17.9 mU/g wet weight, NI= 34.82 ± 9.4 mU/g wet weight, $p=0.07$ by Student's t-test). In the lung homogenate we found no difference of MPO activity between I and NI lungs over time. Mean BALF MPO activity was significantly increased only in the I lungs compared with controls (4.7 ± 1.6 mU/g wet weight; $p=0.04$ by Student's t-test) . MPO activity in BALF was significantly increased at t26 and t28 in the I lungs compared to controls ($p<0,01$ in both case by Dunnett's multiple comparison test, data not shown), while in the NI it was increased only at t26 ($p<0,05$, Dunnett's multiple comparison test, data not shown). No differences were found for the lung homogenates.

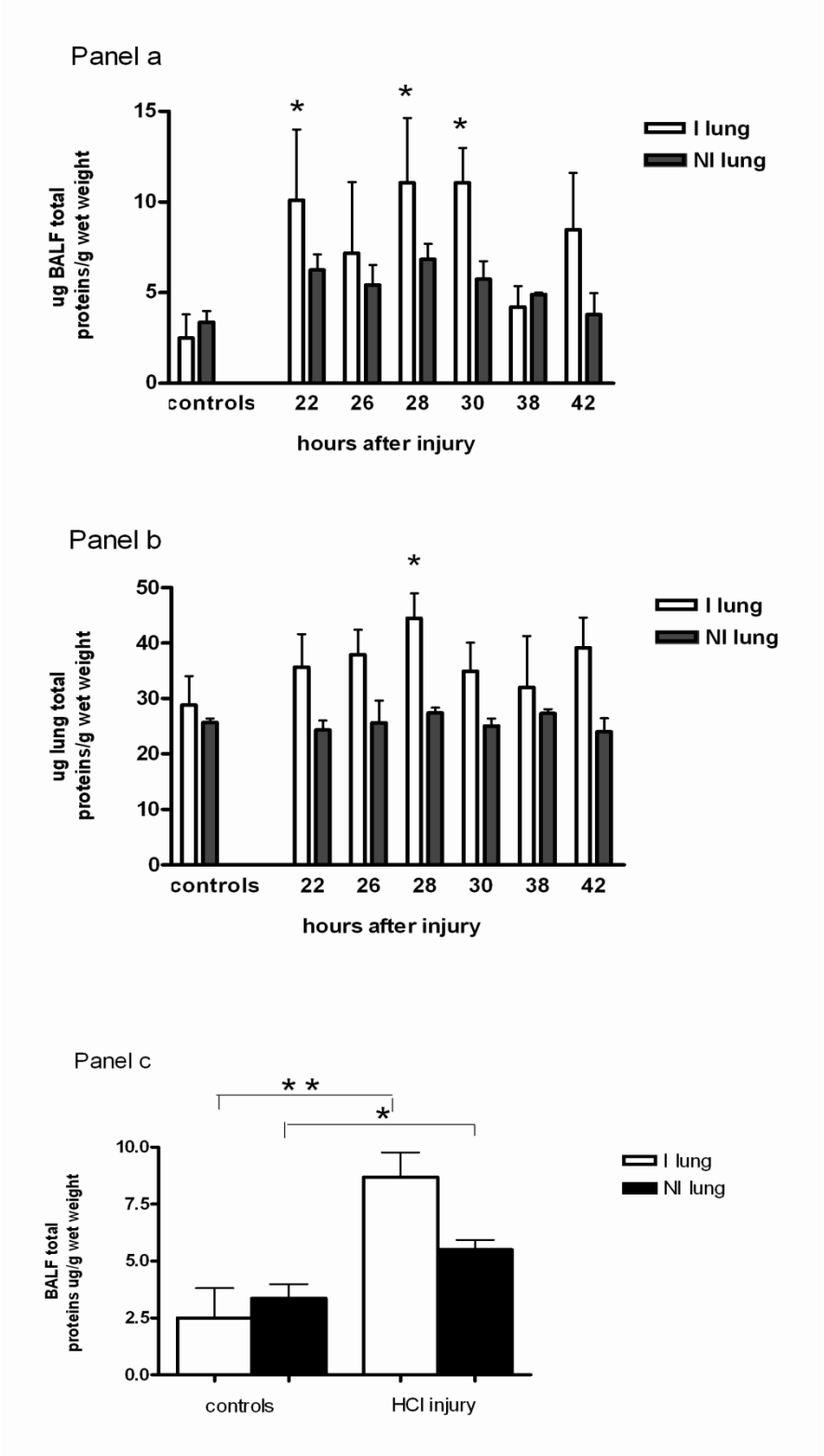

Figure 5. *Determination of total protein content in BALF* **(Panel a)** *and lung homogenate* **(Panel b)** *of injured (I) lungs compared to the respective not injured (NI) lungs.* Time points 22, 26, 28, 30, 38, 42 h from acid instillation. Data are expressed as means ± SD, n=4 mice/time point. *$P<0.05$ compared with control (Dunnett's multiple comparison test). **Panel c** shows the BALF group mean ± SD of all I and NI lung total proteins. Differences were statistically significant (p=0.004 and p=0.02, respectively, by Student's t-test).

DSPC pool size

BALF DSPC pool size was significantly decreased in the I lungs by HCl injury compared to NI lungs (p=0.008, Student's t-test) and tended to be affected by time from the beginning of the study (p=0.056) by two ways ANOVA. Only the I BALF DSPC pool size was significantly decreased compared with controls (p=0.02) by Student's t-test. BALF DSPC of the I lungs was significantly decreased at t28 an t30, p<0,05 and p<0,01 respectively by Dunnett's multiple comparison test (Figure 6).

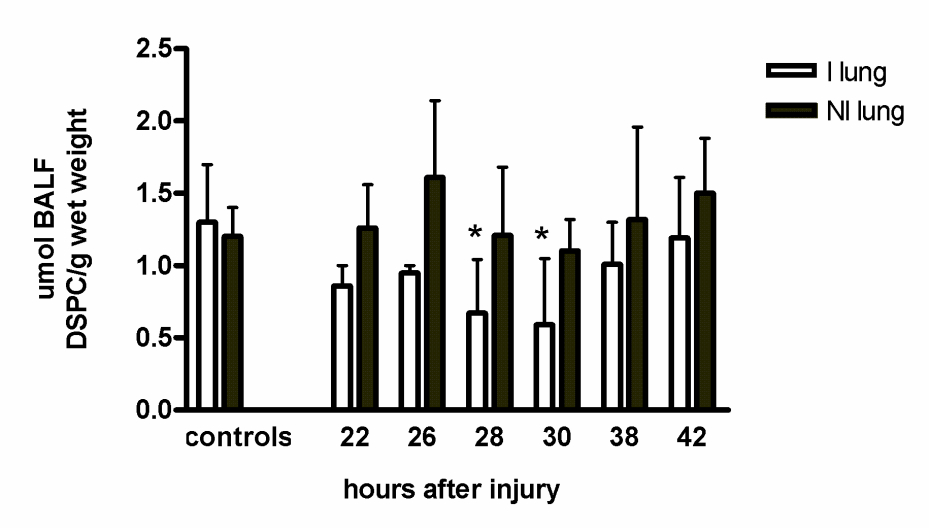

Figure 6: *Pool size of DSPC*. DSPC concentration (I and NI lung) extracted from BALF of I group compared to controls at 22, 26, 28, 30, 38, 42 hours from acid instillation in the right main bronchus. The data are expressed as means ± SD, n=4 mice/time point. BALFs DSPC from I lungs were significantly decreased compared with the NI contralateral lungs (p=0.008, Student's t-test) and tended to be affected also by time (p=0.056) by two ways ANOVA. They were also significantly decreased compared with right control lungs (p=0.02, Student's t-test). *$P<0.05$ compared with control (Dunnett's multiple comparison test).

