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DHA synthesis during pregnancy and markers of lung injury in infants with acute lung diseases

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ABSTRACT

Docosahexaenoic acid (DHA) is an essential constituent of membrane cell phospholipids and a precursor of eicosanoid's synthesis. During pregnancy, DHA is taken up from the maternal bloodstream and supplied to the fetus by placenta transfer. Maternal DHA intake, metabolism, and transfer are therefore crucial for fetal growth and development. In the first part of this thesis we aimed to evaluate the feasibility of measuring DHA endogenous synthesis in pregnant women using the stable isotope natural abundance approach.

Alveolar surfactant is a key player in sustaining lung physiology. It's well established that lack of surfactant, surfactant inhibition as well as changes in surfactant composition, can seriously compromise gas exchange and may lead to mechanical ventilation support. Thus, in the second part of this thesis we studied surfactant composition during acute lung disease in newborn infants.

First we compared term newborns affected by neonatal pneumonia and without lung disease to clarify if surfactant proteins' changes could have a role in the reduced pulmonary compliance observed in these patients. Finally, we studied if and how histological chorioamnionitis and gestational age affect surfactant composition in pre-term infants affected by RDS.

RIASSUNTO

L'acido docoesaenoico è un componente essenziale dei fosfolipidi delle membrane cellulari ed un precursore per la sintesi degli eicosanoidi. Durante la gravidanza il passaggio di DHA dalla circolazione materna al feto è mediata dal passaggio trans-placentare. Assunzione, metabolismo materno e transfer placentare del DHA sono quindi fondamentali per la crescita e lo sviluppo del feto. L'obiettivo della prima parte di questa tesi è stato quello di valutare la fattibilità nel misurare la sintesi endogena di DHA durante la gravidanza utilizzando l'approccio dell'abbondanza naturale degli isotopi stabili.

Il surfattante alveolare è di fondamentale importanza nella fisiologia polmonare. È noto che una carenza di surfattante, una sua inibizione così come dei cambiamenti nella sua composizione, possono compromettere l'efficienza dello scambio gassoso al punto da rendere necessario il supporto della ventilazione meccanica. Nella seconda parte di questa tesi abbiamo quindi studiato la composizione del surfattante nei neonati affetti da malattia polmonare acuta. Prima abbiamo confrontato neonati con polmonite neonatale con neonati privi di patologia polmonare per chiarire il ruolo delle proteine specifiche del surfattante nella ridotta compliance polmonare che si osserva nella fase acuta della polmonite. Infine abbiamo studiato come e se età gestazionale ed esposizione alla corioamniosite istologica influenzano la composizione del surfattante in neonati pretermine affetti da RDS.

Chapter 1

Analytical methods in stable isotopes studies

INTRODUCTION

The number of protons found in an atom's nucleus (atomic number), confers a specific position inside the periodic table thus characterizes univocally every element. All atoms of the same element have the same atomic number but may differ in the mass number (number of nucleons). When element's atoms have different numbers of neutrons they are named isotopes of that element.

Based on the stability of the nucleus, isotopes can be stable or radioactive. The stable isotopes have a stable combination of protons and neutrons thus they do not decay and they are considered to be safe. Instead radioactive isotopes undergo decay, and during this process they can emit different type of rays (alpha, beta or gamma).

Since atomic mass bears influence reactivity, isotopes of the same element exhibit nearly identical chemical behaviour. However, their mass difference can lead to the discrimination from one to another isotope by some physical or chemical processes.
As a consequence of this "*being similar but not identical*", isotopes are important tools with a unique number of applications.

ISOTOPES IN CLINICAL PRACTICE AND IN LIFE SCIENCE RESEARCH

Due to their safety, stable isotopes are used both in clinical practice, as diagnostic tools, and in research to study metabolic processes *in vivo*.
In these fields when we talk about stable isotopes we are not referring to a specific isotope of an element, rather to a molecule in which one or more atoms have been replaced by the heavier isotopes.

The most known example of isotope administered in clinical practice is undoubtedly ^{13}C-urea. The ^{13}C-urea breath test allows for a noninvasively detection of *Helicobacter pylori*[1]. *Helicobacter pylori* is able to survive in the stomach because it produces urease an enzyme that cleavages urea and locally lowers the pH value. After the administration of a dose of ^{13}C-urea, *Helicobacter pylori* produces ammonia and $^{13}CO_2$ that appears only in the expired CO_2 of patients positively affected by the infection. The result of the test is

considered to be positive when the amount of $^{13}CO_2$ is significantly higher than the levels normally observed in the population.

^{13}C breath tests are often used in clinical practice, especially in the field of gastroenterology because of their being non-invasive, safe, simple, and effective. All these tests are based on the same principle of ^{13}C-urea breath test, since they involve the measurement of the $^{13}C/^{12}C$ ratio in breath CO_2 after ingestion of a nutrient, meal, or other substrate containing ^{13}C[2]. Because the increased enrichment in breath $^{13}CO_2$ should reflect digestion, absorption, metabolism, and oxidation rate of the substrate it is possible to obtain specific information about different diseases. Examples of conditions that can be investigated by the use of breath tests are: malabsorption and pancreatic function, liver function and disease, bacterial overgrowth, maldigestion and gastroparesis[3].

Water labelled with ^{2}H or ^{18}O represents another example of isotopes used in clinical practice: by its administration is possible to measure total body water, water turnover and total daily energy expenditure[4].

Stable isotopes labelled molecules are very important also in life science research where they play pivotal roles in metabolic studies. Virtually, any metabolic process involving proteins, lipids, or carbohydrates can be studied using molecules labelled with stable isotopes of carbon (^{13}C), hydrogen (^{2}H deuterium), nitrogen (^{15}N), or oxygen ($^{18}0$)[5]. The replacement of atoms with the heavier isotopes allows to discriminate, to trace, and to detect both the parental compound of interest and its metabolites from the ones that are physiologically found in the living organisms. Because of their ability to trace the metabolic pathway of the molecule of interest these compounds are defined as *tracers*.

ISOTOPES TOXICITY

The use of stable isotopes is generally considered ethically acceptable in humans at all life-cycle stages. Isotopes effects have been observed only when their presence exceeds natural abundance levels, with the most significant expected when compounds are labelled with deuterium, because of the greater mass discrimination between deuterium and hydrogen [6]. High levels of deuterium can impair synthesis of protein and cell division and alter the rate of enzymatic reactions. Observations in adults showed that the threshold for clinically relevant side-effects is between 70–140 g 100% D_2O[6], which corresponds to a dosage of approximately 200–400 mg of deuterium/kg body weight, this threshold is never reached by usual tracer dosages used in clinical studies (deuterium range dosage: 1–80 mg/kg body weight)[7].

Because of the smaller difference in mass between the two isotopes of carbon and oxygen clinical effects are unlikely to be observed. Moreover, as far as carbon, the human

body is already naturally enriched in ^{13}C (the reported pool for ^{13}C is about 2000 mg/kg of body weight) and the amount introduced with tracer studies increments this value by less than 1.5%[7].

STUDY DESIGN OPTIONS IN STABLE ISOTOPES STUDIES

There are two types of metabolic studies: the ones that study the tracer itself without considering metabolic transformations, and those that study the conversion of the tracer as a precursor into one or more products[5]. The type of study to undertake depends on the questions needed to be addressed.

Beside the type of metabolic study there are also different experimental approaches available. The identification of the best study design to use depends on the information needed, sensitivity and accuracy requirements, availability of labelled tracers and finally analytical equipment [8].

Ideally tracers can be introduced by all routes of administration (for example oral, enteral, endotracheal or intravenous); by single dose (bolus), by multiple doses or by constant infusion. Generally, the last two approaches are required in kinetic studies since only after achieving the isotopic steady state some assumption and calculations can be made.

In single-labelled-experimental design only a tracer molecule is administered while the multiple–labelled design consists in the administration of a mixture containing two or more tracers. The advantage of the multiple–labelled design is particularly evident in the metabolic studies of related compounds, not only because more information can be obtained for nearly the same effort but also because the metabolic fate of different related compounds can be directly compared in the same subjects, reducing interpretation problems due to subject variability[8].

Finally, another important issue in study design is the identification of how the molecule has to be labelled in order to provide the information needed. For example, if the purpose of our research is to study the conversion rate of linoleic acid (18:2n-6) into arachidonic acid (20:4n-6) we will administer a single oral dose of U-^{13}C18:2 n-6 as a free fatty acid; conversely if we are interested in studying how triglyceride structure impacts on fatty acids absorption we need to label differently the fatty acids esterified to the backbone of glycerol[9].

Tracers labelled with stable isotopes used in metabolic studies are obtained by chemical synthesis. Cost of isotopes is one of the factors that must be considered when calculating the amount of tracer to use *in vivo* experiment. Usually there's a tradeoff between the lowest dose to administer and the minimum enrichment that has to be reached,

in order to obtain an acceptable accuracy in the results, which ultimately depends on the analytical equipment.

STABLE ISOTOPE NATURAL ABUNDANCE

The "natural carbon ^{13}C diet approach" or "stable isotope natural abundance approach" allows to use, as tracers, molecules characterized by a ^{13}C content that is different from the one usually observed in the population. These tracers are not produced by chemical synthesis but can be obtained by an accurate selection of nutrient sources. In fact the method is based on the measurement of the natural variation in the ^{13}C content of selected nutrients.

Above it has been stated that, due to the different mass, isotopes can be discriminated by some physical or chemical processes. Processes that impact on the relative abundance of isotopes are known as "*isotope fractionation"*. Fractionation can occur, for example, during phase changes. The process of evaporation enriches the liquid water of the heavier isotopes (^{18}O and ^{2}H) while the lighter isotopes (^{16}O and ^{1}H) tend toward the vapour phase. During condensation the heavier isotopes are the first to condense while the lighters tend to remain into the vapour phase. This explains why snow falling at the poles is depleted in ^{2}H and ^{18}O content with respect to rainfall at the equator.

Fractionation can also occur during biological processes, for example during photosynthesis. Because of small differences imparted by the different mass, plants discriminate against ^{13}C [10].

There are three types of plants: C3, C4 and CAM plants. C3 plants use the Calvin-Benson photosynthetic cycle and they fix CO_2 by the action of ribulose phosphate carboxylase. Carboxylation step shows great fractionation and explains the very low content of ^{13}C observed in these plants (δ ^{13}C varies from -22 to -35 $^{0}/_{00}$). Examples of C3 plants are wheat, rice, and rye. C4 plants use the Hatch –Slack photosynthetic cycle: CO_2 is taken up through phosphoenolpyruvate carboxylase and converted into malate or aspartate which are then cleavage again in CO_2 that is finally taken up by ribulose phosphate carboxylase. Since the first process does not show a strong fractionation effect this plants have a higher content of ^{13}C compared to C3 plants (δ ^{13}C varies from -8 to -20 $^{0}/_{00}$). Examples of C4 plants are sugar cane, corn, and tropical grasses plants. The third type of plants, to whom pineapple and cactus belong, use the Crassulacean acid metabolism (CAM). CAM plants during night act as C4 plants while during the afternoon like C3 plants, their content of ^{13}C allows to distinguish them from C3 plants (δ ^{13}C varies from -10 to -20 $^{0}/_{00}$) while the distinction from C4 must be made by other measurements.

Because animals can incorporate carbon only ingesting vegetables or meat the amount of ^{13}C found in their body, as in the expired CO_2, will mirror the isotope ratios of their food sources. European diets are richer in C3 plants while North American diets are richer in C4 plants. As a consequence European people have $^{13}C/^{12}C$ ratios similar to C3 plants and lower ratios compared to people living in North America[11].

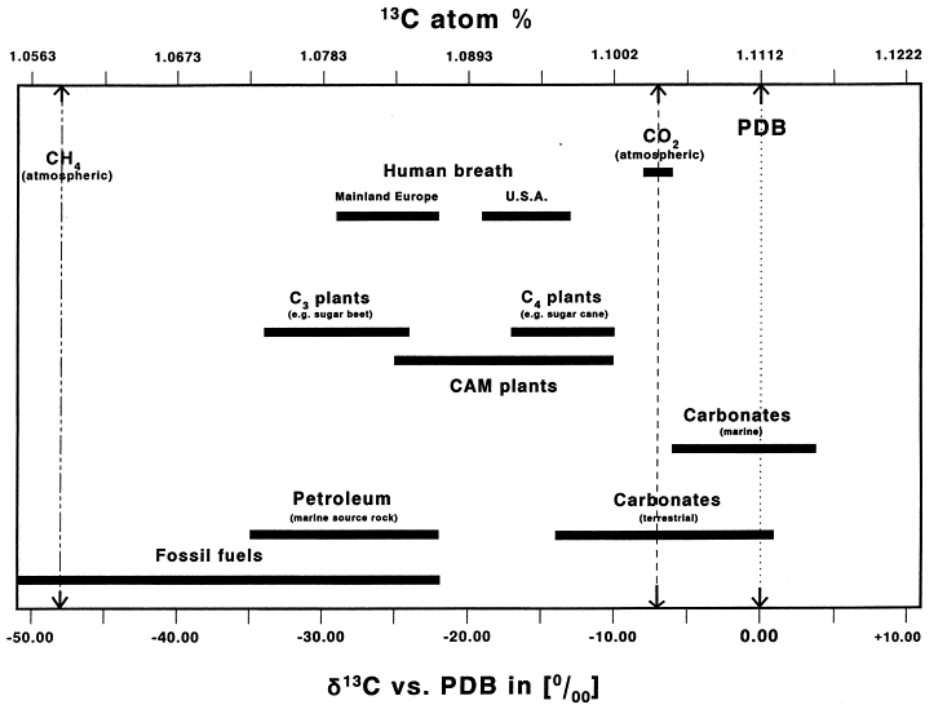

Figure 1. Typical examples of natural δ ^{13}C values.
From Meier-Augenstein *et al.*[12]

The stable isotope natural abundance approach consists in administering a nutrient which $^{13}C/^{12}C$ ratio differs from the one usually found in the population in order to study its metabolic fate. In European population nutrients from C4 plants can be used as natural tracers, since after their administration a change in the isotopic enrichment mean value of the nutrient can be observed. This approach has been used in humans for the first time in 1995 to estimate arachidonic acid synthesis in full term neonates[13]. Our research group in 2007 published a study using the same approach to estimate linoleic acid and α -linoleic conversion into their long chain polyunsaturated fatty acids metabolites[14]

Advantages of stable isotope natural abundance approach are first of all economic, since no isotopes have to be synthesised, moreover they are extremely safe. Major limitations are:

i) impossibility to trace all the metabolic pathways and ii) necessity of analytical instrumentation with a high accuracy in measuring the $^{13}C/^{12}C$ ratio.

MASS SPECTROMETRY

With modern analytical techniques, tracers can consecutively be detected either unchanged or after conversion into their metabolites. Mass spectrometry (MS) is the analytical method most used in stable isotope labelling studies. Because MS separates and quantifies ions on the base of their mass-to-charge ratio (m/z) by this technique it is possible to discriminate between isotopes found in simple and complex mixtures.

The application of MS to biology started in the 1940s, when heavy stable isotopes were used as tracers to study processes such as CO_2 production in animals[15]. Nowadays MS shows a wide range of applications in several fields like proteomics, genomics, drug discovery, environment, forensic and pharmacology.

CONVENTIONAL MASS SPECTROMETRY

Basically, any information gathered from a mass spectrometer comes from the analysis of gas-phase ions[16]. All mass spectrometers are composed by an ion source, a mass analyser and an ion detector and are connected to a computer system, which processes and records the data, and to a vacuum pump to control the pressure within it.

The ion source converts the sample into gas-phase ions. Ions are then accelerated by an electromagnetic field to the mass analyser, where they are separated according to their m/z. Finally, ions hit the detector, causing a cascade of electron emissions that results in a measurable current proportional to the number of ions that reached the detector. This represents the final signal recorded by the computer system.

There are different kinds of ion sources, mass analysers and detectors available. There is no best combination at all, specific devices used in each of the components depend on the type of information desired and on the properties of the sample[15].

In the study of fatty acid metabolism MS is coupled to gas chromatography. By travelling through the length of the chromatographic column fatty acids of the complex biological mixtures are separated before reaching the mass spectrometer where isotopes can be finally distinguished.

ISOTOPE RATIO MASS SPECTROMETRY

Conventional mass spectrometers have a limited accuracy when enrichments are very low, as a consequence differences in natural abundance need to be measured by Isotope Ratio Mass Spectrometry (IRMS).

IRMS thanks to accuracy and precision, beside has become the method of choice to measure isotope distribution at natural abundance level, is also used more and more in biochemical and biomedical application in combination with stable isotope labelled compounds[12].

IRMS instruments accept the analyte in the form of one of a limited number of gases, which must be isotopically representative of the original sample[17]. As in conventional MS, to study fatty acids IRMS requires to be coupled to gas chromatography. Since the analyte must be converted into a gas before entering the ion source of IRMS, a splitter at the end of the chromatographic column leads the effluent into a combustion or a pyrolysis tube for the analysis of carbon and nitrogen, and oxygen and hydrogen, respectively. In organic carbon analyses CO_2 and H_2O are representative of the combusted analyte. After the remove of H_2O by a Nafion™ trap, CO_2 is transported by the helium flow to IRMS. CO_2 passes through the magnetic field in ionised form and then reaches the detector represented by Faraday cups. $^{13}C/^{12}C$ ratio of the sample is obtained by the simultaneous measurement of 44 and 45 m/z signals that ultimately correspond to $^{12}CO_2$ and $^{13}CO_2$.

The enrichment of an isotope in a sample, compared to a standard value, is given as atom % excess (APE). In IRMS very small variations of the heavier isotopes are detected, as a consequence the δ notation has been adopted to report changes in isotopic abundance as a per mil deviation compared to a reference standard[12].

$$\delta_{sample} = (R_{sample} - R_{standard}/R_{standard}) * 1000$$

Where:

R_{sample}= heavy isotope/light isotopes ratio of the sample

$R_{standard}$= heavy isotope/light isotopes ratio of the reference material

If $\delta_{sample} < 0$ the sample has a lower amount, compared to the standard, of the heavier isotope

$\delta_{sample} > 0$ the sample has a higher amount, compared to the standard, of the heavier isotope

Standard materials used to establish $R_{standard}$ usually represent materials highly enriched in the heavy isotopes. Most analyzed substances are depleted in the heavy-isotope relative to the standard and will therefore have negative δ values.

Chapter 2

DHA synthesis during pregnancy using the isotopic natural abundance approach

INTRODUCTION

Long chain polyunsaturated fatty acids (LC-PUFAs) are fatty acids (FAs) characterized by a chain of at least 20 carbons and three, or more, double bonds. Depending on the position of the first double bond from the methyl end of the carbon chain, LC-PUFAs can be divided in two main classes: n-3 and n-6[i].

Despite they can act as an energy source, like all the FAs, their main functions are, undoubtedly, metabolic and structural. LC-PUFAs are precursors of lipid mediators, regulators of physiological functions and inflammatory processes, and they are also important components of cell membrane phospholipids (PLs), maintaining membranes characteristic fluidity, permeability and conformation.

Among LC-PUFAs arachidonic (ARA; 20:4n-6), eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) are of particular biological interest.

SYNTHESIS OF LC-PUFAs

LC-PUFAs found in human blood and tissues can have two different origins: they can be provided by dietary intake[ii] or be the result of endogenous conversion from their metabolic precursors.

Since the human body is not able to introduce a double bond three or six carbons from the methyl end, linoleic (LA; 18:2n-6) and α-linolenic (ALA; 18:3n-3) acids, defined as essential fatty acids (EFAs), must be introduced directly from the diet before being converted in LC-PUFAs.

[i] n-9 represents a further class, but appreciable amounts of these fatty acids occur only in case of n-3 and n-6 deficiency.

[ii] Dietary sources of FAs: ARA can be found in meat, eggs, and offal; EPA and DHA mainly in oily fish and seafood; LA in vegetal oils; ALA in seeds oils.

In the endoplasmatic reticulum, via the mammalian pathway of n-6 and n-3, LA and ALA can then be converted, respectively, in ARA and EPA by consecutive desaturation and chain elongation steps[18]. Further steps and a compartmental translocation to peroxisomes are required to obtain DHA from EPA[18].

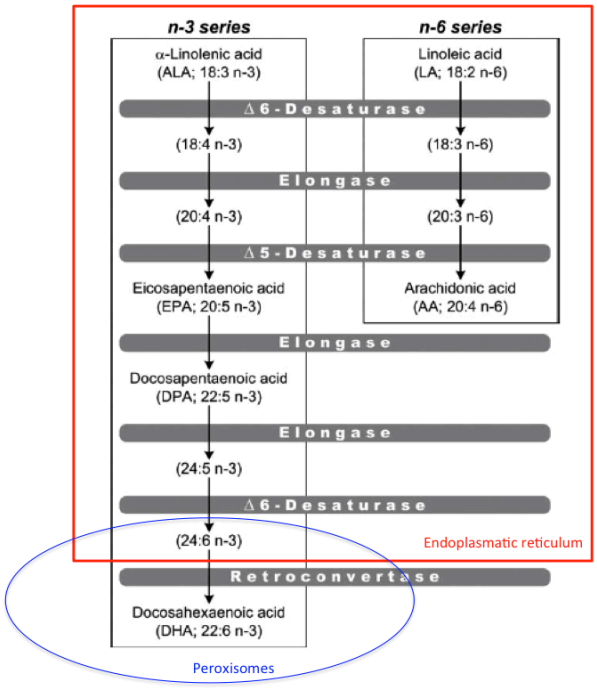

Figure1. Metabolic pathway of n-3 and n-6 LC-PUFAs in humans.
Adapted from Giltay *et al.*[19].

The sharing of the same enzymes causes a competition between LA and ALA metabolism. As a result, in the western diets, where consumption of LA is much higher than that of ALA[20], conversion of the n-6 PUFAs predominates on the n-3 series.

By the administration of EFAs, labelled with stable isotopes, several studies investigated human subjects' ability to convert them into LC-PUFAs products[21–26].
Approximately 22-33% of the administered dose of ALA was used as an energy source and ß-oxidised to CO_2[21,22], and less than 10% was used for DHA synthesis[21,22,25]. These studies pointed out how, in human subjects, gender appears to be a strong determinant in DHA synthesis with women having a much greater ability in the bioconversion of ALA to DHA compared to men[21,22,27], probably because of the effects of estrogens[22,28].
Studies performed in newborns showed that both term and pre-term newborns are capable of synthesizing LC-PUFAs from their 18-carbon precursors[23,26] and that infants with intrauterine growth restriction (IUGR) have a less active DHA formation than those who are

appropriate for gestational age[24]. In pre-term newborns ARA synthesis at 1, 3, and 7 months of age was found to be significantly greater than DHA synthesis and both LC-PUFAs synthesis decreased significantly with time[14].

In the last decade a number of studies have investigated the association between variants in the fatty acid desaturase encoding genes (FADS) and the efficiency of these enzymes in the production of LC-PUFAs from their precursors[29–33]. The higher levels of substrates and lower levels of desaturase products found in the carriers of the minor allele suggested a decline in desaturase activity because of the polymorphism.

BIOLOGICAL ROLE OF LC-PUFAs

LC-PUFAs are the metabolic precursors of several lipids mediators that play key roles in inflammation, in physiologic and pathologic processes.

Acute inflammation response can be divided in 2 phases: initiation and resolution. Eicosanoids, such as prostanoids and leukotrienes, are potent pro-inflammatory mediators derived from the metabolism of ARA by the action, respectively, of cyclooxygenase-2 and 5-lipoxygenase. Since they allow the recruitment of neutrophils and their homing at the site of inflammation they are of particular importance in the initial response phase. During the course of inflammation prostaglandin E_2 switches eicosanoid biosynthesis from predominantly pro-inflammatory arachidonate-derived mediators to the anti-inflammatory and pro-resolution ones, namely lipoxins (LXs)[34]. LPXs, generated from ARA by the action of 15-lipoxygenase, are important in limiting the inflammation cascade, since they inhibit neutrophils recruitment and promote phagocytosis of apoptotic neutrophils by monocyte-derived macrophages[35]. The discovery of LXs and their anti-inflammatory activity "shattered the dogma" that all ARA-derived mediators are solely pro-inflammatory[36].

During the self-limited resolution phase several lipid mediators from n-3 LC-PUFAs are biosynthesized by new pathways. Resolvins, protectins and, the last discovered, maresines, collectively named specialized pro-resolving mediators (SPM) represent these novel pro-resolving local mediators that are characterized, *in vivo*, by anti-inflammatory properties.

The discovery of SPM was the evidence for considering: i) resolution of self-limited inflammation not only a passive process but an active programmed response turned on by neutrophils, and ii) anti-inflammatory effects of n-3 LC-PUFAs not only due to the competition with ARA, in preventing pro-inflammatory eicosanoids biosynthesis, but in the production of active pro-resolving lipid mediators[37].

Eicosanoids are reported to affect and regulate the physiology of cardiovascular system. ARA is the precursor of prostaglandin and thromboxane 2-series, the first has a pro-arrhythmic effect while the second is a potent platelet activator and a vasoconstrictor agent.

Opposite effects are exerted by prostaglandin and thromboxane 3-series, derived from EPA. ARA is than considered to be more pro-inflammatory and to affect negatively the cardiovascular system in respect of EPA. Literature data reported that increased consumption of n−3 LC-PUFAs reduces the risk of cardiovascular disease[38].

Eicosanoids are also important mediators in the pathophysiology of asthma, where the overproduction of leukotrienes is considered one of the major causes of inflammation.

Lipid mediators cannot be stored within cells for later release but are synthesized when required. As a consequence, a balance n-6/n-3 ratio in membrane PLs is required by the organism to produce the pro-inflammatory mediators involved in the initial phase of inflammation, the protective ones that stop inflammation cascade and promote resolution, and the 2- and 3-serie of eicosanoids involved in physiological functions.

As mentioned above LC-PUFAs have also important structural roles. DHA, differently from ARA, which is present in all biological membranes and represents approximately 5-15% of the total FAs in most tissue PLs, has a very specific distribution: brain and retina are among the tissues more enriched in DHA[39].

DHA is the major structural lipid of retinal photoreceptor outer segment membranes. It accounts, approximately, for 30-65% of all the total FAs present in both phosphatidylethanolamine (PE) and phosphatidylserine (PS)[39]. The PLs localized in this neural subcellular component are characterized by having not only the n-2 but also the n-1 position occupied by LC-PUFAs, DHA represents respectively the 75-100% and 5-25% of them[39]. It has been suggested that DHA may operate in signalling cascades to enhance activation of membrane-bound retinal proteins and may also be involved in rhodopsin regeneration[40].

The brain is the fattiest organ in the body. LC-PUFAs represent the 35-40% of brain lipids and DHA accounts for one third of the total FAs content of PE and PS[39]. Synaptic membranes, mitochondria, and microsome are the subcellular fractions with the highest concentration of DHA[41]. Conversely, white matter and myelin fraction contain low levels of PUFAs[39]. The particular conformation of cell membranes highly enriched in DHA is considered to be important in the downstream of signalling events as in the neurotransmitter release.

Structural and metabolic roles of LC-PUFAs are highly connected. They are components of membrane PLs from which they can be released by phospholipase A2 and used for synthesis of lipid mediators, but they also assure the correct function of membrane proteins, ion channels, transport systems and receptors by maintaining membrane permeability, fluidity, and conformation.

Because of LC-PUFAs' ability to interact with transcription factors, they are also involved in the regulation of gene expression. Peroxisome proliferator-activated receptors

and nuclear factor-kappa B represent two of these transcription factors whose activity can be affected by LC-PUFAs.

DHA SUPPLEMENTATION DURING PREGNANCY: EFFECTS ON FETAL DEVELOPMENT AND PREGNANCY OUTCOMES

During the third trimester of pregnancy and during the first month of postnatal life considerable amounts of DHA accumulate in retinal photoreceptors and neuronal cell membranes. Accumulation then continues up until the first 24 months of life, even if at lower rates. An adequate supply, especially during the embryological organogenesis and the first months of post-natal life, seems than to be of critical importance for the normal neurological and visual development.

In the last decade, after observational studies suggested the existence of a positive association between oily fish consumption during pregnancy and cognitive and visual abilities of the offspring, several randomized controlled trials (RCTs) were performed.

Gould *et al.* in 2013 published the first comprehensive systematic review and meta-analysis, including published and unpublished trials, of n-3 LC-PUFAs supplementation in pregnancy with cognitive and visual outcomes[42]. The review highlighted that many of the RCTs have several methodological limitations such as small sample size, systematic bias, poor quality and lack of data; moreover, in some trials supplementation took place during pregnancy and lactation, which made difficult to determine the period during which supplementation is effective if a benefit is found. The authors combined in a meta-analysis the data relative to cognitive development. No differences were observed between infants, toddlers or school age children of supplemented and control groups. Only in the preschool children group (2-5 years), after supplementation during pregnancy and lactation, development standard score and intelligent quotient were found to be higher, despite this effect was from two trials with a high risk of bias. As far as the visual development no meta-analysis was performed because of the use of different tests and ages at assessment. The authors conclude that evidence does not support or refute the hypothesis that n-3 LC-PUFAs supplementation in pregnancy improves child cognitive or visual development.

Effects of n-3 LC-PUFAs supplementation on gestation length and preterm birth rates have been investigated too.

Three meta-analysis of RCTs found that gestational age at delivery was significantly higher in the intervention group supplemented with n-3 LC-PUFA[43–45]. Szajeska *et al.* found a weighted mean difference of 1.57 days (95% CI: 0.35-2.78)[43], Makrides *et al.* of 2.55 days (95% CI: 1.03-4.07)[44] and Salvig *et al.* of 4.5 days (95% CI: 2.3-6.8)[45]. An increase in birth weight was reported too[44,45]. Larqué *et al.* in a systematic review suggested that the

somewhat higher birth weight reported in RCTs, for supplemented pregnancy, was probably due to the greater length of these pregnancies, since in one of the RCTs no differences were observed anymore when using the gestational age as covariable[46].

More controversial are the effects of supplementation in reducing the risk of preterm birth. A recent systematic review and meta-analysis conclude that n-3 LC-PUFAs supplementation during pregnancy does not reduce the incidence of preterm birth or improve neonatal outcome measured as birth weight, neonatal intensive care unit admission, necrotizing enterocolitis, or sepsis[47].

Effects of supplementation during pregnancy on allergic diseases in newborns, preeclampsia, maternal and postpartum depression were addressed too. Data from reviews and meta-analysis do not find differences in pre-eclampsia rates [43,44,48] or advantages in the incidence of maternal and postpartum depression [49].

Global recommendations for EPA and DHA intake, revised in 2014, suggest that pregnant and lactating woman should introduce at least 200 mg DHA/day. As suggested by Larqué *et al.* n-3 LC-PUFA supplementation may not yield any measurable benefit but very low intakes could result in loss of function that could be of major importance in prematurely born babies[46]. In a randomized multicenter trial, fish oil supplementation during pregnancy from gestation week 22 until delivery, beside improving fetal n-3 LC-PUFAs status, was found to attenuate depletion of maternal stores[50]. As observed by Hanebutt *et al.*[51] DHA supplementation counteracts the depletion of maternal DHA stores during the last trimester meeting the increased fetal needs that peak when human brain growth spurts.

FAs TRANSPORT ACROSS HUMAN PLACENTA

FAs are transported across placenta only in form of non-esterified fatty acids (NEFAs). NEFAs taken up by the placenta are derived from maternal circulating NEFAs, bound to albumin, and from triglycerides (TG)[iii].

NEFAs can cross lipid bilayers by simple diffusion or by saturable protein-mediated transport. Proteins identified in NEFAs' transfer across placenta are: plasma membrane fatty acid binding protein (P-FABPpm) and fatty acid transfer proteins (FAT/CD36 and FATP). The existence of this complex and specific FAs-transport system has been proposed to be crucial in facilitating the preferential transport of maternal plasma FAs so that, the requirements of the fetus can be met[51].

Once inside the cytosol of the syncytiotrophoblast NEFAs can be differently routed. They can bind the intracellular fatty acid binding proteins (FABS) and be translocated, across

[iii]The presence, in human placenta, of binding sites for lipoproteins and the lipoprotein lipase activity, which hydrolyzes lipid esters, allow the utilization of maternal TG, as sources of NEFAs.

basal membrane, to fetal circulation by the action of FAT/CD36 or FATP. NEFAs can also be re-esterified and deposited as TG for later release, or used to produce energy, via ß-oxidation, or even to synthesize eicosanoids. Finally they can also modulate gene expression by the activation of nuclear transcription factors.

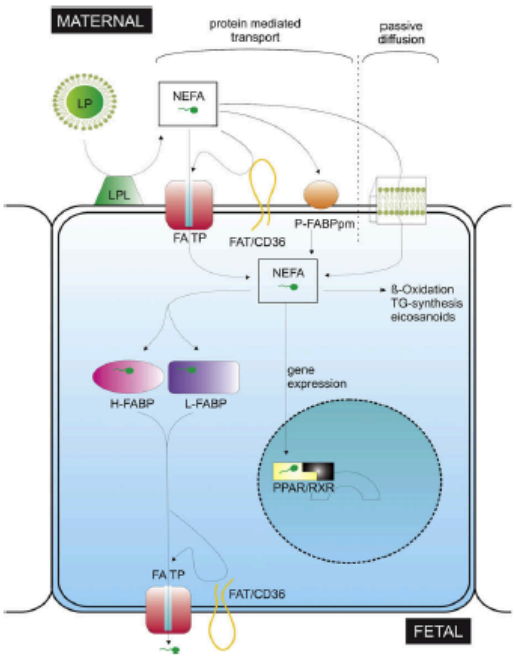

Figure 2. Model of placental fatty acid transport.
LP, lipoprotein; LPL, lipoprotein lipase; NEFA, non-esterified fatty acid; FATP, fatty acid transport protein; FAT, fatty acid translocase; P-FABPpm, placental plasma membrane fatty acid binding protein; L-FABP, liver-fatty acid binding protein; H-FABP, heart-fatty acid binding protein; PPAR, peroxisome proliferator activated receptor; RXR, retinoid X receptor.
From Hanebutt *et al*[51].