Mean DSPC lung homogenate pool sizes did not differ between the I and NI lungs and they were also not different from their respective control lungs (Table 2).

	Right homogenates		Left homogenates		Right BALF		Left BALF	
	Controls	**HCl model (I)**	**Controls**	**HCl model (NI)**	**Controls**	**HCl model (I)**	**Controls**	**HCl model (NI)**
FSR (%synthesis/day)	16.3	23	13.1	26.7	23.8	33.2	23.7	36
ASR (umol/g wet weight/day)	36	42.1	27.1	45.8	30.9	29.2	28.5	46.8
DSPC pool size (umol/g wet weight)	2.2±0.7	1.8±0.3	2.1±0.6	1.7±0.5	1.3±0.4	0.9±0.3	1.2±0.2	1.4±0.4

Table 2. *Kinetics parameters and mean pool size of BALF and lung homogenates from HCL injured and control mice).* Eighteen hours after injury mice received an intraperitoneal dose of deuterated water 10% v/v, 1 ml/kg. Samples were collected at 4, 8, 10, 12, 20, 24 h after isotope dose, that corresponded to 22, 26, 28, 30, 38, 42 h from acid instillation. FSR was calculated using the slope of the enrichment versus time curves of surfactant DSPC-palmitate. ASR was calculated by multiplying FSR by DSPC pool size. I= acid injured lungs; NI= contralateral, non injured lungs. N=4 mice/time point.

DSPC-palmitate synthesis

Deuterium urinary enrichment reached the plateau after 8 hours from the injection and it was maintained during the entire study (data not shown).

DSPC synthesis (lung homogenate ASR) and secretion (BALF ASR) of I, NI and control lungs are reported in Table 1. In lung homogenate DSPC synthesis was respectively 14.3% and 40.8% faster in I and NI lungs compared with the respective control lungs.

In BALF, DSPC secretion of the I lungs was similar to the controls, whereas that of the NI lungs was 37.6% faster than the respective control lungs. The NI lungs synthesized 64.3% more mg/g wet lung/day of DSPC than the I lungs.

The specificity of this synthesis is shown on fig.5, where controls had a lower rate of synthesis compared to BAL and lung of the HCl model.

Discussion

In this study we used body water-derived deuterium incorporation into lipids to trace the synthesis of the most important sub-family of surfactant phospholipids, DSPCs, in a well-characterized model of a unilateral acid-induced lung injury[11]. We wanted to assess if a localized injury influences surfactant regulation only in the afflicted (confined) lung space, or if a regional damage could influence the whole pulmonary surfactant system. In this model, selectivity of the injury was assessed by lung macroscopic examination during the autopsy. Animals with bilateral or left injury were excluded. In almost all animals the injury was confined in the lower lobe bronchus, confirming the goodness of the model.

According to a previous study [11] control mice and NaCl 0.9% (vehicle) treated mice did not show a significant difference in the inflammatory status; hence we used healthy mice as controls for comparison. Local lung injury and endothelial dysfunction were assessed by MPO activity and total protein. MPO tend to be higher in the injured lungs but it's substantially unvaried between injured and not injured lungs both in BALF and lung tissue. Proteins are significantly higher in BALF of I lungs confirming an alveolar tissue damage with subsequent plasma protein leaking. Moreover, proteins tend to be higher also in NI lungs as an effect on alveolar capillary permeability by inflammatory mediators released into the blood stream after the HCl harm in the contralateral lung. The inflammatory status is in accordance with our previously reported description of the model[11].

We measured the synthesis of lung surfactant DSPC-palmitate using a well-established protocol involving stable isotope technique, successfully used also to trace surfactant's components regulation in humans [18, 20]

In lung homogenate DSPC-palmitate synthesis was increased by 14.3% in the I lungs and by 40.8% in the NI lungs compared with control mice (Table 1). The NI lungs DSPC synthesis was remarkably faster compared with the respective I lungs, suggesting a compensatory mechanism occurring in the lung parenchyma contralateral to the injury. A mechanism of systemic and/or organ-mediated inflammatory stimuli originated in I lungs and delivered to NI lungs could trigger an increased DSPC synthesis in NI lungs even if these lungs were unlikely to be

directly involved in the damage. Another explanation could be that inflammatory milieu in damaged lungs is different from the NI lungs. MPO and protein data did not reflect a neutrophil and alveolar permeability difference between I and NI inflammatory state, but some other unidentified factors could inhibit DSPC production and/or enhance DSPC catabolism. One of these could be type II pneumocytes damage that inhibit DSPC synthesis as a consequence of a direct disruption of the only one cell type able to synthesize surfactant. In this scenario a clue is that DSPC absolute quantity is less in I lungs BALF than in all of the other lungs BALF and tissue (DSPC pool size 30% lower than the control values, $p=0.02$, and 36% lower than the respective NI value, $p=0.008$). Moreover a recent study by our research group demonstrated that in ARDS patients, tracheal aspirate DSPC was markedly reduced compared to control patients, its FSR was 3.1 times higher than that of controls [14] attesting that in critical ill patients, a compensatory mechanism is present but inflammation could prevent it to be successful. Finally, hydrolysis of DSPC by phospholipase A2 (PLA2s) released by inflammatory cells requires millimolar calcium concentrations which are reached in the alveolar space during inflammation subsequent to ARDS. In these conditions, the enzyme becomes fully active outside the cellular compartment.[27-28].

In lung homogenates we did not find any difference in DSPC pool size among I and NI lungs as compared with controls. This supports the assumption that the surfactant catabolism is more pronounced in the alveolar compartment where alveolar macrophages and neutrophils play a major role in surfactant catabolism. Shanley et al. demonstrated that macrophage inflammatory protein (MIP-2) was up-regulated in aspiration-induced lung injury in rats, leading to an accumulation of neutrophils via a chemotactic mechanism [7]. A murine model of granulocyte-macrophage colony stimulating factor (GM-CSF) lung deficiency, demonstrated that an increase in pool size of GM-CSF(-/-) mice was due to a lack of surfactant catabolism by alveolar macrophages [29].

It is well established that surfactant DSPC secretion to the alveolar space needs a lipid intracellular transport protein, (ATP-binding cassette A3, ABCA3) and the surfactant specific protein SP-B to aggregate and excrete surfactant by exocytosis.

Recent studies in mice receiving intraperitoneal lipopolisaccaride showed that STAT-3 (signal transducer and activator of transcription-3), activated by members of the IL-6 like group of pro-inflammatory cytokines, increased DSPC and SP-B concentration in lungs and in the alveolar space by a STAT-3 dependent pathway [30]. This induction of surfactant homeostasis improved lung functionality and recovery. In addition, maintenance of surfactant function in ABCA3-deleted mice after birth was associated with compensatory lipid synthesis in non-targeted type II cells, indicating that surfactant homeostasis is a highly regulated process that includes sensing and co-regulation among alveolar type II cells [31].

In summary, combining the efficacy of a well-characterized model of lung injury and the sensibility of stable isotopes/GC-IRMS techniques we showed that after a local injury the entire lung system is involved in responding to the damage , by activating the immune system and the compensatory mechanisms of surfactant synthesis. We speculate that surfactant catabolism and synthesis are two interdependent pathways, and that it is likely that synthesis undergoes a "whole organ" regulation while inflammation/catabolism has its climax locally that gradually decreases away from the injury.