Several studies found significantly higher percentages of ARA and DHA (and lower LA and ALA) in cord blood than in maternal circulation[52–54]. The effect by which the percentages of FAs are found to be higher in cord blood than in maternal one is called "*biomagnification*". Because of delta 5- and delta 6-desaturase activities in fetal liver microsomes are very low and because no activity has been found in placenta[55] a preferential and selective materno-fetal transfer of LC-PUFAs, supported by *in vitro* and *in vivo* studies, has been proposed. Larqué *et al.* studying, *in vivo*, the placental transfer of ^{13}C-labeled FAs, demonstrated a preferential accretion of DHA into the human placenta relative to the other FAs investigated (LA, palmitic and oleic acid)[56]. This result is in line with previous findings

from *in vitro* and perfusion studies that showed higher affinities and binding capacities of P-FABpm for ARA and DHA compared to ALA and oleic acid[57] and a preferential placentar uptake and transfer of DHA[58,59].

In complicated pregnancies alterations in placental transfer of LC-PUFAs have been described. In gestational diabetes mellitus (GDM) there is an higher placental uptake of LC-PUFAs but the transport to fetal circulation is impaired compared to normal pregnancies, as a consequence cord levels of LC-PUFAs are found to be lower than maternal ones [60,61]. In intra-uterine growth restricted (IUGR) pregnancies decreased ARA/LA and DHA/ALA ratios are observed in fetal compared with maternal blood, probably because of the changes in transport occurring in relation to placental insufficiency[62].

In this study with the use of a stable isotope natural abundance approach, we aimed to measure the percentage of endogenous DHA synthesis during pregnancy and to evaluate its transfer from maternal to fetal circulation.

This approach is based on the fact that it's possible to trace a specific nutrient if it has a natural ^{13}C enrichment significantly different both from the one introduced with the diet and from the one endogenously synthesized.

DHA derived from oils of biotechnological origin and produced by single-cell organisms is characterized by a ^{13}C enrichment significantly different from the one produced endogenously by progressive desaturation and elongation of ALA.

Supplementation during pregnancy with this type of oil changes the mean enrichment of maternal DHA.

The most the maternal DHA ^{13}C enrichment resembles the one of supplemented DHA the lower is the endogenous DHA synthesis. The higher the ability of the pregnant woman to produce DHA the most ^{13}C enrichment will be close to the one measured before supplementation's start.

AIM OF THE STUDY

The aim of this study was to evaluate the feasibility of measuring DHA synthesis during pregnancy using the "stable isotope natural abundance" approach.

MATERIAL AND METHODS

Study population

Women eligible for the study were pregnant women at 20 weeks of gestation before starting DHA supplementation.

Twenty women were supplemented with capsule containing DHA of biotechnological origin (Study Group) and 6 with capsule containing DHA of fish origin (Control Group).

Study design

Blood samples that had been collected at times of diagnostic veni-punctures for routine care were used with up to 5 samples per woman. A first blood sample, defined as the baseline (T_0) was collected before the start of the DHA supplementation, at 22 weeks of gestation. A second and a third samples, T_1 and T_2, were collected respectively after 10 days of supplementation and at the 30th week of gestation. At the time of delivery blood samples were also collected from the mother and from the umbilical cord.

Samples collection, storage and analytical methods

Blood samples were drawn in EDTA-containing tubes; plasma was separated from the red blood cells after centrifugation at 1300 x *g* for 10 minutes at room temperature. Plasma was stored in tubes containing pyrogallol as antioxidant at -80°C until analysis.

Isolation and analysis of FAs profile of plasma PLs

Plasma lipids were extracted from 200 µL of plasma, according to Folch *et al.*[63], after the addition of appropriate internal standards for each lipid class containing both nonanoic and heptadecanoic or pentadecanoic acid. The extracts were dried under nitrogen at 37°C. The lipids were dissolved in 100 µL of chloroform/methanol (2:1, v/v) and applied to a Silica Gel TLC plate (20x20). The TLC plate was developed with heptane/diisopropylether/acetic acid (60/40/3, v/v/v). After drying, the lipid classes were visualized by spraying the TLC plate with 1,2-dichlorofluorescein and detected under UV-light. The spots corresponding to the different lipid classes were identified by comparison

with standards, scraped off and collected in glass tubes. Methylation was performed with 3 mL HCl/MeOH, the tubes, after flushing with nitrogen, were capped and incubated for 60 min at 100°C. After cooling, HCl was neutralized by adding 3 mL of 10% K_2CO_3. The methyl esters were extracted with 250 µL of hexane/BHT (50 mg/L). 1 µL of the extract was analysed by using a gas chromatograph with a flame ionization detector (HP-5890, Amstelveen, Olanda). Separation of methyl-esters was achieved on an Omegawax column (30 m x 0.25 mm x 0.25µm film thickness, Supelco, Milano, Italy). Injections were performed in splitless mode at an injector temperature of 280° C.

FAs, from C8 to C24 carbon atom, were identified by comparing retention times with reference standards (Nu-Check Prep, Inc., Elysian, MN). Data for plasma FAs are expressed in moles percent (mol%).

Samples were analysed in single.

^{13}C DHA plasmatic enrichment and percentage of DHA endogenous synthesis

The ^{13}C enrichment of DHA methyl ester derived from plasmatic PLs was analyzed by using gas chromatograph-combustion interphase isotope ratio–mass spectrometer (Delta Plus XL; Finningan MAT, Bremen, Germany). Separation of methyl-esters was achieved on a DB225 column (30 m x 0.25 mm x 0.25 µm film thickness; Agilent J & W, Folsom, CA). Injections were performed in splitless mode at an injector temperature of 250° C. The ^{13}C enrichment of DHA was expressed as δ^{13}C values relative to Pee Dee Belemnite (PDB) carbonate[64].

δ^{13}C was calculated by the use of this formula:

$$\delta^{13}\mathrm{C} = [(R_x/R_s) - 1] * 1000$$

Where:

R_x = isotopic ratio of the sample

R_s = isotopic ratio of the reference standard. The defined reference standard for ^{13}C has been PDB with ^{13}C: ^{12}C of 0.0112372.

To determine ^{13}C values relative to the PDB reference, CO_2 samples with a known δ^{13}C value (-61.5079) were introduced at appropriate time points during chromatography.

All samples were analysed in duplicate.

The following equation was used to calculate the percentage of DHA endogenous synthesis at T_2 and at $T_{delivery}$:

Percentage DHA endogenous synthesis $= 100 - [(A - B/C - B) * 100]$

Where:

A= $\delta^{13}C$ value of the plasma phospholipid DHA at T_2 or $T_{delivery}$

B = $\delta^{13}C$ value of the plasma phospholipid DHA at T_0

C = $\delta^{13}C$ value of the DHA present in the capsule

Capsule ^{13}C DHA enrichment and oil FAs composition

The oil present inside the capsule was analysed both for ^{13}C DHA enrichment and for its composition in FAs.

100 µL of diluted oil (1:50 in chloroform) after the addition of appropriate internal standards (nonanoic and pentadecanoic acid) were extracted according to Folch *et al.*[63]. The extracts were dried under nitrogen at 37°C. Methylation was performed as described above for FAs derived from plasma PLs. The methyl esters were extracted with 1 mL of hexane/BHT (50 mg/L). The extract was analysed as previously described for plasma PLs in order to obtain information about relative amounts of FAs present in the oil and ^{13}C enrichment of DHA methyl ester.

Statistical analysis

Data are presented as mean ± SD.

Statistical analysis was performed using PASW Statistics 18.0 for Windows (SPSS Inc., Chicago, IL).

Mann-Whitney U test was used to compare clinical data and plasma FAs composition at different time-points between the two study groups.

Friedman's test 2-way ANOVA was used to evaluate, during the course of the study, the effect of: i) DHA supplementation on plasmatic DHA enrichment and ii) the effect of gestation on plasma FAs composition.

Wilcoxon ranked test was used to compare plasmatic DHA enrichment and plasma fatty acids composition between maternal and cord plasma.

A $p < 0.05$ was considered as statistically significant.

RESULTS

Fifteen out of 26 women had been studied until delivery. Totally 8 women reported to have quit the assumption or not to have taken daily the capsule, samples collected later were then excluded from the analysis. Three women are still on study.

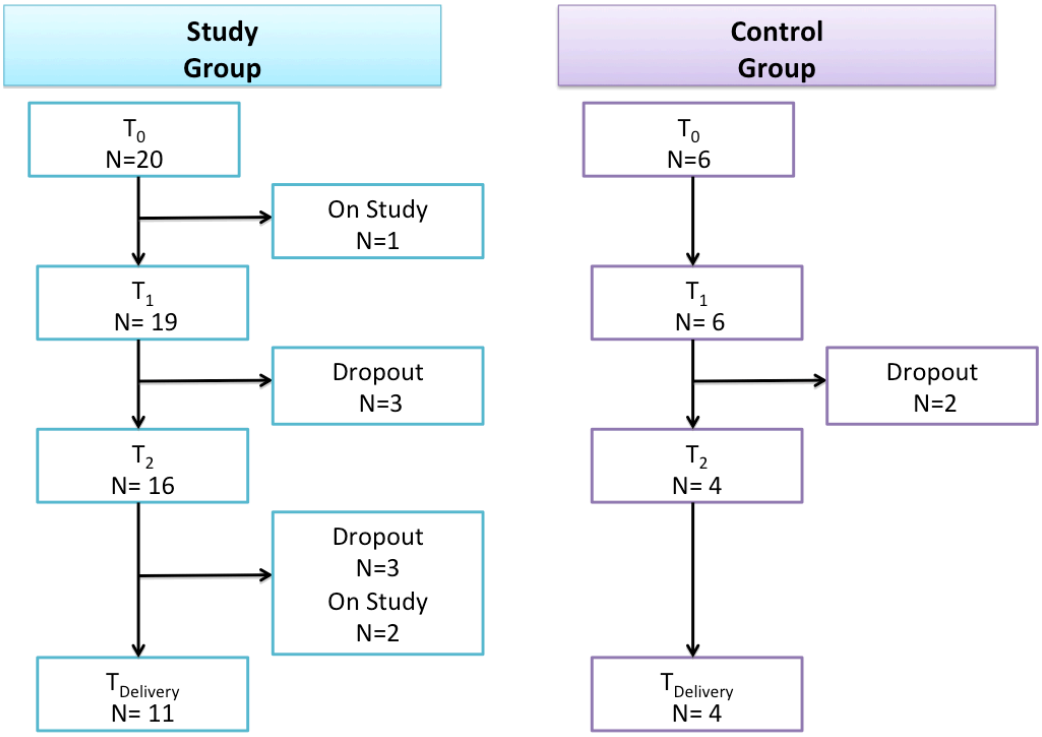

Figure 3: Diagram of the study population and dropout at every study time point.

Table 1 reports clinical data about pregnancy of the 15 women studied until delivery and newborn characteristics at birth. All women had a normal pregnancy, no cases of GDM neither of IUGR were observed in the two study groups.

	Study Group	**Control Group**	***p*[1]**
Length of Gestation (wks)	39.48 ± 1.23 (N=11)	39.79 ± 0.30 (N=2)	0.923
Weight Increment between T_0 and $T_{delivery}$ (kg)	10.33 ± 2.23 (N=11)	13.50 ± 4.12 (N=4)	0.212
Newborn Birth weight (gr)	3417 ± 286 (N=9)	3536 ± 557 (N=4)	0.825
Newborn Birth length (cm)	49.78 ± 0.83 (N=9)	50.25 ± 2.06 (N=4)	0.825

Table 1: Clinical data about pregnancy in the 15 women studied until delivery and newborns characteristics at birth.

[1] By Mann-Whitney U test.

Capsule ^{13}C DHA enrichment and oil FAs composition

^{13}C enrichment of DHA present in the two capsules is shown in table 2; table 3 reports the composition in FAs of the two oils.

	$\delta^{13}C$ vs PBD
DHA of biotechnological origin	-15.8
DHA of fish origin	-26.0

Table 2: DHA ^{13}C enrichment of biotechnological and fish origin contained in the two capsules.

FA (mol%)	Capsule DHA of biotechnological origin	Capsule DHA of fish origin
C8	1.09	1.13
C10	0.51	0.65
C14	10.56	3.91
C16	26.56	17.07
C16:1n-7	0.42	4.80
C18	1.09	4.78
C18:1n-9	13.62	10.56
C18:2n-6	1.50	1.56
C18:3n-6	0.36	0.74
C18:3n-3	0.16	0.53
C20	1.80	0.69
C20:3n-6	0.56	0.41
C20:4n-6	0.54	3.77
C20:3n-3	0.15	0.16
C20:5n-3	0	7.09
C22	0.17	0.41
C22:4n-6	0.15	0.52
C22:6n-3	40.33	45.27

Table 3: Composition in FAs of the two oils.

Effect of DHA supplementation on ^{13}C DHA plasmatic enrichment

As expected by the design of the study, ^{13}C DHA plasmatic enrichment changed significantly in the study group during the course of the time, no differences were observed in the control group.

	T_0 $\delta^{13}C$ vs PBD	T_1 $\delta^{13}C$ vs PBD	T_2 $\delta^{13}C$ vs PBD	$T_{Delivery}$ $\delta^{13}C$ vs PBD	p^1
Study Group	-28,06 ± 1.49^{a} N=16	-25.29 ± 2.52^{abc} N=15	-23.14 ± 2.64^{bc} N=12	-22.50 ± 1.73^{bc} N=9	0.0001
Control Group	-28,27 ± 0.48 N=6	-27.95 ± 0.52 N=6	27.25 ± 0.21 N=4	-27.60 ± 1.09 N=4	0.127

Table 4: Maternal ^{13}C DHA plasmatic enrichment in the two study groups measured at the different study time points.

[1] By Friedman's test 2-way ANOVA. Means with different superscripts are significantly different (p<0.005).

As reported in table 5 at delivery, in both the study groups, no differences were observed between maternal and cord ^{13}C DHA plasmatic enrichment.

	$\delta^{13}C$ vs PBD Maternal plasma	**$\delta^{13}C$ vs PBD Cord plasma**	***p*[1]**
Study Group (N=9)	-22.50 ± 1.73	-23.17 ± 2.37	0.139
Control Group (N=4)	-27.60 ± 1.09	-27.60 ± 1.22	1.000

Table 5: Maternal and cord ^{13}C DHA plasmatic enrichment in the two study groups at the time of delivery.

[1] By Wilcoxon Ranked Test.

Percentage of DHA endogenous synthesis

In the study group the percentage of DHA endogenous synthesis at T_2 and at $T_{delivery}$ were 54.7 ±13.0% and 53.1 ± 11.0%, respectively.

Effect of pregnancy on FA composition of plasma PLs

Between the two groups no differences were observed in maternal FA composition of plasma PLs before starting the supplementation and at the different study time points, neither between cords. The groups were then pulled together to evaluate the effect of gestation on FA composition of plasma PLs. To test for differences maternal and cord composition were compared.

The FA profiles of plasma PLs are shown in table 6 and 7.

FA (mol%)	T_0 (N=26)	T_1 (N=25)	T_2 (N=20)	$T_{Delivery}$ (N=15)	p^1
C14	0.71 ± 0.33	0.75 ± 0.35	0.64 ± 0.19	0.62 ± 0.22	0.257
C16	35.69 ± 1.20^{a}	35.57 ± 2.45^{ab}	35.90 ± 1.46^{ab}	37.18 ± 1.67^{b}	0.086
C16:1n-7	0.85 ± 0.31	0.82 ± 0.33	0.79 ± 0.19	0.94 ± 0.34	0.724
C18	9.54 ± 1.50	9.30 ± 1.52	8.78 ± 1.16	8.62 ± 1.11	0.763
C18:1n-9	8.53 ± 1.19^{ab}	8.29 ± 1.30^{b}	8.83 ± 0.98^{ab}	9.54 ± 0.94^{a}	0.012
C18:2n-6	19.97 ± 2.99	19.98 ± 4.58	20.28 ± 2.14	19.43 ± 2.00	0.615
C20	0.40 ± 0.12	0.39 ± 0.14	0.43 ± 0.16	0.41 ± 0.16	0.878
C20:2n-6	0.34±0.11	0.36±0.11	0.35 ±0.08	0.29 ±0.14	0.224
C20:3n-6	3.25 ± 0.81	3.22 ± 0.79	3.16 ± 0.61	3.21 ± 0.69	0.878
C20:4n-6	9.98 ±1.81	9.85 ±1.69	9.09 ±1.29	9.10 ±1.53	0.008
C22	0.73 ± 0.24	0.76 ± 0.29	0.85 ± 0.24	0.75 ± 0.30	0.687
C22:4n-6	0.31± 0.13^{a}	0.31 ± 0.12^{abc}	0.28± 0.11^{bc}	0.27 ± 0.12^{bc}	0.020
C22:6n-3	4.13 ± 1.28	4.79 ± 1.27	4.91 ± 1.35	4.54 ± 0.81	0.013
C24	0.51±0.20	0.51 ±0.23	0.59 ±0.19	0.44 ±0.24	0.184
C24:1n-9	1.50 ± 0.43	1.61 ± 0.48	1.73 ± 0.60	1.76 ± 0.47	0.140

Table 6: FA profile of plasma PLs, expressed in mol%, during the course of pregnancy.