Future studies are needed to assess the behaviour of other surfactant components like specific proteins and changes in the ventilation parameters of a single lung.

References

1. Nemzek JA, Call DR, Ebong SJ, Newcomb DE, Bolgos GL, Remick DG. Immunopathology of a two-hit murine model of acid aspiration lung injury. *Am J Physiol Lung Cell Mol Physiol* 2000: 278(3): L512-520.
2. Marik PE. Aspiration pneumonitis and aspiration pneumonia. *N Engl J Med* 2001: 344(9): 665-671.
3. Kudoh I, Miyazaki H, Ohara M, Fukushima J, Tazawa T, Yamada H. Activation of alveolar macrophages in acid-injured lung in rats: different effects of pentoxifylline on tumor necrosis factor-alpha and nitric oxide production. *Crit Care Med* 2001: 29(8): 1621-1625.
4. Lienhart A, Auroy Y, Pequignot F, Benhamou D, Warszawski J, Bovet M, Jougla E. Survey of anesthesia-related mortality in France. *Anesthesiology* 2006: 105(6): 1087-1097.
5. Kennedy TP, Johnson KJ, Kunkel RG, Ward PA, Knight PR, Finch JS. Acute acid aspiration lung injury in the rat: biphasic pathogenesis. *Anesth Analg* 1989: 69(1): 87-92.
6. Goldman G, Welbourn R, Klausner JM, Paterson IS, Kobzik L, Valeri CR, Shepro D, Hechtman HB. Ischemia activates neutrophils but inhibits their local and remote diapedesis. *Ann Surg* 1990: 211(2): 196-201.
7. Shanley TP, Davidson BA, Nader ND, Bless N, Vasi N, Ward PA, Johnson KJ, Knight PR. Role of macrophage inflammatory protein-2 in aspiration-induced lung injury. *Crit Care Med* 2000: 28(7): 2437-2444.
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(2): 95-101.
9. 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(3): L468-476.
10. Schmidt R, Meier U, Yabut-Perez M, Walmrath D, Grimminger F, Seeger W, Gunther A. Alteration of fatty acid profiles in different pulmonary surfactant phospholipids in acute respiratory distress syndrome and severe pneumonia. *Am J Respir Crit Care Med* 2001: 163(1): 95-100.

11. Amigoni M, Bellani G, Scanziani M, Masson S, Bertoli E, Radaelli E, Patroniti N, Di Lelio A, Pesenti A, Latini R. Lung injury and recovery in a murine model of unilateral acid aspiration: functional, biochemical, and morphologic characterization. *Anesthesiology* 2008: 108(6): 1037-1046.

12. 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(1): 323-329.

13. Cogo PE, Simonato M, Danhaive O, Verlato G, Cobellis G, Savignoni F, Peca D, Baritussio A, Carnielli VP. Impaired surfactant protein b synthesis in infants with congenital diaphragmatic hernia. *Eur Respir J* 2012.

14. Simonato M, Baritussio A, Ori C, Vedovelli L, Rossi S, Dalla Massara L, Rizzi S, Carnielli VP, Cogo PE. Disaturated-phosphatidylcholine and surfactant protein-B turnover in human acute lung injury and in control patients. *Respir Res* 2011: 12: 36.

15. Zambelli V, Di Grigoli G, Scanziani M, Valtorta S, Amigoni M, Belloli S, Messa C, Pesenti A, Fazio F, Bellani G, Moresco RM. Time course of metabolic activity and cellular infiltration in a murine model of acid-induced lung injury. *Intensive Care Med* 2012: 38(4): 694-701.

16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951: 193(1): 265-275.

17. Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* 1982: 78(3): 206-209.

18. Cogo PE, Simonato M, Mariatoffolo G, Stefanutti G, Chierici M, Cobelli C, Ori C, Carnielli VP. Dexamethasone therapy in preterm infants developing bronchopulmonary dysplasia: effect on pulmonary surfactant disaturated-phosphatidylcholine kinetics. *Pediatr Res* 2008: 63(4): 433-437.

19. Mason RJ, Nellenbogen J, Clements JA. Isolation of disaturated phosphatidylcholine with osmium tetroxide. *J Lipid Res* 1976: 17(3): 281-284.

20. Cogo PE, Carnielli VP, Bunt JE, Badon T, Giordano G, Zacchello F, Sauer PJ, Zimmermann LJ. Endogenous surfactant metabolism in critically ill infants

measured with stable isotope labeled fatty acids. *Pediatr Res* 1999: 45(2): 242-246.

21. Torresin M, Zimmermann LJ, Cogo PE, Cavicchioli P, Badon T, Giordano G, Zacchello F, Sauer PJ, Carnielli VP. Exogenous surfactant kinetics in infant respiratory distress syndrome: A novel method with stable isotopes. *Am J Respir Crit Care Med* 2000: 161(5): 1584-1589.

22. Carnielli VP, Pederzini F, Vittorangeli R, Luijendijk IH, Boomaars WE, Pedrotti D, Sauer PJ. Plasma and red blood cell fatty acid of very low birth weight infants fed exclusively with expressed preterm human milk. *Pediatr Res* 1996: 39(4 Pt 1): 671-679.

23. Vedovelli L, Baritussio A, Carnielli VP, Simonato M, Giusti P, Cogo PE. Simultaneous measurement of phosphatidylglycerol and disaturated-phosphatidylcholine palmitate kinetics from alveolar surfactant. Study in infants with stable isotope tracer, coupled with isotope ratio mass spectrometry. *J Mass Spectrom* 2011: 46(10): 986-992.

24. Cogo P, Baritussio A, Rosso F, Gucciardi A, Moretti V, Badon T, Duner E, Zimmernann L, Carnielli VP. Surfactant-associated protein B kinetics in vivo in newborn infants by stable isotopes. *Pediatr Res* 2005: 57(4): 519-522.

25. Gucciardi A, Cogo PE, Traldi U, Eaton S, Darch T, Simonato M, Ori C, Carnielli VP. 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(13): 2097-2103.

26. Wadke M, Brunengraber H, Lowenstein JM, Dolhun JJ, Arsenault GP. Fatty acid synthesis by liver perfused with deuterated and tritiated water. *Biochemistry* 1973: 12(14): 2619-2624.

27. Arbibe L, Koumanov K, Vial D, Rougeot C, Faure G, Havet N, Longacre S, Vargaftig BB, Bereziat G, Voelker DR, Wolf C, Touqui L. Generation of lyso-phospholipids from surfactant in acute lung injury is mediated by type-II phospholipase A2 and inhibited by a direct surfactant protein A-phospholipase A2 protein interaction. *J Clin Invest* 1998: 102(6): 1152-1160.

28. Arbibe L, Vial D, Rosinski-Chupin I, Havet N, Huerre M, Vargaftig BB, Touqui L. Endotoxin induces expression of type II phospholipase A2 in

macrophages during acute lung injury in guinea pigs: involvement of TNF-alpha in lipopolysaccharide-induced type II phospholipase A2 synthesis. *J Immunol* 1997: 159(1): 391-400.

29. Ikegami M, Jobe AH, Huffman Reed JA, Whitsett JA. Surfactant metabolic consequences of overexpression of GM-CSF in the epithelium of GM-CSF-deficient mice. *Am J Physiol* 1997: 273(4 Pt 1): L709-714.

30. Ikegami M, Falcone A, Whitsett JA. STAT-3 regulates surfactant phospholipid homeostasis in normal lung and during endotoxin-mediated lung injury. *J Appl Physiol* 2008: 104(6): 1753-1760.

31. Besnard V, Matsuzaki Y, Clark J, Xu Y, Wert SE, Ikegami M, Stahlman MT, Weaver TE, Hunt AN, Postle AD, Whitsett JA. Conditional deletion of Abca3 in alveolar type II cells alters surfactant homeostasis in newborn and adult mice. *Am J Physiol Lung Cell Mol Physiol* 2010: 298(5): L646-659.

Chapter Four

Synthesis of surfactant protein B and C *in vivo* by stable isotopes

The most important role of pulmonary surfactant is to lower surface tension in the alveoli and to stabilize these structures to prevent alveolar collapse[1]. Surface tension is reduced by a phospholipid (PL) film that is stable during alveolar expansion and compression associated with breathing.

This film is composed mainly by phosphatidylcholine, and phosphatidylglicerol, two PLs that at body temperature have a rigid conformation. On the other hand, the surfactant film on the alveolar surface has to assume a rapid formation and decompression of the PL film. This property is mainly due to the presence of two hydrophobic proteins, surfactant protein B (SP-B) and surfactant protein C (SP-C).[2]. No information is available on SP-C synthesis in humans

Our lab has previously described a method to measure SP-B kinetics in vivo in humans [3]. This work presents a optimized method to measure surfactant protein B kinetics and a novel method to measure surfactant protein C kinetics.

Surfactant protein B

Surfactant protein B is a 18 kDa hydrophobic peptide composed of 79 amino acid (Figure 1) [4], expressed in Clara cells and alveolar type II cells.

SP-B is encoded by a single 9.5-kb gene on human chromosome 2. The SP-B cDNA is highly conserved, sharing 70% homology in human, rabbit, and murine species at the amino acid level.

Human SP-B mRNA is translated into a 40 kDa preproprotein, that is cleaved to a 42-kDa proprotein. This proprotein is then cleaved at the amino terminus by cathepsin D or a cathepsin D-like protease, resulting in a 25-kDa intermediate protein. At the end, the mature, active peptide is obtained by removing the carboxyl-terminal peptide [5].

Figure 1: Structure of pro SP-B and SP-B

Surfactant protein B peptide and mRNA are detected as early as 14- to 15-wk gestation in the human fetal lung, and they are localized in epithelial cells of bronchi and bronchioles.

After 25-wk, the active peptide is only detected in alveolar type II cells, in the alveolus and in alveolar macrophages. Pro-SP-B is detected in bronchiolar and alveolar cells, suggesting that distinct processing of SP-B occurs in proximal versus distal airway cells [6].

Surfactant protein B has several functions in lung surfactant system, the most important one is its ability in enhancing the rate of adsorption and surface spreading of the phospholipids.

Surfactant protein B interacts primarily with the head group region of the lipid bilayer. The positively charged amino acid residues of SP-B interact at the surface of surfactant lipids – together with S-PC - forming stable monolayers and bilayers that reduce surface tension and enhance the stability and spreading of the lipid film (Figure 2) [2].

SP-B can interact with lipid vesicles, generating phospholipids sheets necessary for the formation of lamellar bodies.

SP-B has also an important role in the extracellular environment, playing a critical part in surfactant homeostasis. In fact, it can promote the adsorption of lipid molecules into the expanding surface sheet and enhance the stability of the film during the respiratory cycle [7].

A study in mice by Clark et al. lacking of the gene encoding for SP-B demonstrated that this deletion resulted in respiratory failure immediately after birth, although the composition of surfactant phospholipids was normal. Epithelial cells contained, instead of lamellar bodies, aberrant multivesicular bodies with small-accumulated lipid vesicles. It was demonstrated the importance of surfactant protein B in the packaging of surfactant phospholipids into lamellar bodies [8]. SP-B also contributes to recycling of surfactant from the alveolar space, enhancing the uptake of phospholipids by type II epithelial cells.

In addition, a deficiency of surfactant protein B interrupts the processing of surfactant protein C precursor, with the formation of an abnormal fragment of 35 amino acids plus an amino-terminal extension [9].

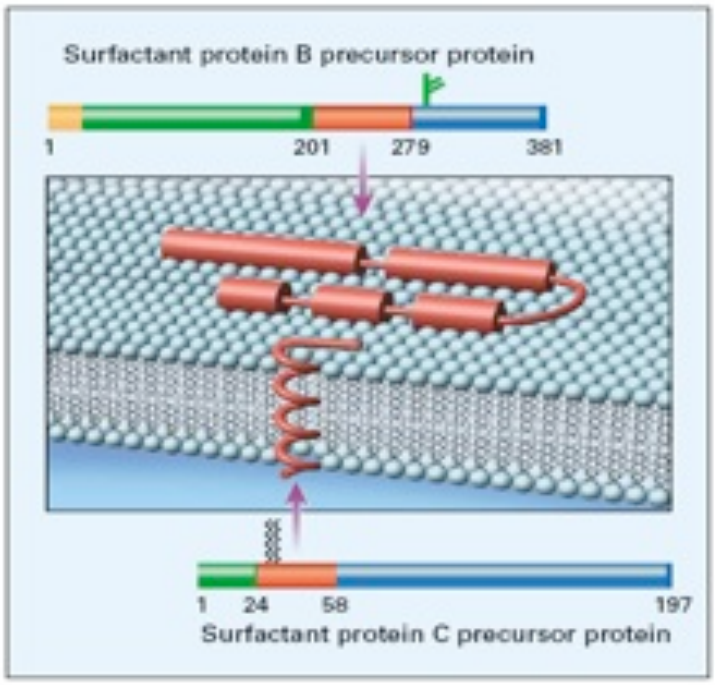

Figure 2: disposition of surfactant protein B and C within the surfactant bilayer

Surfactant protein c

Surfactant protein C is one of the most hydrophobic proteins in the proteome, accounting for approximately 4 percent of surfactant by weight. It is a 4.2 kDa lipopeptide consisting of a 35 amino acid polypeptide rich in valine, leucine and isoleucine (figure 3). The central hydrophobic domain of SP-C is configured as a trans-membrane spanning alpha elix with covalent attachment of palmitoyl groups at two adjacent amino- terminal cysteine residues.

It is expressed only in alveolar type II cells, and its mature form is produced from a multistep cleavage of C-terminal and N-terminal regions of a larger 21 kDa precursor called pro-SPC [10]. This precursor is encoded by a six exons, 2.4 kb gene (SFTPC), located on the short arm of the chromosome 8.

Figure 3: Structure of pro SP-C and SP-C

Surfactant protein C is the only known protein synthesized as an integral transmembrane precursor protein and then secreted as a luminal peptide. SP-C has an important role in the surfactant system, and most of its properties are strictly linked to its extreme idrophobicity. SP-C, inserted in the PL film, disrupts lipid packing promoting lipids movements between sheets of membranes [11].

SP-C isolated from pulmonary surfactant is not a unique molecule, but it is composed of a mixture of isoforms, where one is quantitatively the most present in vivo. The number of known modifications appears to increase in parallel with

increases analytical sensitivity, and probably additional SP-C forms will be discovered in the future. At the state of the art, we know that the major form of SP-C is presumably the biologically active molecule.

It is anyway important to discover the minor isoforms structure, to better understand the proteolytic formation of SP-C, its metabolism and the relationship between structure and activity.