[1] By Friedman's test 2-way ANOVA. Means with different superscripts are significantly different (p<0.005).

FA (mol%)	T Delivery (N=15)	Cord plasma (N=15)	p[1]
C14	0.62 ± 0.22	0.73 ± 0.28	0.256
C16	37.18 ± 1.67	36.13 ± 1.77	0.100
C16:1n-7	0.94 ± 0.34	1.16 ± 0.67	0.256
C18	8.62 ± 1.11	13.22 ± 2.91	0.001
C18:1n-9	9.54 ± 0.94	6.89 ± 1.88	0.001
C18:2n-6	19.43 ±2.00	6.41 ±1.35	0.001
C20	0.41 ± 0.16	0.66 ± 0.19	0.001
C20:2n-6	0.29 ± 0.14	0.28 ± 0.19	0.910
C20:3n-6	3.21 ±0.69	4.07 ±1.42	0.041
C20:4n-6	9.10 ±1.53	16.24 ±2.61	0.001
C22	0.75 ± 0.30	0.80 ± 0.38	0.730
C22:4n-6	0.27 ±0.12	0.72 ± 0.30	0.001
C22:6n-3	4.54 ±0.81	6.50±2.02	0.009
C24	0.44 ± 0.24	0.77 ±0.59	0.053
C24:1n-9	1.76 ± 0.47	1.44 ± 0.71	0.112

Table 7: FA profile of maternal and cord plasma PL, expressed in mol%, at the time of delivery.

[1] By Wilcoxon Ranked Test.

DISCUSSION

The first aim of this study was to evaluate the feasibility of measuring DHA endogenous synthesis using the "stable isotope natural abundance" approach.
The same approach was used by our research group to measure endogenous ARA and DHA synthesis over long periods in preterm infants fed formula containing LC-PUFAs[14].
Our results demonstrate that this method can also be used in pregnant woman following n-3 LC-PUFAs supplementation with 200 mg/die of DHA of biotechnological origin. In this way there is no need for administering highly enriched and expensive tracers or for altering the diet, moreover the recommended daily dose of DHA for pregnant and lacting woman resulted adequate for study's purpose.

Analysis of DHA enrichment of biotechnological origin was found to be different both from the one endogenously synthesized and from the one introduced by the diet, acting as a natural tracer. On the other hand, and as expected, DHA enrichment of fish origin was comparable to the values of DHA derived from plasma PLs at baseline in all study population. As a consequence, after the start of supplementation with n-3 LC-PUFAs, ^{13}C DHA enrichment of maternal plasma PLs did not change in the control group, while in the study group values measured at T_2 and $T_{delivery}$ were found to be significantly different from the values measured at T_0.

The percentage of endogenous DHA synthesis was calculated at T_2 and at $T_{delivery}$. Calculation was not feasible at T_1 because ^{13}C DHA enrichment in plasma PLs has not yet reached equilibrium (T_0 and T_1 were not statistically different). The mean value found at both times was about 50±10%. This means that half of the DHA found in plasma PLs comes from *de novo* synthesis. Nevertheless this value might be a little over-estimated. DHA introduced by the diet and the one derived from lipolysis of adipose tissue can not be discriminated from the one endogenously synthesized since their ^{13}C enrichment should not differ.

As an evidence of the well known maternal transfer of LC-PUFAs across placenta, DHA enrichment of cord plasma PLs was found to be not significantly different from maternal values, in both the study groups.

To test if plasma PLs' FAs composition changes significantly during the course of gestation the profile at T_0, T_1, T_2 and $T_{delivery}$ were compared by Friedman's ANOVA. Oleic acid (C18:1n-9) significantly increased during gestation, adrenic acid (C22:4n-6) steadily diminished while ARA decreased significantly from T_1 to T_2. These findings are in line with previous data by Al *et al*. who studied maternal FAs patterns during normal pregnancy, from the 10^{th} to the 40^{th} week of gestation[52]. In our study population the start of supplementation with n-3 LC-PUFAs increased the relative amount of DHA, being T_0 and T_1 significantly

different. Al *et al*. reported that DHA increased until 18th week of gestation and then diminished, since women were not supplemented during pregnancy this might explain our different findings.

Data obtained from comparisons between maternal and cord plasma PLs' Fas compositions are in agreement with literature data[53,54]. Percentages of saturated FAs (stearic and arachidic acid, lignoceric acid showed only a tendency to significance) and LC-PUFAs (ARA, dihomo-gamma-linolenic acid, C22:4n-6, and DHA) were found to be higher in cord samples, the opposite was observed for LA and C18:1n-9.

Possible future developments

To the best of our knowledge no data are available on women's ability to synthesise DHA during the course of gestation. Literature data suggest that estrogens positively influence DHA synthesis[22,28]. Estrogen levels are significantly higher in pregnant women compared to non-pregnant ones. By the use of this method we can than evaluate: i) if DHA endogenous synthesis is higher during pregnancy, and ii) if it changes during the course of pregnancy as a response to the increased estrogen levels observed in the last trimester.

Comparison of maternal and cord ^{13}C DHA enrichment is an index of fetus' dependency on maternal LC-PUFAs transfer. In complicated pregnancies where placental transfer of LC-PUFAs seems to be impaired[61,62], evaluation of ^{13}C DHA enrichment values as of relative amounts of DHA found in maternal and cord samples may help in understanding if non physiologic pregnancies affect fetus' ability to synthesise DHA.

Chapter 3

The pulmonary surfactant system

LUNG ALVEOLAR SYSTEM AND SURFACTANT

In humans, lung alveolar system represents the largest surface exposed to the environment covering up, in adults, 70-120 m^2. This area is necessary to allow an adequate level of gas exchange with blood to support the metabolic functions. The alveolus, the basic respiratory exchange unit, consists of an epithelial layer and extracellular matrix surrounded by capillaries; it has a radius of about 0.1 mm and a wall thickness of about 0.2 μm[65]. Three types of cells are found in alveoli: pneumocytes I or Type I alveolar epithelial cells, pneumocytes II or Type II alveolar epithelial cells, and macrophages. Type I cells, which cover approximately 95% of the alveolus surface[66], have a flattened morphology, with organelles clustered around the nucleus, perfectly designed for an efficient gas exchange. Type II cells have a cuboidal shape and even if they account only for the remaining 5% of the alveolus surface they represent the 60% of alveolar epithelial cells. Type II cells are known to have different functions and the main one is the secretion of lung surfactant. They also contribute to epithelial repair after injury, since they can proliferate and differentiate into Type I cells, and, finally, they are involved in host defence secreting cytokines[66]. Macrophages localized in the alveoli remove particles and microorganisms coming with the air and degrade part of the spent surfactant.

The surface tension at the air-water interface, together with the shape and the small dimension of alveoli, explains their tendency to collapse opposing lung inflation and thereafter respiration[65].

Surfactant, secreted by cells Type II, forms a lipid monolayer at the air-water interphase that reduces the surface tension, hence stabilizing the alveoli and preventing their collapse at the end of expiration. Consequently, it also reduces the work of breathing during lung expansion, and prevents alveolar oedema.

Surfactant exerts also a critical role in host defence representing an efficient barrier against environmental insults, like pathogens.

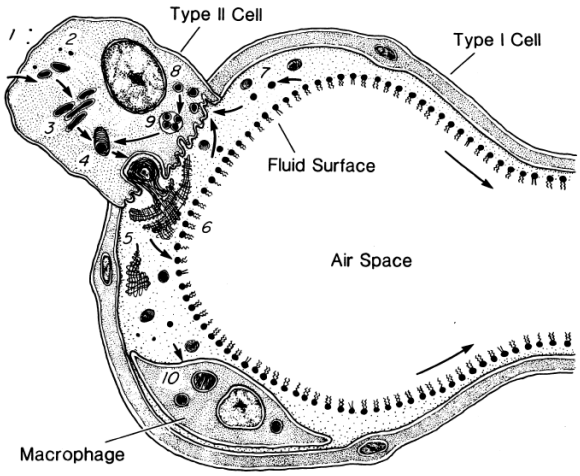

Figure 1. Structure of alveoli.
From Hawgood *et al*.[67]

PULMONARY SURFACTANT COMPONENTS

Pulmonary surfactant is a complex mixture that consists of roughly 90% of lipids and 10% of proteins.

80-90% of the whole lipid portion is represented by phospholipids (PLs), while cholesterol comprises the largest amount of neutral lipids[iv].

Phosphatidylcholine (PC) makes up the major part (70-80%) of the PLs and generally around 50-60% of PC molecules contain two saturated fatty-acyl moieties (DSPC). Dipalmitoyl-PC (DPPC) is the main type of DSPC[68]. Though DPPC is not the sole component of surfactant in lowering the surface tension, it is the major surface-active component. DPPC alone is capable of generating low surface tensions approaching values of about 0 mN/m[69].

The second most abundant PL in surfactant is phosphatidylglycerol (PG) that is present at 7-15% of the total PLs; other surfactant minor PLs include phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylinositol (PI), and phosphatidylserine (PS)[70].

The precise role of PG, rather than PI, are not completely understood[69] but evidences suggest that PG may play an important role in regulating innate immunity and respiratory syncytial virus infection[71,72]. Other minor PLs are unlikely to have surface active properties and speculations suggest possible roles in metabolism and in its signalling events[69].

[iv] Free fatty acids and glycerides are found only in trace amounts.

Cholesterol, the most abundant of neutral lipids, when added to DPPC alone or DPPC and PG, increases adsorbtion rates[69], however, when present in improper amounts, it has been associated with impaired surface activity[73].

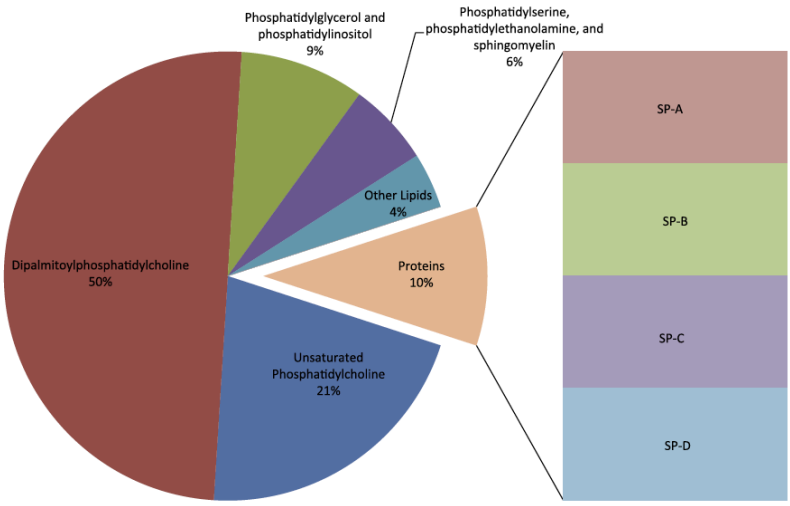

Figure2. Composition of pulmonary surfactant
From Agassandian *et al.*[70]

The four surfactant-associated proteins (SPs) A, B, C, and D constitute the remaining 10% of pulmonary surfactant. SP-A and SP-D have hydrophilic structures that consist of large macromolecular assemblies and belong to the family of collectins[74] while SP-B and SP-C are small proteins with an extremely hydrophobic nature. All of these proteins participate to surfactant functions and formation. Thanks to protein biochemistry and genetic techniques it has been possible to identify the specific roles of each SP.

SP-A has been the first of the SPs to be purified[75] and analysed for its primary structure[76]. SP-A has four domains: i) an amino terminus, ii) a collagen-like domain, iii) a neck region, and iv) a carbohydrate recognition domain. SP-A, once secreted, is assembled into a large oligomer of 18 monomers, grouped in six trimeric subunits, whose alignment pattern resembles that of a "bunch of tulips"[67].

Thanks to a carbohydrate recognition domain, SP-A recognizes oligosaccharide moieties on various lung pathogens, thus opsonizes microorganisms, enhances their uptake by phagocytes, and stimulates the production of free oxygen radicals[77,78]. SP-A also inhibits directly the proliferation of Gram-negative bacteria by increasing the permeability of the microbial cell membrane[77].

Beside its role in the innate defence of the lung[79,80], SP-A is important for the formation of tubular myelin, a structure formed by surfactant as soon as it is secreted from type II cells and thought to be the structure from which the surface films forms *in vivo*[74]. SP-A is not absolutely required for breathing: in a murine model a null mutation of the SP-A gene was found to interfere with the formation of tubular myelin but did not alter postnatal survival, pulmonary function, and alveolar PLs pool size[81]. Surface activities of surfactant isolated from SP-A over-expressing mice and SP-A knockout mice are reported to be more and less resistant to serum protein inhibitors, respectively[81,82]. A number of studies used models of SP-A knockout mice to investigate its function as a collectin, the most frequent phenotypic characteristic observed in SP-A –/– mice was a pulmonary host defence defect[83–87].

SP-D is structurally similar to SP-A but it consists of a large oligomer of 12 monomers, grouped in four trimeric subunits, assembled in a cruciform-like structure.
Also SP-D is necessary, like SP-A, in host defence against lung pathogens. SP-A and SP-D functions have been described to be distinct, as well as complementary[80,88].
Unlike the SP-A –/– mice, the SP-D –/– mice manifest surprisingly abnormal lung structure and impaired surfactant metabolism. Targeted inactivation of the SP-D gene causes: i) marked accumulation of surfactant[89] and ii) inflammation and emphysema associated with an increased number of activated alveolar macrophages[90,91].

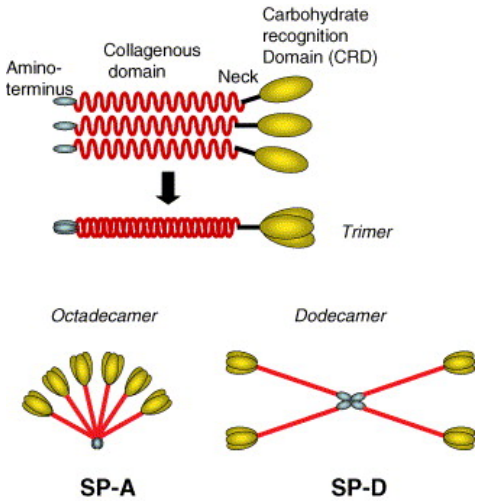

Figure 3. SP-A and SP-D structures
From Sano *et al.*[88]

SP-B is a relatively small homodimeric protein, comprising two sub-units held together by a disulphide bond and it is found to be closely associated with surfactant PLs. It is expressed in a cell specific manner by Type I and Type II cells[92]. SP-B has several functions primarily related to surface tension reduction and metabolism of pulmonary surfactant[93]. When added to mixture of surfactant-like PLs, SP-B greatly enhances their surface tension-reducing properties[94], facilitates lipid insertion from lipid vesicles into the air-liquid monolayer[95,96], and counteracts the inhibition of surfactant function due to plasma-derived proteins, that is assumed to occur under conditions of alveolar protein leakage[97].

In the presence of calcium and PLs, SP-B also interacts with SP-A to form tubular myelin and is essential for the genesis of lamellar bodies[98], unique organelles of Type II cells in which intracellular surfactant is stored.