The human, porcine and rabbit SP-C polypeptide chain contains 35 amino acids residues, but canine and bovine SP-C are one residue shorter, and this is the result of a different extents of N-terminal truncation. SP-C is post-translationally modified by addition of one (canine and mink) or two (human, porcine, rabbit) palmitoyl groups via thioester bonds. Increased amounts of non- and monopalmitoylated SP-C are found in human alveolar proteinosis surfactant [12] .

It was shown by FTIR spectroscopy that SP-C is oriented in a transmembrane way in dipalmitoylphopsphatidylcholine –phophatidylglycerol bilayers [12]. The exact localization of SP-C in pulmonary surfactant remains an open question, and from the available data, it is possible that the peptide is incorporated in both PL bilayers and monolayers.

SP-C and inflammation

Inflammatory signals can influence SP-C expression, as it has been well demonstrated in animals model of lung injury. To simulate respiratory distress and fibrosis, rats were instilled with bleomycin and Savani et al. found a significant decrease in both SP-C transcription and protein expression levels [13]. Another study by Matthew et al. demonstrated that hyperoxia, which causes accumulation of inflammatory mediators within lung, reduces SP-C expression [14]. Also, in a murine model of Aspergillus fumigatus SP-C expression was downregulate [15].

The intratracheal administration of TNF-α in adult mice downregulates SP-C mRNA, and a *in vitro* study in murine cells showed that TNF- α inhibits SP-C transcription [16]. Chaby et al. demonstrated that the mature form of SP-C can interact with LPS, while the immature form of SP-C does not.

Also, analysis of binding inhibition with synthetic analogs of lipid A, indicate an important role in contrasting LPS for the alpha configuration of the terminal phosphate group [10].

When associated with vesicles of dipalmitoylphosphatidylcholine, SP-C inhibits the binding of radiolabeled LPS to a macrophage cell line (RAW 264.7); also, it demonstrated to inhibit TNF-α production stimulated by LPS. Furthermore LPS-binding capacity of SP-C is resistant to peroxynitrite, a mediator of acute lung injury formed by reaction of nitric oxide with superoxide anions.

SP-C can also interact with CD-14, a receptor on the surface of phagocytes that can recognize LPS [17]. In fact, SP-C can enhance the binding between CD-14 and LPS modifying the conformation of CD-14. Also SP-A can improve this binding in the same way.

These data suggest that SP-C can contribute to the host defence, together with SP-A and SP-D; also, the binding with CD-14 demonstrates the role of SP-C in innate immunity is not limited to gram negative bacteria, but also to other pathogenic microorganisms.

SP-C: lung development and genetics

SP-C expression is initiated early in the embryonic period of lung formation, and the protein's transcripts are detected in epithelial cells lining the developing airways. SP-C expression decreased in cells of the proximal conducting portion of the lung, and maintained in the periphery of the developing respiratory tubules as the branching tubules elongate. Finally, alveoli are formed from respiratory tubules and SP-C mRNA is detected only in alveolar type II cells of the mature lung.

To understand the role of surfactant protein C, Glasser et al. inactivated SFTPC in embryonic stem cells to obtain mice lacking SP-C [18]. These mice demonstrated to have the same content of surfactant lipids and proteins, but the physical and mechanics properties were affected.

There is an association between interstitial lung diseases (ILDs) in newborn infants and adults and mutation in the *SFTPC* gene.

The ILDs are a heterogeneous collection of over 100 different diseases, characterized by pulmonary fibrosis. Idiopathic pulmonary fibrosis (IPF) is the prototypic ILD [19]. Nogee et al. reported a mutation located in the intronic region of *SFTPC* with ILD presentation in both a full term infants and mother. This mutation caused the production of a truncated precursor protein, with absence of detectable mature SP-C [20].

Thomas et colleagues studied a family in 5 generations that included 11 individuals with pulmonary fibrosis and 3 affected with nonspecific interstitial pneumonia. (NSIP). People were from 4 months to 57 years old. A mutation consisting in the change of leucine in position 188 to a glutamine, was present in all affected members. They also found an abnormal staining of SP-C in the lung tissue of the affected people and abnormal lamellar bodies in the alveolar type II cells [21].

SP-C mutations can disrupt ATII cells function, and it can be an etiological basis for the development of ILD. Abnormalities in ATII cells caused by the *SFTPC* gene mutations don't create a significant change at alveolar level, also during an acute lung injury. However, a prolonged alveolar insult can enhance a condition of altered metabolism and abnormal secretion by these cells. In addition to a lack of SP-C, extracellular matrix disturbances can lead to epithelial cell disorder, apoptosis, fibrosis, that can deeply change the alveolar environment [11].

There is also a strong relationship between SP-B deficiency and decreased concentration of mature SP-C and accumulation of a larger inactive SP-C derived peptide, called $SP\text{-}C_i$, which is not observed under normal condition. This peptide has a poor ability to promote PL absorption, and doesn't bind LPS *in vitro*. This, in combination with the almost complete absence of mature SP-C, implies that SP-B-deficient children lack active forms of both membrane-associated surfactant protein [22].

Material and methods

Adult patients were admitted to the department of Anaesthesia and Critical Care, University of Padova, Italy. Children were admitted in Neonatal Intensive Care Unit of the Department of Pediatrics, University of Padova. The local ethical committee approved the study and for children informed consent was obtained from both parents.

Study design

Patients received a 24 h constant intravenous infusion of 1 g 1-^{13}C Leucine for adults, and 2 mg/kg/h for infants (Cambridge Isotope Laboratories, Andover, MA) dissolved in saline.

Blood and tracheal aspirates were collected every 6 hours until 72 h. Tracheal aspirates and blood samples were centrifuged at 400g and 1300g respectively and supernatants were stored at -80° C until analysis.

Isolation of SP-B and SP-C from tracheal aspirates

Phospholipids' phosphorus was measured according to Bartlett [23]

Lipids from tracheal aspirates (TA) were extracted according to a modified Bligh and Dyer method [24]. An aliquot corresponding to 200 ug of PL were mixed with 2 volumes of MeOH:$CHCl_3$:HCl (2:3:0.005N), vortexed for 10 min, centrifuged for 10 min at 400g and the organic phase recovered. The procedure was repeated twice.

The organic phases were evaporated to dryness under N_2 stream, resuspended in chloroform and applied to a Bond Elute NH_2 column containing 100mg resin (Supelco, Milan, Italy) preconditioned with 3-5 ml chloroform. After loading the sample, columns were eluted sequentially with 3 ml of the following chloroform/ methanol/ acetic acid mixtures: 20:1:0; 9:1:0; 4:1:0; 4:1:0.025; 3:2:0; 1:4:0; 1:9:0. Surfactant protein B were eluted with the 4:1:0 and 4:1:0.025 fractions, whereas SP-C started to be recovered with the mixture 3:2:0 [3].

SPB derivatization

SP-B fractions (4:1:0 and 4:1:0.025) were pooled together, dried under nitrogen and hydrolysed to free amino acids by 0.5 ml of HCl 6N for 24 h at 110°C.[3].

Individual amino acids were converted into their N-acetyl-n-propil derivates with an optimized procedure [25]. The hydrolysed samples were dried at 40°C under a nitrogen stream and propylated with 1 ml of a solution of n-propanol and acetyl chloride (1:4 v/v) at 100°C for 1 hour. The tubes were then put in an ice bath to stop the reaction. Solvents were removed under a nitrogen stream at 40°C , and dichloromethane (2x 0.25ml) was added and evaporated at room temperature to remove the excess of isopropanol and water. The n-propyl esters were converted in N-acetyl-n-propyl esters by adding 1 ml (1:2:5 v/v) of acetic anhydride, triethylamine (Sigma-Aldrich, Milan) and acetone for 10 min at 60°C. The acetylates were dried under a gentle stream of nitrogen at room temperature and dissolved in 2 ml of ethil acetate, 1 ml of saturated solution of NaCl was added to obtain a phase separation.

After vortexing, the organic phase was dried under nitrogen stream, added of dichloromethane (2x0.25ml) and any remaining reagent removed under nitrogen. The derivatized amino acids were dissolved in ethyl acetate and stored at -20°C until analysis.

^{13}C enrichment of leucine was measured on a gas chromatograph (Agilent Techologies Italia SPA, Cernusco sul Naviglio, Italy) coupled with an Agilent mass spectrometer operated in positive chemical ionization (PCI) mode. Leucine was separated on a 50 m x 0.20 mm x 0.33 μm Ultra 2 fused silica capillary column.

The ion monitored were m/z 216 for leucine and 217 for $1^{13}C$-leucine. Results were expressed as mole percent excess (MPE), referring to a calibration curve for $1^{13}C$ leucine.

SP-C derivatization

For studying the incorporation of ^{13}C leucine into SP-C, 1:4:0 and 1:9:0 fractions were pooled together, evaporated to dryness and hydrolyzed to free amino acids for 24 h at 100°C in 500 µl of 6N HCl.

Amino acids from acid hydrolysis were derivatized into their oxazolinone derivatives [26]. Briefly, samples were evaporated to dryness under nitrogen stream and a 100ul (50:50 v/v) mixture of trifluoroacetic acid / trifluoroacetic anhydride (Sigma-Aldrich, Milan) was added. Then, samples were heated at 110°C for 5 min. Benzene (500ul) and water (1 ml) were added, tubes vortexed and centrifuged. The benzene layer was collected for analysis.

GC-MS analysis

Both SP-B and SP-C leucine enrichments were measured on a 6890N gas chromatographer coupled to an Agilent 5973i mass spectrometer (Agilent Technologies Italia SpA, Cernusco sul Naviglio, Italy). Leucine was separated on a 50 m x 0.20 mm x 0.33 µm Ultra 2 fused silica capillary column (Agilent technologies, Italia, SpA). The oven temperature was programmed for 1 min at 80°C, increased from 80°C to 180°C at 6°Cmin^{-1}; increased from 180°C to 280°C at 30°Cmin^{-1}; held at 280°C for 3 min.

The ion monitored for SP-B leucine was m/z 216, and 217 for1^{13}C-leucine, while the ions for SP-C leucine were m/z 209 and 210 for 1^{13}C-leucine.

Results were expressed as mole percent excess referring to a calibration curve for 1^{13}C-leucine.

Plasma leucine enrichment

One hundred ul of plasma was deproteinized with sulphosalicilic acid (10% wt/vol), and plasma amino acids were derivatized according to Husek (Husek 1991) as N (O,S) ethoxycarbonyl esters in a aqueous solution of ethil chloroformate. Free plasma leucine enrichment was measured by gas chromatography mass spectrometry (GC/MS). Results were expressed as MPE referring to a calibration curve for 1^{13}C leucine.

Kinetic parameters

All kinetic measurements were performed assuming a steady state: in all patients plasma $1^{13}C$ leucine reached steady state within 6 hours from the isotope infusion. Also, the slope of the enrichment curve over time did not deviate significantly from zero between time 6 and 24 h for plasma leucine.

Fractional synthesis rate (FSR) was calculated by dividing the slope of the linear increase of the enrichment of SP-B and SP-C by the plasma steady state enrichment of free $1^{13}C$ leucine.

Secretion time (ST) was defined as the time lag between the start of the infusion of the precursor and the appearance of the enriched product. This was calculated by plotting the regression line for the linear increasing part of the enrichment versus time curve and extrapolating it to baseline enrichment.

Peak time is the time of maximum enrichment of surfactant SP-B after the isotope infusion.

Kinetic data are presented as median individual and group values and range.

Results

Patients

No side effects were observed after the administration of ^{13}C leucine. Patients' clinical characteristics are reported in Table 1.a and 1.b.

Five adults and four children were studied. Clinical characteristics are reported in table 1.a and 1.b

Three adults were recovered for acute respiratory distress syndrome or acute lung injury (ARDS/ALI), while controls were recovered for neurological failure.

Two infants had no lung disease (hydrocephalous and myotonic dystrophy), while two had congenital diaphragmatic hernia (CDH).

Patients	Sex	Age (y)	Weight (kg)	Intubation (d)	Ventilator mode	Group
1	M	55	120	28	PCV	ARDS
2	F	81	70	19	PCV	ARDS
3	F	41	62	18	PCV	ARDS
4	M	67	70	408	tracheostomy	control
5	F	26	50	15	PCV	control

Table 1.a: *Clinical characteristics of the study adults*

Patients	Sex	Gestational Age (wk)	Age (h)	Weight (kg)	Intubation (d)	Ventilator mode	Group
1	M	35	19	3.5	7.5	HFOV	CDH
2	F	38.6	77	3.4	6	HFOV	CDH
3	M	36	624	3.05	13	SIMV	control
4	F	37	696	2.5	3	SIMV	control

Table 1.b: *Clinical characteristics of the study infants*

Surfactant protein B and surfactant protein C kinetics

SP-B and SP-C kinetics were successfully measured in all study adults and infants (fig. 4.a and 4.b)