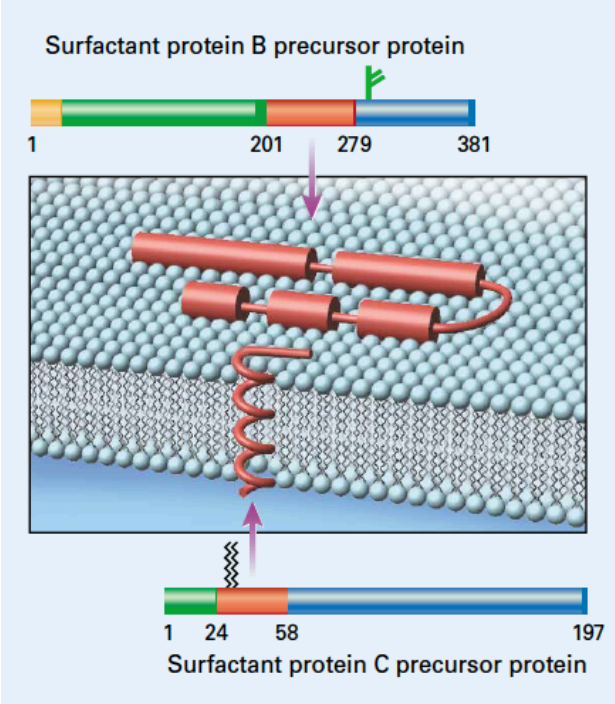

Figure 4. Proteolytic processing of SP-B and SP-C into mature forms and their interactions with PLs bilayers. SP-B is represented as a monomeric structure. From Whitsett *et al.*[99]

Targeted disruption of the SP-B gene resulted in lethal postnatal respiratory failure associated with pulmonary actelectasis[100]. SP-B −/− mice showed a normal composition in surfactant PLs but no lamellar bodies and tubular myelin were detected. The phenotype of SP-B deficient mice, with a marked decrease in fully processed SP-C and an accumulation of

the pro-protein, indicated SP-B critical role into the appropriate processing of SP-C precursor[99–101].

Consistently with the lung epithelial-cell specific expression of SP-B, abnormalities observed in SP-B −/− mice and in infants with hereditary SP-B deficiency were confined to the respiratory tract[100].

SP-C is one of the most hydrophobic proteins in the proteome[99] and it is expressed only in Type II cells. It consists in a very regular and rigid alpha-helix that covers approximately two thirds of its sequence and a N-terminal domain with two palmitolyated cysteine residues[65,74]. SP-C activities largely overlap those of SP-B: when added to PLs mixture SP-C enhances the spreading and stability of PLs in a similar but less active manner to that of SP-B[95,99]. SP-C contributes to respiratory homeostasis and it may also play a fundamental role in pulmonary immunology, both during acute lung infection and chronic lung disease[102]. SP-C −/− mice develop a severe progressive pulmonary disorder with histologic features associated with emphysema, monocytic infiltrates, epithelial cell dysplasia, and atypical accumulations of intracellular lipids in Type II cells and alveolar macrophages[103].

In agreement with the proposed immunological and immunomodulatory role of SP-C, in SP-C knockout mice the pulmonary clearance of RSV was found to be diminished and associated with increased and prolonged inflammation[104].

SURFACTANT METABOLISM: SYNTHESIS, SECRETION AND CATABOLISM

The secretion and re-utilization cycle of surfactant involves a number of complex regulated processes: synthesis, packaging into lamellar bodies, transport and secretion into the alveolar lining fluid and finally degradation and recycling back into the Type II cells.

Surfactant components are synthesised in the endoplasmatic reticulum, then packaged and stored in the form of tightly packed membranes in lysosome–related organelles called lamellar bodies.

As described above, surfactant PLs are particularly enriched in saturated fatty acids, with DSPC being the most abundant and important component.

The *de novo* synthesis of PC occurs by the cytidine diphosphocholine (or Kennedy) pathway, the predominant one in most tissues[70]. It is a pathway of three reactions, by which fatty acids, synthesised *de novo* by the lungs, are incorporated into newly synthesised PC. The α isoform of CTP:phosphocholine cytylytransferase (CCTα) represents the rate-limiting and rate-regulatory enzyme of PC synthesis in mammalian cells[105]. Initially, PC is enriched in unsaturated species, but, thanks to acyl remodelling and selective transport mechanisms, the final composition of active functional surfactant can be achieved inside lamellar

bodies[106]. PL transfer proteins mediate surfactant PLs transport from endoplasmatic reticulum to the lamellar bodies, where membrane transporters proteins, like the ATP-binding cassette (ABC) transporter A3 (ABCA3[v]), finally catalyse the trans-membrane movement of PLs.

Hydrophobic proteins B and C are synthesised in the endoplasmatic reticulum as large precursors, then they are transported from the trans-Golgi to the lamellar bodies through the multivesicular bodies. Proteolytic processing of the pro-proteins to their respective mature peptides occurs in an acidic compartment distal to the Golgi, consistent with processing in the multivescicular bodies[92]. Multivesicular bodies are organelles containing numerous internal unilamellar vesicles and are believed to precede the final assembly and storage of surfactant.

In vitro studies and SP-B knockout mice models suggest that the newly processed SP-B may associate with the internal vesicles of the multivesicular bodies and promote vesicle fusion, which, in turn, may facilitate formation of the membrane lamellae[92].

Once mature, lamellar bodies move intracellularly to the apical area of Type II cells, fuse with the plasma membrane and ultimately secrete surfactant into alveolar space by exocytosis.

Both synthesis and secretion of surfactant are influenced by chemical or physical mechanisms. Synthesis of surfactant is stimulated by glucorticoids, thyroid hormones and other growth factors, their mechanism generally increases enzymes activity or up-regulates the expression of enzymes involved in surfactant components synthesis. Glucorticoids, for example, stimulate surfactant synthesis at two different steps. They increase *de novo* synthesis of fatty acids, by direct activation of Fatty Acids Synthase[107,108], and that of PC by regulating CCTα activity, controlling mRNA stability[109] or the transcriptional levels in the lung[110].

The secretion of lamellar bodies can be mediated by a transient rise in intracellular calcium or by a cyclic AMP-dependent pathway. In the intact lung, surfactant secretion can be stimulated by hyperventilation (the physical stretch generates a transient increase in cytosolic calcium), by second messengers, and by agonists that directly stimulate type II cells called secretagogues[111]. Since the effects of agonists are modest compared to hyperventilation it appears that *in vivo* regulation may be controlled by physical stretch rather than circulating or paracrine mediators [111].

[v] ABCA3 is a lipid transport protein required for normal synthesis and storage of pulmonary surfactant in alveolar Type II cells[70].

The mechanisms that take place from the unpacking of secreted lamellar bodies to the formation of surface active film have been extensively studied.

The fusion of lamellar bodies with plasma membrane causes a connection between internal medium of lamellar bodies and extracellular environment and this is considered to be the trigger for the unpacking of secreted lamellar bodies[74]. The secreted surfactant membranes, in presence of saturated PLs, SP-A, SP-B, and calcium, rearrange into tubular myelin: a highly ordered surfactant structure, lung specific and produced in the alveolar airspaces[112].

SP-A, as SP-D, is secreted by alternative pathways to that of lamellar bodies and it is the essential key player in tubular myelin formation, as proved by knockout SP-A mice models[81]. Moreover membrane spacing in tubular myelin appears to be determined by the collagen-like domain of SP-A[113].

SP-B and SP-C finally promote the rapid adsorption of tubular myelin and form the air-water pulmonary interface film.

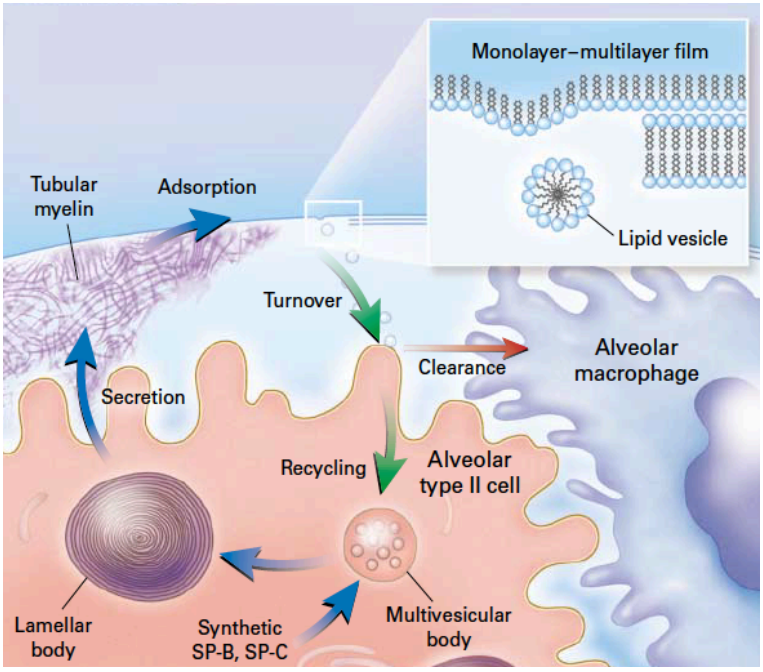

Figure 5. Surfactant metabolism cycle

From Whitsett *et al.*[99]

Compressions and expansions of surfactant caused by the breathing cycle transform surfactant into a non-surface-active form, so, after functioning as a surface tension-reducing agent, surfactant must be removed from the airspaces to balance its active secretion, since the pool size has to be maintained constant[114,115].

Type II cells and macrophages represent the two distinct mechanisms by which surfactant is cleared from the airways, both of them contributes equally[115]. There are two main differences in these mechanism: i) surfactant internalized by macrophages is completely catabolized, while surfactant taken up by type II cells is either recycled or catabolised, ii) taken up mechanism by type II cells is more selective depending on the presence of SPs and on the physical structure of surfactant, which is influenced by SP-D[115].

SURFACTANT DYSFUNCTION AND LUNG DISEASE

Surfactant dysfunction is related to several types of lung diseases both in pediatric and in adult patients.

Respiratory distress syndrome (RDS) and acute respiratory distress syndrome (ARDS) represent two emblematic conditions that prove how an active surface film is necessary for normal breathing and for an efficient gas-exchange.

RDS is a syndrome caused by lack of surfactant into the airways of prematurely born infants while ARDS can affect all age groups and is characterized by Type II epithelial cells injury and surfactant inactivation, a mechanism by which surface activity is decreased or completely abolished by chemical or physical inhibitors.

Chemical inhibitors degrade surfactant components while physical inhibitors compete with surfactant components to adsorb at the air-water interface. Reactive oxygen species and lytic enzymes like phospholipases or proteases are examples of chemical inhibitors while plasma proteins, haemoglobin, cell membrane lipids, free fatty acids, meconium, and many other blood- and tissue-derived compounds are physical inhibitors.

Endothelial and alveolar lining cells injuries increase the permeability of alveolar-capillary barrier allowing plasma components to reach the airways and inactivate surfactant. Sepsis, haemorrhage, and other forms of lung injury (such as pulmonary contusion) represent causes of ARDS development in adults while in children the most common cause is represented by infection[116].

The two following chapters of this thesis will deal with surfactant composition during acute lung disease in newborn infants.

First we studied surfactant composition in term newborns affected by pneumonia and compared to that of newborns without lung disease in order to comprehend if alterations in the level of SPs could contribute to the reduced pulmonary compliance observed in these patients. Then we studied a population of pre-term infants affected by RDS and we try to understand if and how histological chorioamnionitis and different gestational age can impact on surfactant composition.

Chapter 4

Surfactant protein B and A concentrations in neonatal pneumonia

INTRODUCTION

Neonatal pneumonia represents a significant health concern in neonatal medicine, especially in developing countries.

In 2012 an updated systematic analysis reported that 64% of children who died in the first 5 years of their life died of infectious causes, and pneumonia was the leading cause representing almost 20% of total deaths worldwide[117]. Most of these deaths (>95%) occur in developing countries since pneumonia is more common and more likely to be fatal in children with severe malnutrition[118]. In the developed countries, pneumonia annual incidence is estimated to be 33 per 10000 in children younger than five years[119] and it is a leading cause of hospitalization among children in the United States[120].

In order to eradicate infection and provide an adequate support of gas exchange, administration of antibiotic agents and mechanical ventilation represent the standard therapy for severe pneumonia.

Bacterial pneumonia in newborn babies can cause problems with the functioning of pulmonary surfactant, thus, for these infants breathing can be very difficult.

A well functioning surfactant system is essential for lung stability, a good respiratory compliance and an efficient gas exchange. Surfactant homeostasis and functions depend on the presence and the relative amounts of its components. A lack or an altered amount of one or more surfactant components can have dramatic consequences.

Adult patients affected by ARDS and severe pneumonia requiring mechanical ventilation show a decreased respiratory compliance and an impaired gas exchange. In these patients a marked impairment in the composition and in the biophysical function of surfactant have been documented[121–129]. SPs were decreased[123,126,127], the relative PC palmitic acid content was reduced[121,128], and a reduction in PG or PC could be observed throughout[121–123,125,129]. These reductions were generally associated with an increase in the

relative amounts of minor lipids such as phosphatidylinositol, phosphatidylethanolamine, phosphatidylserine, and sphingomyelin.

In pediatric patients while it's well established that surfactant deficiency causes respiratory failure in newborns with RDS[130], the reduced pulmonary compliance observed in term newborns at the onset of pneumonia still does not have a clear explanation.

Understanding of SPs changes in children with lung infection is limited and sometimes discordant. Reduced amounts of SP-A have been found in infants with acute viral bronchiolitis, including forms due to Respiratory Syncytial Virus (RSV)[131,132] and bacterial and viral pneumonia[133]. Kerr *et al.*[132] found decreased levels of SP-B in the bronchoalveolar lavage fluid (BALF) of children with severe RSV bronchiolitis, while Le Vine *et al.* found concentrations that did not differ from controls in infants with bacterial and viral pneumonia[133]. Moreover, a recent study in children with acute lung injury, showed no change in either BALF SP-B and SP-A concentrations[134].

AIM OF THE STUDY

The aim of this study was to understand SPs role and changes during neonatal pneumonia.

We therefore studied SP-B and SP-A amounts in tracheal aspirates (TAs) of term newborns during pneumonia clinical course, from the peak of the disease until the clinical improvement just before extubation and compared with those of newborns with healthy lungs, in order to comprehend if alterations in the level of SPs could contribute to the reduced pulmonary compliance observed in patients with pneumonia.

We also investigated if SP-B and SP-A levels correlate with the degree of respiratory failure and with DSPC turnover rate, that is known to be increased, with shorter half-life ($t_{1/2}$) values, during various acute lung diseases[135,136].

MATERIALS AND METHODS

Study population

From January 2011 to December 2013 we prospectively recruited 13 term newborns (37 to 41 weeks gestational age), up to 10 days of life with neonatal pneumonia. We also recruited 15 newborns with no lung disease who required mechanical ventilation for elective surgery or neurological impairment. All newborns were admitted to the Neonatal Intensive Care Units of the University of Padua or of the Polytechnic University of Marche, Ancona, Italy.

The diagnosis of pneumonia was based on the 2008 CDC/NHSN (Centers for Disease Control and Prevention/National Healthcare Safety Network) criteria for pneumonia in infants ≤1 year of age. These criteria are basically limited to clinically defined pneumonia and are: i) chest x-ray showing new or progressive or persistent infiltrate, consolidation or cavitation or pneumatoceles; ii) worsening gas exchange (desaturation or rise in oxygen requirement or rise in ventilation demand); iii) at least 3 of the following: temperature instability with no other recognized cause; leucopenia (<4000 WBC/mm^3) or leucocytosis (≥15000 WBC/mm^3) with left shift (≥10% band cells); new onset of purulent sputum, or change in character of sputum, or increased respiratory secretions, or increased suctioning requirements; apnea, tachypnea, nasal flaring with retraction of chest wall or grunting; wheezing, rales or rhonchi; cough; bradycardia (<100 beats/min) or tachycardia (>170beats/min). Diagnosis of bacterial pneumonia was based on these indirect clinical criteria and on the response given by every infant to the antibiotic therapy.

Inclusion criteria were: respiratory failure (newborns with pneumonia) requiring mechanical ventilation with a FiO_2>0.35, a Mean Airway Pressure>7 cm H_2O, and a prediction to be mechanically ventilated for at least 48 h.

Control newborns had no lung disease (no clinical and laboratory signs of infection, normal chest x ray, FiO_2<0.30) but required mechanical ventilation as a result of major surgery or neurological impairment leading to poor airway control.

Another inclusion criteria for both groups was the presence, at the start of the study, of an arterial line, placed for invasive arterial blood pressure monitoring and/or arterial blood gas analysis.

Exclusion criteria were: i) severe congenital malformations; ii) chromosomal abnormalities; iii) exogenous surfactant administration in the last 48 h before the start of the study.

The study protocol was approved by the Local Ethics Committee of the University of Padua and by the Ethics Committee of the Polytechnic University of Marche, and written informed consent was obtained from both parents.

Study design

We collected TAs every 6 h for the first 72 h and then every 12 h until extubation in newborn with pneumonia and in controls; 0.5 ml of blood was drawn at the start of the study in EDTA-containing tubes to perform arterial gas analysis and plasma urea determination. If additional parental consent was obtained, we also measured DSPC turnover with a tracer dose of (U-^{13}C-PA) dipalmitoyl-phosphatidylcholine (U-^{13}C-PA DPPC) mixed with 2 to 5 mg/kg of Curosurf, used as a spreading agent.