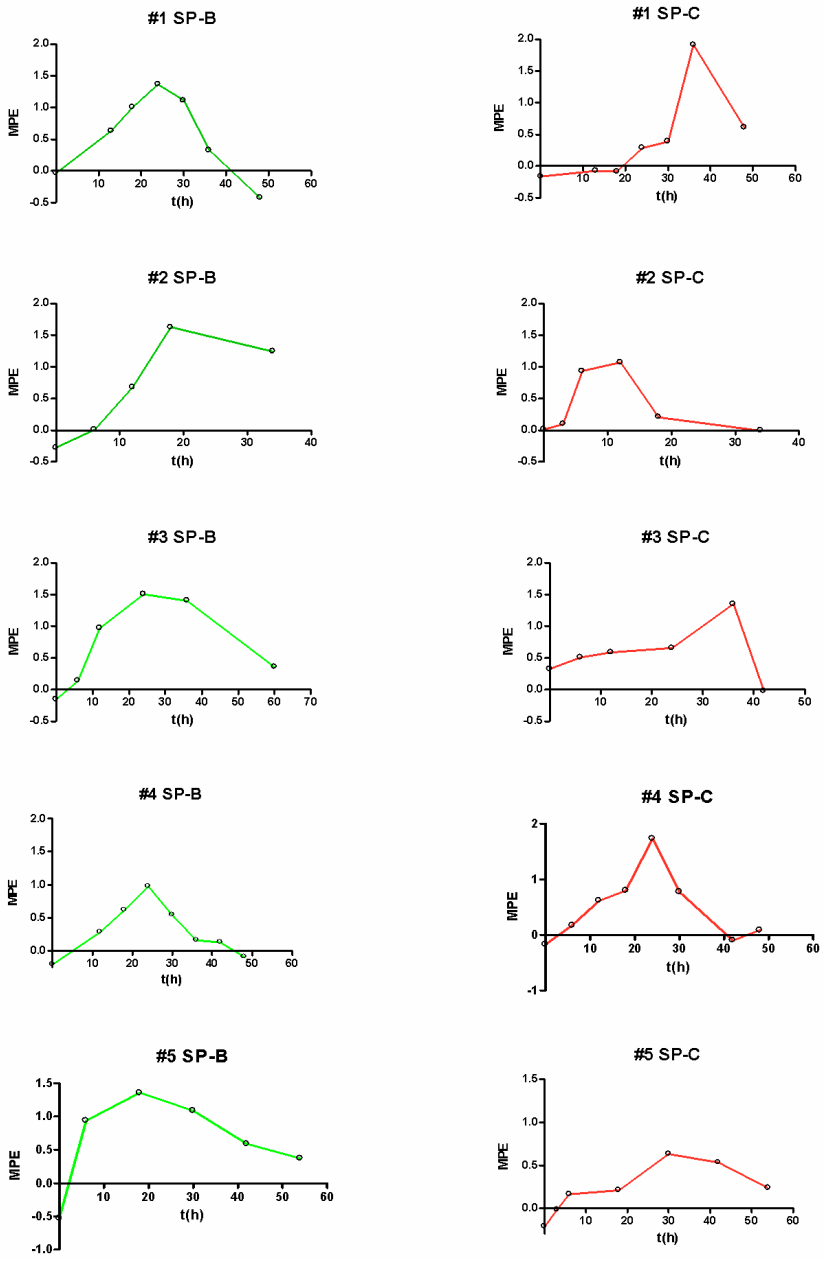

Figure 4a: Enrichment curves for SP-B and SP-C of all study adults.

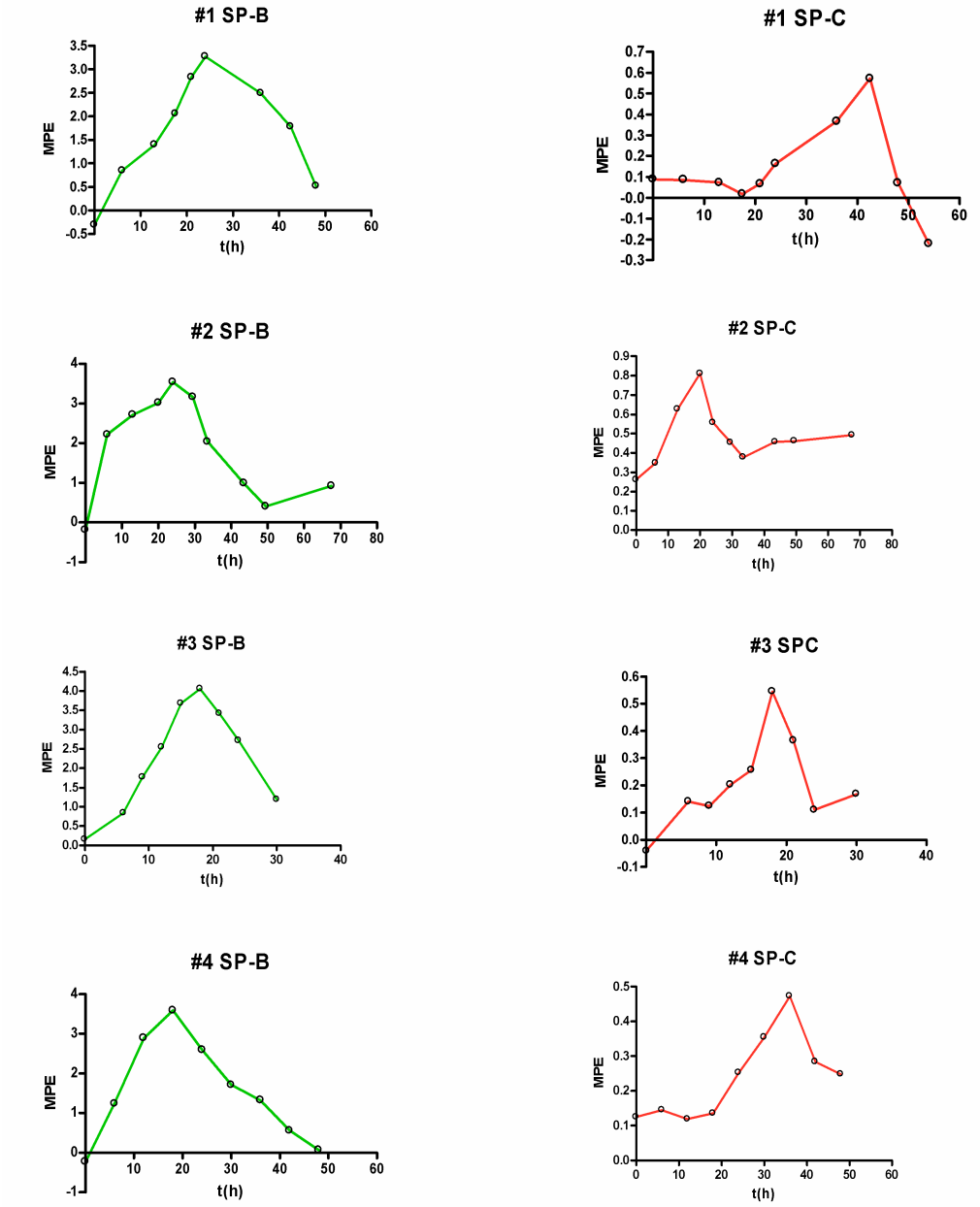

Figure 4.b: Enrichment curves of all study infants.

Individual and median SP-B and SP-C kinetics parameters are reported in Table 2.a and 2.b

	SP-B			SP-C		
Patients	**FSR (% day)**	**ST (h)**	**Peak time (h)**	**FSR (% day)**	**ST (h)**	**Peak time (h)**
1	64	3	24	30	17	36
2	71	6	18	51	1	12
3	73	2	24	13	30	24
4	47	7	24	66	5	24
5	54	2	18	12	2	30
Median	63.9	3.2	24	30	5	24
Range	47-73	2-7	18-24	12-66	1-30	12-36

Table 2.a: Adults kinetics parameters

	SP-B			SP-C		
Patients	**FSR (% day)**	**ST (h)**	**Peak time (h)**	**FSR (% day)**	**ST (h)**	**Peak time (h)**
1	43	1.2	24	7	17	42.5
2	34	26	24	16	5	20
3	88	3	18	14	7	18
4	88.5	1	18	5	3	36
Median	65.5	2	21	10	6	28
Range	34-88.5	1-26	18-24	5-16	3-17	18-42.5

Table 2.b: Infants kinetics parameters

Discussion

In this study, we report two main results:

1- an optimized method to measure surfactant protein B kinetics
2- a novel method to measure surfactant protein C kinetics

Our group had recently published the method of SP-B purification from newborn infants' TA [27].

The optimization of the derivatization procedure has been achieved extending the propylation time (from 20 min to 1 h at 100°C). A standard solution of 50 ng/ul of L-Leucine was derivatized, using both mehods, and analysed by a GC-FID. The area obtained by the new extended procedure was increased twice.

The second goal was to isolate surfactant protein C from TA and to measured its kinetics in both adults and studied infants.

As shown in tables 2.a and 2.b, median FSR, ST and PT for SP-B and SP-C were successfully measured in all patients.

SP-B and SP-C are very hydrophobic proteins and their analytical separation is difficult. We chose to separate and isolate these proteins by sorbent chromatography.

We previously did not find contamination of SP-B by SP-C [27].

We also performed an experiment to evaluate the effective distribution of the proteins studied in the eluted fractions by LTQ Orbitrap at Chiesi Farmaceutici, Parma, Italy (data not shown).

In this study, there is a very low amount of patients and it is not possible to make any meaningful clinical speculation. Our goal was to identify a method to measure the kinetics of SP-B and SP-C *in vivo*.

It is well established that low amounts of surfactant protein B and surfactant protein C lead to respiratory failure [2]. Since the first use of surfactant in human neonates over two decades ago, a large number of surfactant preparation have been developed, including animal- derived products and non-protein-containing synthetic products. Surfactants available for daily clinical practice have

dramatically decreased morbidity and mortality in human premature neonates [28].

However, the levels of SP-B and SP-C found in these commercial preparations are reduced compared to levels found in native pulmonary surfactant, and they could be too low to sustain the adsorption of the phospholipids at the air-liquid interface. Synthetic surfactant proteins could be a solution to standardize protein composition and enhance surfactant function.

Within this frame, it is important to understand surfactant proteins' kinetics in different clinical situation to improve surfactant composition, and consequently the patients' outcome.

References

1. Glasser JR, Mallampalli RK. Surfactant and its role in the pathobiology of pulmonary infection. *Microbes Infect* 2012: 14(1): 17-25.
2. Whitsett JA, Weaver TE. Hydrophobic surfactant proteins in lung function and disease. *N Engl J Med* 2002: 347(26): 2141-2148.
3. Cogo P, Baritussio A, Rosso F, Gucciardi A, Moretti V, Badon T, Duner E, Zimmernann L, Carnielli VP. Surfactant-associated protein B kinetics in vivo in newborn infants by stable isotopes. *Pediatr Res* 2005: 57(4): 519-522.
4. Hawgood S. Surfactant protein B: structure and function. *Biol Neonate* 2004: 85(4): 285-289.
5. Pryhuber GS. Regulation and function of pulmonary surfactant protein B. *Mol Genet Metab* 1998: 64(4): 217-228.
6. Whitsett JA, Nogee LM, Weaver TE, Horowitz AD. Human surfactant protein B: structure, function, regulation, and genetic disease. *Physiol Rev* 1995: 75(4): 749-757.
7. Hawgood S, Derrick M, Poulain F. Structure and properties of surfactant protein B. *Biochim Biophys Acta* 1998: 1408(2-3): 150-160.
8. Clark JC, Wert SE, Bachurski CJ, Stahlman MT, Stripp BR, Weaver TE, Whitsett JA. Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. *Proc Natl Acad Sci U S A* 1995: 92(17): 7794-7798.
9. Vorbroker DK, Profitt SA, Nogee LM, Whitsett JA. Aberrant processing of surfactant protein C in hereditary SP-B deficiency. *Am J Physiol* 1995: 268(4 Pt 1): L647-656.
10. Chaby R, Garcia-Verdugo I, Espinassous Q, Augusto LA. Interactions between LPS and lung surfactant proteins. *J Endotoxin Res* 2005: 11(3): 181-185.
11. Mulugeta S, Beers MF. Surfactant protein C: its unique properties and emerging immunomodulatory role in the lung. *Microbes Infect* 2006: 8(8): 2317-2323.
12. Johansson J. Structure and properties of surfactant protein C. *Biochim Biophys Acta* 1998: 1408(2-3): 161-172.
13. Savani RC, Godinez RI, Godinez MH, Wentz E, Zaman A, Cui Z, Pooler PM, Guttentag SH, Beers MF, Gonzales LW, Ballard PL. Respiratory distress after intratracheal bleomycin: selective deficiency of surfactant proteins B and C. *Am J Physiol Lung Cell Mol Physiol* 2001: 281(3): L685-696.
14. Matthew E, Kutcher L, Dedman J. Protection of lungs from hyperoxic injury: gene expression analysis of cyclosporin A therapy. *Physiol Genomics* 2003: 14(2): 129-138.
15. Haczku A, Atochina EN, Tomer Y, Chen H, Scanlon ST, Russo S, Xu J, Panettieri RA, Jr., Beers MF. Aspergillus fumigatus-induced allergic airway inflammation alters surfactant homeostasis and lung function in BALB/c mice. *Am J Respir Cell Mol Biol* 2001: 25(1): 45-50.
16. Bachurski CJ, Pryhuber GS, Glasser SW, Kelly SE, Whitsett JA. Tumor necrosis factor-alpha inhibits surfactant protein C gene transcription. *J Biol Chem* 1995: 270(33): 19402-19407.
17. Augusto LA, Synguelakis M, Johansson J, Pedron T, Girard R, Chaby R. Interaction of pulmonary surfactant protein C with CD14 and lipopolysaccharide.

Infect Immun 2003: 71(1): 61-67.
18. Glasser SW, Burhans MS, Korfhagen TR, Na CL, Sly PD, Ross GF, Ikegami M, Whitsett JA. Altered stability of pulmonary surfactant in SP-C-deficient mice. *Proc Natl Acad Sci U S A* 2001: 98(11): 6366-6371.
19. Garcia CK. Idiopathic pulmonary fibrosis: update on genetic discoveries. *Proc Am Thorac Soc* 2011: 8(2): 158-162.
20. Nogee LM, Dunbar AE, 3rd, Wert SE, Askin F, Hamvas A, Whitsett JA. A mutation in the surfactant protein C gene associated with familial interstitial lung disease. *N Engl J Med* 2001: 344(8): 573-579.
21. Thomas AQ, Lane K, Phillips J, 3rd, Prince M, Markin C, Speer M, Schwartz DA, Gaddipati R, Marney A, Johnson J, Roberts R, Haines J, Stahlman M, Loyd JE. Heterozygosity for a surfactant protein C gene mutation associated with usual interstitial pneumonitis and cellular nonspecific interstitial pneumonitis in one kindred. *Am J Respir Crit Care Med* 2002: 165(9): 1322-1328.
22. Li J, Ikegami M, Na CL, Hamvas A, Espinassous Q, Chaby R, Nogee LM, Weaver TE, Johansson J. N-terminally extended surfactant protein (SP) C isolated from SP-B-deficient children has reduced surface activity and inhibited lipopolysaccharide binding. *Biochemistry* 2004: 43(13): 3891-3898.
23. Bartlett GR. Phosphorus assay in column chromatography. *J Biol Chem* 1959: 234(3): 466-468.
24. Paschen C, Griese M. Quantitation of surfactant protein B by HPLC in bronchoalveolar lavage fluid. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005: 814(2): 325-330.
25. Corr LT, Berstan R, Evershed RP. Development of N-acetyl methyl ester derivatives for the determination of delta13C values of amino acids using gas chromatography-combustion- isotope ratio mass spectrometry. *Anal Chem* 2007: 79(23): 9082-9090.
26. Dwyer KP, Barrett PH, Chan D, Foo JI, Watts GF, Croft KD. Oxazolinone derivative of leucine for GC-MS: a sensitive and robust method for stable isotope kinetic studies of lipoproteins. *J Lipid Res* 2002: 43(2): 344-349.
27. 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(1): 323-329.
28. Horbar JD, Wright EC, Onstad L. Decreasing mortality associated with the introduction of surfactant therapy: an observational study of neonates weighing 601 to 1300 grams at birth. The Members of the National Institute of Child Health and Human Development Neonatal Research Network. *Pediatrics* 1993: 92(2): 191-196.