Administration of tracing, TAs collection and processing were standardized and performed as previously reported[136,137]. The tracer was administered via a small catheter inserted through the endotracheal tube at the carina level. TAs were performed as follows: 1 ml of 0.9% saline was instilled into the endotracheal tube and, after the neonate was ventilated by hand bagging, secretions were collected through a Lukens trap. Samples were kept at +4° C and processed within 3 h. TAs were brought to a final volume of 2 mL with saline, gently vortexed and then centrifuged t 400 x *g* for 10 minutes to discard mucus and cells. The supernatant was divided in aliquots and stored at -80° C until analysis. Fifty µl of each tracer dose mixed with the spreading agent were stored at -80° C too, in order to determine U-^{13}C-PA DPPC enrichment.

We used TAs instead of BALF because of ethical concerns, since TA suctioning is a routine procedure to clean endotracheal tube whereas BALF are seldom performed in neonatal intensive care. Moreover a recent study suggested that TAs surfactant phospholipid composition is similar to that obtained from BALF[138].

Vital and ventilator parameters were recorded every hour. Arterial blood gas analysis were recorded at the start of the study and then at least every 12 h, according to the policy of the two units or when requested by the attending physician. Respiratory failure was assessed by: PaO_2/FiO_2, oxygenation index (OI), and alveolar-arterial oxygen gradient ($AaDO_2$), calculated as $((MAP*FiO_2/PaO_2)*100)$ and $((FiO_2*(760-47))-(PaCO_2/0.8))-PaO_2$, respectively.

Analytical Methods

Quantification of SP-A and SP-B

SP-B and SP-A amounts in TAs were measured by ELISA using anti-human SP-B and SP-A antibodies raised in rabbits, produced in our laboratory, and using SP-B and SP-A proteins isolated from BALF of patients with alveolar proteinosis as standards [139,140].

SP-B was measured according to a previously described ELISA technique[141].
TAs were mixed with propanol 1/1 (v/v), transfer to polystyrene plates, and dried at 37° C, then trifluoroethanol was added and dried too at 37° C. Plates were added with methanol and incubate for 20 minutes at room temperature, the organics were decanted and the plates were washed with phosphate-buffered saline (PBS) 0.5% (v/v) Tween 20 (PBST 0.5%). This step was repeated 2 times. Plates were saturated with BSA 1% (w/v) in PBS for 2 hr at room temperature and then washed with PBST 0.5%. A solution of anti SP-B (1:10000 in 1% BSA/PBS) was applied for 12 hr at room temperature. After washing, the biotinylated anti-rabbit antibody was added and plates were gentle shaked for 2 hr at room temperature. Plates were washed to remove the secondary antibody excess and then a solution containing 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) and H_2O_2 in sodium phosphate-acetate buffer (pH 4.2) was added and incubated for 4 hr. After the addition of a solution of SDS 1% (w/v) a stable green solution was obtained and optical density was than read at 450 nm. Amount of SP-B was calculated from a standard calibration curve from 0 to 30 ng of SP-B.

SP-A content in TAs was determined by a competitive ELISA developed by our research group[139]. Briefly, polystyrene plates were coated for 1.5 h at room temperature with a solution of purified human SP-A solubilized in TRIS, then washed with a phosphate buffer saline 0.1% (v/v) Triton X-100 solution (PBST 0.1%). Plates were blocked for 1 h with 3% BSA in PBST 0.1%. Standards and TAs samples were incubated for 2 h at 37°C with 3% BSA in PBST 0.1% in the presence of the primary antibody diluted 1:30 (rabbit anti-human SP-A). Plates were then turned upside down to completely remove the blocking solution and standards and TAs samples, previously incubated, were added to each well. After 2 h of gentle shaking at room temperature, plates were washed with PBST 0.1% and incubated for 1 hr at room temperature with gentle shaking with a solution of biotinylated rabbit secondary antibody diluted 1:1000. The secondary antibody excess was removed by PBST 0.1% washing and then a solution of Ortho-Phenylenediamine and H_2O_2in citrate buffer (pH= 4.65) was added to each well. Plates were kept in the dark for 15 minutes, after the addition of H_2SO_4 2M a stable orange solution was obtained and optical density was than

read at 492 nm. Amount of SP-A was calculated from a standard calibration curve from 0 to 5 ng of SP-A.

Isolation, quantification, and enrichment of DSPC

DSPC was extracted from TAs according to the method of Bligh and Dyer[142] after the addition of internal standard (pentadecanoylphosphatidylcholine). The lipid extract was dried under nitrogen at 37° C and then reacted with osmium tetroxide. Surfactant DSPC was then isolated by thin layer chromatography using a mixture of cholorform/methanol/potassium chloride/triethylammine (40/12/33/8/24, v/v/v/v/v). After drying DSPC spot was visualized spraying the TLC plate with 1,2-dichlorofluorescein and detected under UV-light. The spot was scraped off and collected in glass tubes. DSPC fatty acids were hydrolyzed and derivatized by incubation for 60 min at 100°C with 3 mL HCl/MeOH. After cooling, HCl was neutralized by adding 3 mL of 10% K_2CO_3. Methyl esters were extracted with 250 µL of hexane. To quantify the amount of DSPC the extract was analysed by using a gas chromatograph with a flame ionization detector (HP-5890, Amstelveen, Olanda)[143].

The same extract was analyzed by gas-chromatography-mass-spectrometry (GC-MS, 6890N - 5973 inert, Agilent Technologies, Milan, Italy) to measure U-^{13}C-palmitate enrichment. The gas chromatograph was coupled with a quadrupole mass spectrometer operating in electron impact ionization mode.

The results were expressed as mole percent excess (MPE) referring to a calibration curve[137,144]. MPE represents the increase in the mole percentage of each palmitic acid (U-^{13}C-palmitate) above the baseline value obtained at the start of the study before tracer's administration.

Quantification of PLs

PLs were extracted from TAs according to the method of Bligh and Dyer[142] and the extract, dried under nitrogen at 37°C, was used for the measurement of phospholipid phosphorus as described by Bartlett[145]. Briefly, the dried extract was incubated for 60 min at 200°C with H_2SO_4 5M. After cooling H_2O_2 was added and the glass tubes were incubated again at 200°C until H_2O_2 had completely evaporated.

The tubes were then placed in a boiling water bath for 20 minutes after adding deionized water, ammonium molibdate 5% (w/v) and Fiske and Subbarow reagent. The absorbance of cool samples was read at 830 nm and the amount of phosphorous was calculated from a standard calibration curve from 0 to 2 µg of phosphorous. To get the total amount of PLs in TAs the measured value of phosphorous was multiplied by 25.

Urea determination in plasma and TAs

A commercially available colorimetric assay was used to measure urea in plasma and in TAs to normalize SP-B, SP-A, DSPC and PLs for the epithelial lining fluid (ELF) volume[146].

Calculation and Statistics

SP amounts in TAs were expressed as % of PL or as concentrations in ELF[146]. DSPC $t_{1/2}$ and pool size were calculated as reported in previous studies published by our group[137,144]. Briefly after conversion of the enrichment, measured at different times, into tracer/trace ratio (TTR), the TTR values were fitted to a one or two phase exponential decay and the equation was used to calculate DPPC kinetics parameters. The pool size in which the tracer distributed was calculated on the basis of Fick principle, while the half-life of (U-^{13}C-PA) DPPC was calculated from TTR decay[vi].

Data are presented as mean ± SD or median (IQR) based on the variable distribution. Comparisons within the groups were assessed by Wilcoxon signed-rank test. Comparisons between the two study groups were assessed by Student t test or Mann-Whitney U test, according to the variable distribution. Significance was set at $p \leq 0.05$. Pearson's correlation analysis was performed to test the association between SP-B (%PL) ratio, SP-B ELF, SP-A (%PL) ratio and SP-A ELF measured at the start of the study, with the OI and DSPC $t_{1/2}$.

Statistical analysis was performed using PASW Statistics 18.0 for Windows (SPSS Inc, Chicago, IL).

RESULTS

We studied 13 newborns with pneumonia and 15 controls with no lung disease. Eight controls were extubated immediately after surgery and therefore, for these patients, we collected only one TA sample.

ELF SP-B, SP-A, DSPC, and total PLs concentrations at the start of the study could be measured in 11 out of 13 newborns with pneumonia and in 14 out of 15 control newborns.

Close-to-extubation, and at least after 6 h from the previous TA, SP-B, SP-A, DSPC, and PL could be measured in 11 out of 13 newborns with pneumonia and in 7 out of 15 controls. At

[vi] In biexponentional models the decay used to calculate the half-life was the one of the second part of the curve.

this time point we could not express the amount of SPs as weight/volume of ELF because the arterial line had already been removed.

DPPC kinetic was measured in 19 newborns from whom additional parental consent was obtained, 12 of the pneumonia group and 7 controls. In three newborns (2 with pneumonia and 1 with no lung disease) only DPPC kinetics was measured because the sample was too scarce to perform SPs' analysis.

Clinical characteristics and respiratory parameters of the study patients, are reported in Table 1. None of the study patients received exogenous surfactant.

The two study groups were comparable for gestational age, birth weight, age, and hours of mechanical ventilation at the study start, while significant differences were observed in C-Reactive Protein, fraction of inspired oxygen (FiO_2), OI, $AaDO_2$, PaO_2/FiO_2 ratio, peak inspiratory pressure (PIP), positive end expiratory pressure (PEEP) and mean airway pressure (MAP) at the start of the study. A tendency to lower values was observed for PaO_2 in the pneumonia group compared to controls.

By paired analysis, FiO_2, OI, $AaDO_2$, PIP and MAP decreased significantly during the study in the pneumonia group: from 0.50 (0.25-0.60) to 0.25 (0.22-0.29) (p=0.008); from 5.7 (3.1-7.5) to 2.2 (1.8-2.7) (p=0.002), from 194.5 (75.6-307.2) to 45.6 (37.9-77.1) (p=0.006), from 20.8 ± 3.6 to 17.2 ± 2.2 (p=0.005) cm H_2O, from 8.7 ± 3.1 to 6.1 ± 0.7 (p=0.003) cm H_2O respectively. PaO_2/FiO_2 increased from 173.9 ± 84.3 to 273.8 ± 89.4 (p=0.017); no statistical differences were observed in the control group.

PaO_2 and PEEP did not change significantly during the study in both groups.

	Pneumonia group	Control group	p^1
Gestational Age (wks)	38.9 ± 1.4	38.6 ± 1.4	0.586
Birth Weight (g)	3226 ± 605	3064 ± 650	0.504
Age at study start (days)	2.3 (1.0-5.4)	2.4 (1.0-8.0)	0.730
C-reactive Protein at study start (mg/l)	26.1 (10.4-92.9)	3.3 (2.9-4.9)	0.003
Mechanical Ventilation at study start (h)	44 (12-69)	24 (18-52)	0.593
FiO_2 at study start	0.50 (0.25-0.60)	0.21 (0.21-0.23)	0.001
PaO_2 at study start	67.8 ± 15.4	79.7 ± 17.7	0.060
OI at study start	5.7 (3.1-7.5)	1.4 (1.2-1.7)	<0.001
$AaDO_2$ at study start	194.5 (75.6-307.2)	20.1 (16.6-36.6)	<0.001
PaO_2/FiO_2 at study start	173.9 ± 84.3	361.7 ± 110.6	<0.001
PIP at study start (cm H_2O)	20.8 ± 3.6	15.8 ± 2.4	0.001
PEEP at study start (cm H_2O)	4.0 ± 0.6	3.2 ± 0.9	0.035
MAP at study start (cm H_2O)	8.7 ± 3.1	5.2 ± 1.2	<0.001

Table 1: Clinical characteristics and respiratory parameters in pneumonia and control group.

[1] By Student t test or Mann-Whitney U test, according to the variable distribution

Figure 1 represents changes in OI between the start and the end of the study of all newborns with pneumonia and of those controls who had at least 2 TA samples.

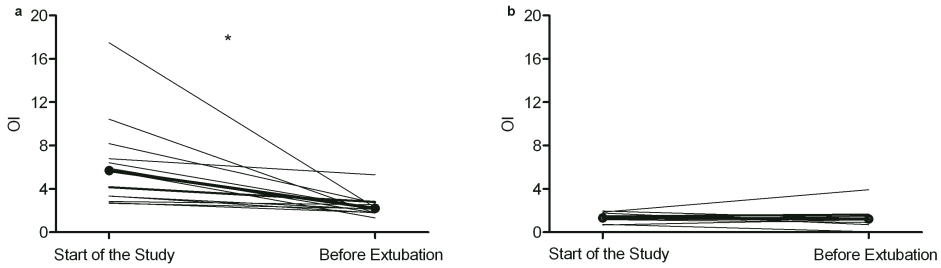

Figure 1: Changes in OI in the two study groups.

SP-B, SP-A, DSPC and PL amounts are reported in Table 2.

	Pneumonia group	**Control group**	p^1
ELF DSPC start of the study (mg/ml)	1.90 (0.63-4.10) (n=11)	4.13 (1.11-5.76) (n=14)	0.286
ELF PL start of the study (mg/ml)	3.21 (1.41-6.85) (n=11)	6.82 (2.25-10.46) (n=14)	0.193
ELF SP-B start of the study (µg/ml)	22.53 (2.12-56.29) (n=11)	7.86 (3.16-23.07) (n=14)	0.367
ELF SP-A start of the study (µg/ml)	16.99 (11.95-44.19) (n=11)	15.99 (11.34-41.48) (n=14)	0.815
SP-B (%PL) start of the study	0.99 (0.20-1.33) (n=11)	0.28 (0.03-0.58) (n=14)	0.029
SP-A (%PL) start of the study	0.59 (0.39-1.23) (n=11)	0.43(0.23-0.70) (n=14)	0.171
SP-B (%PL) pre-extubation	0.05 (0.02-0.10) (n=11)	0.06 (0.03-0.09) (n=7)	0.821
SP-A (%PL) pre-extubation	0.26 (0.13-0.50) (n=11)	0.40 (0.14-0.79) (n=7)	0.497

Table 2: ELF DSPC, PL, SP-B and SP-A measured at the start of the study; SP-B and SP-A measured at the start of the study and before extubation, expressed as % of PL, in the two study groups

[1] By Mann-Whitney U test.

At the start of the study SP-B, expressed as percentage of PL (SP-B (%PL)), was significantly higher in pneumonia compared with controls (p=0.029). We also observed a tendency to a higher ELF SP-B and a lower ELF DSPC and ELF PL.

SPs concentration changes between the first and the last collected TAs, expressed as %PL, are depicted in Figure 2.

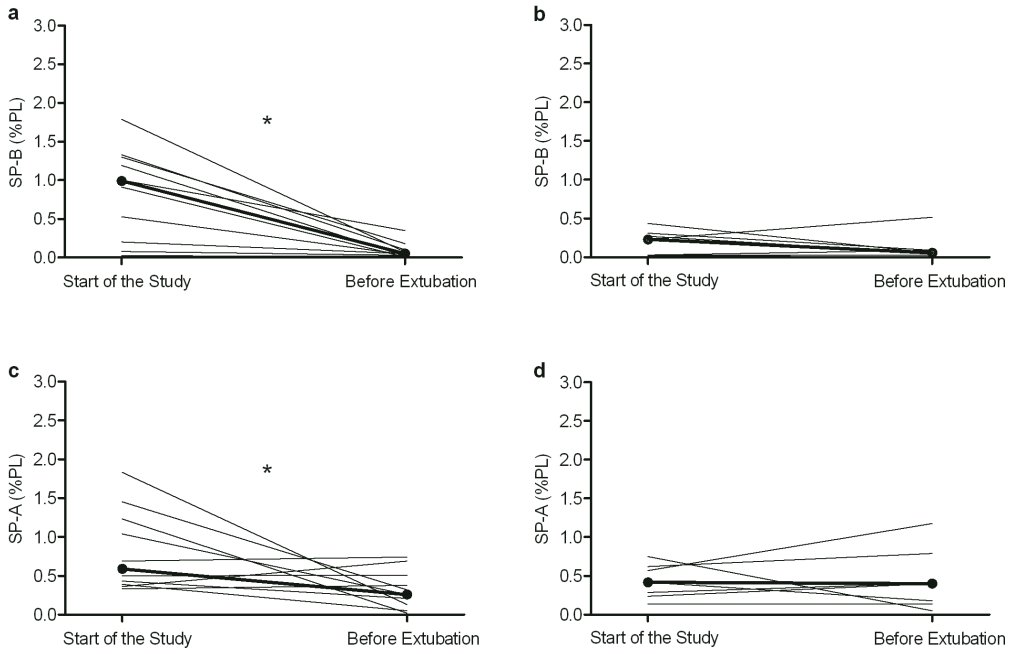

Figure 2: Changes in SP-B and SP-A in the two study groups.
Panel a and c refer to Pneumonia group, b and d to Control group.

In patients with pneumonia SP-B, expressed as % of PL, was significantly higher at the start of the study compared to the percentage found before extubation (*Panel a*, 0.99 (0.20-1.33)% and 0.05 (0.02-0.10)% respectively, p=0.003). Similarly, SP-A (%PL) ratio was higher at study start than before extubation (*Panel c*, 0.59 (0.39-1.23)% and 0.26 (0.13-0.50)% respectively, p=0.050).

In control newborns, from whom SPs could be measured at least two times (n=7), SP-B (%PL) did not differ during the study period, (*Panel b*, 0.23 (0.03-0.31)% and 0.06 (0.03-0.09)%, p=0.398) nor did SP-A (%PL) (*Panel d*, 0.42 (0.23-0.62)% and 0.40 (0.14-0.79)%, p=0.866).

DSPC $t_{1/2}$ was significantly shorter in the pneumonia group (11.8 (5.5-19.8) h) than in the control group (26.6 (19.3-63.6) h) (p=0.011). Median DSPC pool size was lower in newborns with pneumonia compared to newborns with no lung disease (10.6 (4.3-30.0) and 36.0 (29.5-84.0) mg/kg body weight, p=0.031).

No significant correlations were found between SP amounts at the start of the study and the DSPC $t_{1/2}$, pool size or OI, both in the pneumonia and control groups.

DISCUSSION

In this study SP-B and SP-A amounts in TAs of term newborns with pneumonia were compared with those of newborns with healthy lungs. We also studied the SPs profile during pneumonia clinical course, from the peak of the disease until the clinical improvement and extubation.

Few studies have explored changes in TA surfactant protein concentrations during pneumonia in children and no one have presented data corrected for TA dilution (% of PL and ELF) and/or have related the TA SPs amounts to the severity of respiratory failure[133,134]. Expressing surfactant protein levels as % of PL, we found that in patients with pneumonia, SP-B was significantly higher than in controls at study start and that both SP-B and SP-A decreased, as pulmonary function improved. Expressing data as μg/ml ELF, we found no significant differences between pneumonia and control patients, although the levels of SP-B tended to be higher and those of DSPC and PL tended to be lower at the start of the study in the pneumonia group. We also found that DSPC was turned over at a faster rate in pneumonia and that DSPC pool size was significantly decreased in the pneumonia group.

Based on literature data[130,147,148], we expected to find lower levels of SP-B at the onset of pneumonia and to observe increasing SP-B levels during recovery. Despite the heterogenity of data, we were instead struck by finding at the onset of pneumonia an excess of SP-B, with respect to PL. It is possible that SP-B excess represents "spent surfactant", that could contribute with factors, like a leak of plasma proteins in the alveolar space[125], or SP-C deficiency[102], to the decreased lung compliance observed at the onset of the pneumonia. The observed SP-B excess could, however, be explained by other mechanisms like increased secretion of SP-B, a slower clearance or a combination of these mechanisms. Finally it is possible that the excess of SP-B may reflect an accumulation of surfactant subfractions particularly enriched in SP-B[139]. The decrease of the SP-B (%PL) ratio along with clinical and respiratory improvements, were reasonably related with a better oxygenation status, which may represent the result of an improved clearance or a decreased secretion of SP-B. Our data are in line with previous results obtained both in animal studies[149,150] and in newborns after lung injury[151,152]. Ikegami *et al*. found that in healthy mice (with normal lungs) the induction of lung injury by the intra-tracheal injection of LPS resulted in an increase of DSPC and SP-B, mediated by STAT-3, which is consistent with an acute response to the lung injury[149]. Moreover, our research group observed how, in a murine model of unilateral lung injury, DSPC-palmitate synthesis was faster both in the injured and in the non-injured lungs, compared to the naïve control lungs. In this way we proved that, after a local instillation of acid in one bronchus, it is the entire lung system that is involved in responding to the

damage and not only a local area of the lung[150]. Other mechanisms may have contributed to the elevation of SP-B levels. Increased expression of SPs has been observed both in animals exposed to high levels of inspired oxygen[153] and in mechanically ventilated ones[154], however we hypothesized that a main role could have been played by the infection and be mediated by STAT-3 pathway.

A study performed by Epaud *et al.* found that elevated levels of mature SP-B peptide in the airspaces of transgenic mice were associated with decreased inflammation following exposure to endotoxins[155]. Based on these findings a larger study could help us to understand if, in term newborns with pneumonia, higher levels of SP-B are associated with a better clinical outcome and a faster recovery.

The decreased SP-A (%PL) ratio during resolution of pneumonia could be related either to differences in the turnover of surfactant or to other phenomena related to the non-respiratory properties of SP-A.

Only two reports have previously described surfactant composition changes during bacterial pneumonia in infants[133,134]. Both studies corrected the tracheal aspirate dilution by TA total protein amount; this method is less reliable than the ELF method because of the risk to underestimate the SPs amounts due to the increased alveolar capillary leak during the disease's course[156]. Kerr *et al.*[132] measured SP levels in infants ventilated for respiratory failure resulting from severe RSV bronchiolitis but tracheal aspirate dilution was, also in this case, corrected by TA total protein amount.

In our study we used urea to correct for surfactant dilution[146] in the first TA sample, and we expressed SP concentrations as percentage by weight of total PLs in absence of available plasmatic urea level in the second TA. At the end of the study, after the removal of the arterial line, we could not express SP as ELF, because ethical concerns precluded invasive and unnecessary procedures, such as the collection of plasma to correct for surfactant dilution. We therefore chose to express SPs values as % of PL because the amount of total PL recovered from TAs did not significantly differ from the start to the end of the study both in the pneumonia and in the control groups (pneumonia: 0.14 (0.07-0.25) and 0.17 (0.09-0.97) mg/ml, p=0.110; controls: 0.50 (0.20-0.75) and 0.39 (0.06-0.56) mg/ml, p=0.237). This implied that they did not affect the SPs (%PL) ratio and they did not introduce a bias in the comparison within the study groups. A previous study performed in adults with pneumonia, ARDS or cardiogenic edema showed that in adults with pneumonia the BALF PLs and PC content were not significantly different compared to controls[125]. It is conceivable that the TAs unsaturated-PC derived from inflammatory cells may have contributed to maintain the same amount of TAs PL content in our newborns with

pneumonia together with the increase of other surfactant PL classes, either than the sole DSPC[134].

Regarding SP-A (%PL) ratio, Dargaville *et al.*[131] expressed SP-A content as weight/volume of ELF, and as opposed to our study, they found that ELF SP-A in infants with RSV bronchiolitis was, at the peak of the disease, significantly lower compared to controls. This conflicting finding can be explained by the different etiology of the disease or by the fact that they measured ELF SP-A in infants with gestational age ranging between 24-41 weeks.

The administration of a tracer dose of (U-^{13}C-PA) DPPC allowed us to measure the DSPC kinetics, confirming previous findings of our research group[136]. We found that in newborns with pneumonia DSPC pool size and $t_{1/2}$ are, respectively, lower and shorter compared to controls. The faster turnover of TA DSPC in these patients likely reflects the hyperventilation associated with pneumonia and/or the increased DSPC synthesis. The increased catabolism of DSPC observed in children with pneumonia supports the hypothesis that increasing SP-B levels could represent a compensatory mechanisms of the lung to a damage, as we earlier described in a murine model of unilateral lung injury[150].

Our study has four major limitations: first, subject number; second, term newborns who served as controls were on mechanical ventilation for a median value of 24 hours, which may have altered the surfactant homeostasis, hiding possible significant differences between healthy and ill newborns; third, ethical limitations prevented the plasma collection close to the extubation and the calculation of SPs in ELF. Finally, to measure DSPC kinetics, we used (U-^{13}C-PA) DPPC as tracer, in agreement with previous animal studies[140] but without the possibility to prove the actual mixing with the endogenous surfactant pool.

In conclusion, in term newborns with pneumonia SP-B increased with respect to PL and DSPC turned over at a faster rate. Resolution of the disease was associated with restoration of the normal ratio between SP-B and PLs.

Chapter 5

Markers of acute lung disease in preterm infants with respiratory distress syndrome

INTRODUCTION

Lung development

Human lung formation is divided into five distinct periods on the basis of the anatomic changes that occur in lung architecture: the embryonic, the pseudoglandular, the canalicular, the saccular, and the alveolar period[157].

The morphogenesis of the lung begins from the anterior foregut endoderm. During the embryonic and pseudoglandular stages, the two lung buds undergo a highly regulated branching process called "*branching morphogenesis*" crucial for generating the structural airways as well as the terminal alveolar compartments in which gas exchange occurs[158].

During the canalicular period, that goes from the 16th to the 26th week of gestation, the respiratory epithelium begins to differentiate and surfactant components are first observed. The following stage is the saccular one and encompasses period from 26 weeks until term, which is considered at 37 weeks of gestation. Continued proliferation and expansion of the acinar tubules, decreased prominence of the interstitial tissue and a marked thinning of the airspace walls characterise this period[157,159]. In humans the formation of the highly septated and alveolarized structures, comprising the extensive alveolar surfaces mediating efficient gas exchange, occurs during the alveolar stage that takes place from late gestation to the postnatal period[160,161].

Immediately after birth, lungs are filled with inhaled gases and the survival is entirely dependent on gas exchange provided by the lung. The generation of a normal lung structure and a sufficient amount of pulmonary surfactant are of critical importance in the perinatal adaptation to air breathing.

Abnormalities in lung formation associate with different types of diseases depending on the timing at which the formation was disrupted. Tracheal-esophageal atresia, for example, is observed if defects in lung formation occur during the embryonic period, while diaphragmatic hernia can occur because of defects in closure of the diaphragm during the pseudoglandular period[157].

Despite surfactant secretion starts nearly at 24 weeks of gestation, usually only infants born after the end of the 35nd week have adequate amounts of surfactant to breath normally. In preterm infants lung morphogenesis has proceeded normally until birth but in untreated newborns the survival is limited by the lack of pulmonary surfactant.

Respiratory Distress Syndrome

Avery and Mead, in 1959, firstly recognized surfactant deficiency as the main aetiological factor of respiratory distress syndrome[130].

Newborns affected by RDS show apnea, cyanosis, grunting, inspiratory stridor, nasal flaring, poor feeding and tachypnea, shortly after birth, the radiological findings, consistent with alveolar atelectasis, include a diffuse reticulogranular "ground glass" appearance[65].

RDS represented a major cause of mortality and morbidity in neonates, particularly before supplementation with exogenous surfactant preparations was established as a routine therapeutical practice[74].

In 2008 up to 13% of all live deliveries in developed countries were preterm[162]. In a cohort of infants with gestational ages of 22 to 28 weeks and birth weights of 401 to 1500 g the reported incidence of RDS was 93%[163].

In many locations the rate of preterm birth increased, predominantly because of increasing medically indicated preterm births and preterm delivery of multiple gestations associated with assisted reproductive technologies[162].

Despite the human fetal lung is not clinically mature until after approximately 35 weeks of gestation, the clinical experience is that RDS decreases in incidence after 32 weeks of gestation[65,164]. In a very recent analysis conducted in a cohort of about 8000 preterm deliveries comprising all live born non-anomalous singleton from 23 to 37 weeks of gestation, the frequency of all morbidities was found to fell after 32 weeks[165]. Table 1 reports RDS incidence at different gestational ages founded in the cohort study by Manuck *et al.*[165].

Gestational Age [weeks]	23	25	27	29	32	33	34
RDS incidence	79%	60%	59%	54%	44%	37%	30%

Table1: RDS incidence at different gestational age
Data from Manuck *et al*.[165].

The aetiology of RDS has been proposed as multifactorial and/or multigenic[166], the primary deficiency of a functional surfactant system may be associated with other pre-natal or post-natal factors affecting both surfactant synthesis and activity.

Antenatal steroids in combination with postnatal surfactant remains the mainstay of prevention and therapy for RDS in preterm infants[167]. Enhoring and Robertson in 1972 demonstrated the effectiveness of natural lung surfactant administration in an immature rabbit model of RDS[168]. Systematic reviews of randomized controlled trials and meta-analyses confirmed that exogenous surfactant treatment in preterm newborns with RDS reduces mortality, decreases the incidence of pulmonary air leak, and lowers the risk of chronic lung disease or death at 28 days of age[169,170].

The rationale of surfactant replacement therapy is to provide surfactant and counterbalance its inactivation or inhibition by increasing the pool size. In surfactant replacement therapy exogenous surfactant is given at doses between 10 and 20 times the normal pool size to approximate the pool size of term infants[171].

Antenatal steroids are administered to pregnant woman to induce lung maturity in case of imminent preterm delivery, because of their effects on type II cells differentiation and surfactant production.

Clinical data support evidence from animal models of RDS in which the combination of antenatal steroids and postnatal surfactant improves lung function more than either treatment alone[172–175].

Bronchopulmonary dysplasia

BPD is defined as a continuing requirement for supplemental oxygen and/or positive pressure ventilator support at 36 weeks of postmenstrual age[176]. BPD was described for the first time by Northway, in 1967, as a chronic lung disease in late preterm infants, caused by lung injury induced by mechanical ventilation and oxygen toxicity[177].

After the 1990's with the availability of surfactant treatments and the general use of antenatal corticosteroids that further increased survival of extremely low birth weight infants, BPD became the major pulmonary complication of prematurity[178]. Lungs of infants suffering of BPD are now characterized by less fibrosis and inflammation than those of the past but with simplified gas exchange structures. Alveoli are fewer and larger because of the arrest in alveolar and vascular development occurred at the time of delivery. Thus, oxygen and barotrauma are not anymore the only factors initiating much of the lung injury in very preterm infants.

The aetiology of BPD is multifactorial, and all factors occur before lung development has completed. Prenatal and postnatal injuries are represented, respectively, by exposure to intrauterine infection and to oxygen and mechanical ventilation.

BPD occurs approximately in 30% of infants with birth weight < 1000g and in 40% of those born at less than 28 weeks of gestation[179] and associates with long-term pulmonary and/or neurodevelopmental disability and in severe cases with death[178]. Despite antenatal steroids reduced the incidence of RDS they have not significantly decreased BPD incidence[174,175].

There are no pharmacological treatments available. Despite Vitamin A, caffeine and postnatal corticosteroids seem to reduce BPD the only way to decrease the severity and the incidence of BPD is represented by the use of CPAP and low levels of supplemental oxygen[178].

Chorioamniositis

Chorioamnionitis is an intrauterine inflammation of the fetal membranes, the chorion and the amnion. It can be divided into clinic and histological. The first is characterized by maternal fever and a systemic inflammatory response while the second shows no maternal symptoms and can be diagnosed only by histological examination, which also allows classification into acute or chronic chorioamnionitis.

The acute form represents the acute inflammatory response of the mother and the fetus to extracellular microorganisms that gain access to the gestational sac.

Chorioamnionitis is an important cause of preterm birth, its prevalence is inversely proportional to gestational age and affects up to 60% of extremely low preterm infants[vii].

Watterberg, in 1996, was the first to infer the existence of an ambivalent association between chorioamnionitis and respiratory morbidity. Infants exposed to prenatal inflammation showed a lower incidence of RDS but were more likely to develop BPD[180].

After these observations many research groups conducted cohort studies on respiratory outcome in preterm newborns, exposed and not exposed to histological chorioamnionitis, to evaluate incidence of RDS and BPD.

[vii] Preterm births can be classified according to gestational age: extreme prematurity (GA< 28 weeks), severe prematurity (28<GA< 31 weeks), moderate prematurity (31<GA< 32 weeks), and near term (34<GA< 36 weeks).

Birth weight is another parameter used to classify newborns into two groups: extremely low birth weight infants BW < 1000 g and very low birth weight infants BW < 1500 g
Most extremely low birth weight infants are also the youngest of premature newborns, usually born at 27 weeks of gestational age or younger.

Clinical and experimental data from humans and animals agree on the fact that exposure to intra-amniotic inflammation reduces incidence of RDS[181–183]. Lung maturation and surfactant synthesis are likely to be mediated by increased levels of interleukine-1 and of other pro-inflammatory cytokines[184–187].

However, correlation between intra-amniotic inflammation and BPD is more controversial. Chorioamnionitis has been considered essential for BPD development because it increases the likelihood of very premature birth which represents the single most important risk factor for the disease[188].

AIM OF THE STUDY

The aim of the study was to evaluate if and how gestational age and maternal infection affect surfactant composition, lung inflammation, and respiratory outcome in preterm newborns affected by RDS.

We therefore quantify DSPC, PLs, total proteins (Tot PP), SP-B, SP-A, and myeloperoxidase activity (MPO) in TAs collected before the exogenous surfactant's administration, we also recorded ventilator parameters and clinical data of the study cohort.

Based on the presence of maternal infection and gestational age, newborns were divided into three different groups. The groups were pairwise compared to evaluate: i) differences among preterm infants with the same gestational age but exposed or not to maternal infection during intra-uterine life and ii) differences among preterm infants not exposed to maternal infection but with different gestational ages.

We also investigated if the amount of each surfactant component correlates with respiratory parameters recorded at the time of TAs collection, with total amount of surfactant received and with mechanical ventilation length.

MATERIALS AND METHODS

Study population

We retrospectively studied preterm newborns affected by RDS who were admitted to the Neonatal Intensive Care Units of the Department of Pediatrics, University of Padua and of the Division of Neonatology "G. Salesi" Children's Hospital, Ancona.

Eligible infants were preterm newborns (24 to 32 weeks completed gestation) with postnatal age of 24 hr or less and diagnosis of RDS.

Histological chorioamnionitis was diagnosed by histological examination of the placenta and fetal membranes.

BPD was assessed at 36 weeks of postmenstrual age and was defined as the requirement for supplemental oxygen and/or positive pressure ventilator support.

Inclusion criteria were: i) PaO_2 <50mmHg at ambient oxygen ii) central cyanosis at ambient oxygen and need of supplemental oxygen to maintain a PaO_2 >50mmHg, iii) a chest radiography with diffuse reticulogranular appearance.

Exclusion criteria were: i) severe congenital malformations; ii) chromosomal abnormalities; iii) exogenous surfactant administration before collection of TAs, iv) lack of histological examination report.

The study protocol was approved by the Local Ethics Committee of the University of Padua and by the Ethics Committee of the Polytechnic University of Marche, and written informed consent was obtained from both parents.

Study design

TAs were collected up to 24 hours of life before administration of exogenous surfactant, 0.5 ml of blood were drawn at the time of TAs collection in EDTA-containing tubes to perform plasma urea determination.

TAs collection and processing were performed as previously described in Chapter 4.

Vital and ventilator parameters were recorded at the time of TAs collection. Arterial blood gas analysis, if performed at the time of TAs collection, was recorded.

Respiratory failure was assessed by: PaO_2/FiO_2, FiO_2, MAP, oxygenation index (OI), and alveolar-arterial oxygen gradient ($AaDO_2$), calculated as $((MAP*FiO_2/PaO_2)*100)$ and $((FiO_2*(760-47))-(PaCO_2/0.8))-PaO_2$, respectively.

ANALYTICAL METHODS

Quantification of surfactant components

Quantification of SP-A, SP-B, DPSC, PLs, and urea were performed as detailed reported in Chapter 4.

Tot PP in TAs were assayed according to the method of Lowry[189]. Tot PP amount was calculated from a standard calibration curve from 0 to 32 μg of BSA.

Pulmonary neutrophils sequestration was quantified by measurement of MPO activity. Briefly, 100 μL of TAs were added to 2.9 mL of a 50 mM phosphate buffer (pH 6.0) containing 0.53 mM O-dianisidine hydrochloride and 0.0005% hydrogen peroxide. MPO activity was followed spectrophotometrically at 25° C at a wavelength of 460 nm[190]. One unit of MPO activity was defined as that degrading 1 mmol of hydrogen peroxide per minute at 25° C.

Calculation and Statistics

DSPC, PL, Tot PP amounts and MPO activity in TAs were expressed as concentrations in ELF[146]. SPs were expressed as concentrations in ELF [146], as % of PLs, and as % of DSPC.

Data are presented as mean ± SD or median (IQR) based on the variable distribution. Pairwise comparisons between the study groups were assessed by Mann-Whitney U test. Significance was set at $p < 0.05$. Spearmann's correlation analysis was performed to test the association between the amount of each surfactant component and respiratory parameters at study start, total amount of surfactant received and mechanical ventilation length.

Statistical analysis was performed using PASW Statistics 18.0 for Windows (SPSS Inc, Chicago, IL).

RESULTS

We retrospectively studied 46 preterm newborns affected by RDS. These newborns fulfilled the inclusion criteria and had been firstly enrolled for surfactant kinetic studies[191,192].

Nine newborns were born from mothers with a positive diagnosis of histological chorioamnionitis (*Chorio +*) and 37 from mothers with a negative diagnosis. Sixteen out of 37 had a gestational age lower than 28 weeks (*GA≤28 Chorio* -), 21 had a gestational age from 28 to 32 weeks (*28<GA≤32 Chorio* -).

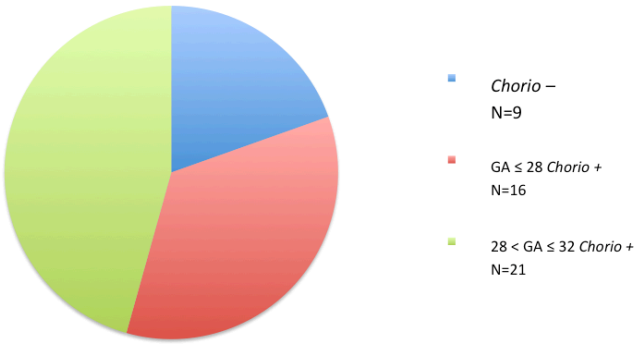

Figure 1.Pie graph of study groups.

TAs were analyzed to quantify DSPC, PLs, Tot PP, SP-A, SP-B, urea, and MPO activity.

Calculation of dilution was not feasible for all samples because of the lack of plasma sample or because of a high dilution of the TA which makes the correction factor not reliable.

In *Chorio +* group amounts of surfactant components were expressed as ELF in 6 out of 9 TAs, in *GA<28 Chorio* – group in 6 out of 16, and in *28<GA<32 Chorio* – group in 13 out of 21.

Since PLs quantification is not reliable when amounts of PLs are too scarce, TAs with a crude value of DSPC lower than 0.01 mg/mL were not analyzed for PLs content.

Table 2 and table 3 report, respectively, clinical and ventilator data and surfactant composition of *Chorio +* and *GA<28 Chorio* – groups.

	Chorio + **(N=9)**	***GA<28 Chorio –*** **(N=16)**	***p*[1]**
Body Weight (grams)	785 ± 209	789 ± 184	0.677
Gestational Age (wks)	25.53 ± 1.53	26.13 ± 1.28	0.187
Age at TAs collection (hr)	2.17 [0.63-22.71]	2.93 [1.03-7.61]	0.803
PCR	4.02 [2.90-24.15]	2.90 [2.90 -2.90]	0.051
PaO_2	50.25 [37.27-53.14]	43.17 [36.69-45.80]	0.181
OI	4.75 [3.24-6.77]	8.00 [7.04-8.90]	0.017
MAP	6.97 [6.50-8.25]	8.80 [6.35-9.35]	0.277
FiO_2	0.29 [0.21-0.50]	0.45 [0.35-0.50]	0.055
$AaDO_2$	120.47 [82.19-245.73]	179.94 [127.73-245.69]	0.345
PaO_2 / FiO_2	124.75 [100.18-200.11]	102.82 [90.56-146.89]	0.491
Lenght of Mechanical Ventilation(days)	4.87 [1.98-7.07]	8.40 [1.42-19.13]	0.442
Total Amount of Surfactant(mg/Kg)	200 [110-205]	287 [200-300]	0.037
Incidence of BPD	50%	46%	0.867

Table 2. Clinical and ventilator data at the time of TAs collection of *Chorio +* group compared to *GA<28 Chorio* – group.

[1] By Mann-Whitney U test

	Chorio + **(N=9)**	***GA<28 Chorio –*** **(N=16)**	***p*[1]**
ELF DSPC (mg/mL)	4.29[2.16-7.47]	0.64 [0.29-4.59]	0.065
ELF Tot PP (mg/mL)	24.01 [5.45-73.32]	30.01 [26.00-41.17]	0.818
ELF SP-A (µg/mL)	30.37 [20.70-65.72]	27.28 [10.01-54.93]	0.818
ELF SP-B (µg/mL)	13.35[5.43-34.92]	5.57 [3.36-10.64]	0.310
ELF MPO (mU/mL)	1983.02[475.41-21071.73]	1.51 [0.55-246.30]	0.017
SP-A%PL	0.35[0.16-1.24]	0.26 [0.18-1.23]	0.876
SP-A%DPSC	1.00[0.42-2.16]	2.82 [0.77-5.77]	0.048
SP-B%PL	0.11[0.03-0.40]	0.15 [0.08-1.73]	0.432
SP-B%DPSC	0.36[0.08-0.97]	0.83 [0.18-1.93]	0.388

Table 3. Surfactant composition in *Chorio +* group compared to *GA<28 Chorio* – group.

[1] By Mann-Whitney U test

The two groups were found to differ significantly in: OI, total amount of surfactant received, MPO activity, and SP-A%DSPC. PCR, FiO_2 and ELF DPSC showed a tendency to significance.

Table 4 and table 5 report, respectively, the clinical and ventilator data and surfactant composition of *GA<28 Chorio* – group compared to *28<GA<32 Chorio* – group.

	***GA<28 Chorio* – (N=16)**	***28<GA<32 Chorio* – (N=21)**	***p*[1]**
Body Weight (grams)	789 ± 184	1157 ± 340	0.0001
Gestational Age (wks)	26.13 ± 1.28	29.97 ± 1.34	0.0001
Age at TAs collection (hr)	2.93 [1.03-7.61]	3.58[1.75-9.20]	0.370
PCR	2.90 [2.90 -2.90]	2.90 [2.90-3.28]	0.483
PaO_2	43.17 [36.69-45.80]	43.17 [39.80-68.80]	0.332
OI	8.00[7.04-8.90]	6.52 [3.83-11.47]	0.244
MAP	8.80 [6.35-9.35]	8.27 [7.15-8.97]	0.948
FiO_2	0.45 [0.35-0.50]	0.40[0.30-0.50]	0.256
$AaDO_2$	179.94 [127.73-245.69]	175.28 [105.00-268.87]	0.976
PaO_2 / FiO_2	102.82 [90.56-146.89]	118.89 [82.08-220.38]	0.742
Lenght of Mechanical Ventilation(days)	8.40 [1.42-19.13]	3.12 [1.30-6.66]	0.080
Total Amount of Surfactant (mg/Kg)	287 [200-300]	200 [155-220]	0.089
Incidence of BPD	46%	40%	0.426

Table 4. Clinical and ventilator data at the time of TAs collection of *GA<28 Chorio* – group compared to that of *28<GA<32 Chorio* –.

[1] By Mann-Whitney U test

	GA<28 Chorio – **(N=16)**	***28<GA<32 Chorio –*** **(N=21)**	***p*[1]**
ELF DSPC (mg/mL)	0.64 [0.29-4.59]	0.75 [0.42-1.03]	0.831
ELF Tot PP (mg/mL)	30.01 [26.00-41.17]	16.12 [11.02-22.77]	0.007
ELF SP-A (µg/mL)	27.28 [10.01-54.93]	17.49[7.91-37.60]	0.831
ELF SP-B (µg/mL)	5.57 [3.36-10.64]	4.87 [2.91-7.70]	0.579
ELF MPO (mU/mL)	1.51 [0.55-246.30]	1.98[0.78-75.78]	0.701
SP-A%PL	0.26 [0.18-1.23]	0.93[0.29-2.16]	0.247
SP-A%DPSC	2.82 [0.77-5.77]	3.21[1.05-6.79]	0.899
SP-B%PL	0.15 [0.08-1.73]	0.26 [0.10-0.48]	0.792
SP-B%DPSC	0.83 [0.18-1.93]	0.68[0.21-1.29]	0.705

Table 5. Surfactant composition in *GA<28 Chorio* – group compared to that of *28<GA<32 Chorio* –.

[1] By Mann-Whitney U test

As expected the two groups differ significantly in gestational age and birth weight. Length of mechanical ventilation and total amount of surfactant tended to be lower in the *28<GA<32 Chorio* – group.

Tot PP were found to be significantly higher in the *GA<28 Chorio* – group compared to *28<GA<32 Chorio* – group.

To evaluate the correlation among surfactant components, respiratory parameters recorded at the time of TAs collection, total amount of surfactant received and mechanical ventilation length groups *GA<28 Chorio* – and 28<*GA<32 Chorio* – were pulled together (*Chorio* –). Correlations in *Chorio +* group were assessed separately.

In the *Chorio +* group total amount of surfactant received was found to correlate significantly with FiO_2 (r=0.857 p=0.003).

In the *Chorio* - group a significant correlation was found between total amount of surfactant received and mechanical ventilation length (r=0.488 p=0.010), and between ELF SP-B and PaO_2/FiO_2 (r=-0.705 p=0.005).

DISCUSSION

The first aim of this study was to evaluate the effect of exposition to inflammation during intra-uterine life on surfactant composition, respiratory status at birth and respiratory outcome.

We therefore compared preterm newborns, with the same gestational age, born from mothers with positive or negative diagnosis of histological chorioamnionitis.

ELF DSPC tended to be lower in *GA<28 Chorio* – than in *Chorio +* group. No differences were observed in SPs and Tot PP expressed as concentrations in ELF, despite the median value of SP-B in the *Chorio +* group was almost three times the value found in the *GA<28 Chorio* – group.

To the best of our knowledge there are no data about human surfactant composition of preterms born from mothers affected by histological chorioamnionitis. Several animal studies investigated the effect of exposure to intrauterine inflammation on lung function and surfactant composition[184–186]. In fetal sheep intra-amniotic LPS injection increases the amount of SPs recovered from BALF after birth by the induction of SPs mRNA[184], and exposure to *Ureaplasma* improves lung function that associated with an increase in surfactant lipids as well in the expression of SPs mRNA[185]. In a study performed on fetal rabbits intra-amniotic administration of interleukin-1α (IL-1 α) was found to increase BALF concentrations of SPs and DSPC[186].

These mechanisms described in animals studies are likely to explain the tendency to the higher amounts of ELF DSPC found in the *Chorio +* group.

Based on the effects observed in animals after fetal exposure to LPS[184] and in animals with normal lungs after LPS intratracheal injection[149] we expected to find significantly higher amounts of SP-B too. The lack of significance in SPs concentration is likely to be due to the high variance of ELF SP-B values and to the very small sample size.

ELF SP-A did not differ between the two study groups, the significant lower value found in the *GA<28 Chorio* – group when expressed as percentage of DSPC is a consequence of the higher levels of DSPC.

As a response to inflammation exposure during intra-uterine life, newborns from mothers with histological chorioamnionitis tended to have higher plasmatic levels of PCR at study start. The significantly higher activity of MPO measured in the *Chorio +* TAs is due to neutrophils entering the lung, in response to pulmonary expression of proinflammatory cytokines.

Finally, in line with literature data which support a better respiratory status both in animals[181,182] and human newborns[183,193] exposed to infection/inflammation before birth, we observed significant lower values of OI and lower doses of total amount of surfactant administered in the *Chorio +* group compared to those of *GA<28 Chorio* – group; a tendency for lower values of FiO_2 was observed too.

Despite literature data suggest also higher incidence rates of BPD in infants born from mothers affected by histological chorioamnionitis[180,188,193,194], in our study cohort no differences were observed in the incidence of BPD at 36 weeks of postmenstrual age.

The second aim of the study was to understand the role played by gestational age on surfactant composition, respiratory status at birth and respiratory outcome.

We divided newborns from mothers with a negative diagnosis of histological chorioamnionitis into two groups: those with a gestational age smaller or equal to 28 weeks and those with a gestational age comprised between 28 and 32 weeks.

There were no such evident differences between the two groups both in surfactant composition and in respiratory status.

The only significant difference was represented by the concentration of total proteins which was found to be significantly lower in *28<GA<32 Chorio* – group. The higher levels of proteins found in TAs of the *GA<28 Chorio* – group suggest an alveolar-capillary membrane more permeable and a more massive protein leak.

The inactivation of surfactant, due to physical inhibitors, it's likely to cause the tendency in higher amounts of exogenous surfactant and longer mechanical ventilation required by the *GA<28 Chorio* – group to sustain lung function and mechanics.

In the *Chorio* – group amounts of surfactant received and length of mechanical ventilation were found to correlate significantly.

Another significant, but negative, correlation was found between ELF SP-B and PaO_2/FiO_2, the worst the respiratory status the higher the amount of SP-B found in TAs. This finding is in agreement with our previous study performed in term newborns affected by neonatal pneumonia in which higher levels of SP-B, expressed as percentage of DSPC, were found at the peak of the disease. Considering the surface active properties of SP-B we speculate that the higher amount of SP-B could represent a response or a mechanism of the lung to improve gas exchange.

In the *Chorio +* group a positive correlation was observed between FiO_2 and the total amount of surfactant received.

Our study has two major limitations: i) the small sample size and does not allow to highlight differences in the variables considered especially in presence of high values of standard deviation; ii) the collection of samples causes a dilution which is of particular importance in

newborns affected by RDS, because of the high final dilution amount of surfactant components can not be expressed as ELF.
As a consequence, in order to confirm these findings and to highlight other differences among study groups a higher number of infants is needed.

Possible future developments

By the analysis and comparisons between TAs collected before exogenous surfactant administration and those collected just before extubation we could be able to describe which changes associate with a lung "ready" to sustain the physiological process of breathing without the support of mechanical ventilation.

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