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CICLO XXIX

Levels of leptin and ghrelin in exhaled breath condensate (EBC) as potential markers of bronchopulmonary dysplasia in ventilated preterm newborns

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Sweet child in time
you'll see the line
the line that's drawn between
good and bad.

Deep Purple, 1970

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ABSTRACT

Bronchopulmonary dysplasia (BPD) was first described in 1967 in a group of preterm infants as chronic inflammatory lung disease associated with arrested pulmonary development and supplemental oxygen needed. In humans is the earliest onset and probably the longest disease, and presents multifactorial origins. Nowadays, in preterm newborns the gold standard diagnostic methods for evaluating predisposition to develop BPD is airway lavage and tracheal aspirate, and less invasive for assessing immunological settings and biomarkers, is blood sample. Recently, non-invasive method based on cooling and condensing of exhaled breath condensate (EBC) containing several classes of compounds and biomarkers was proposed. Numerous researches demonstrate that in EBC high levels of leptin, a pro-inflammatory marker involved in lung injury, are associated with chronic lung disease such as asthma and COPD, but BPD was never evaluated. In the present study we assess EBC levels of leptin and its antagonist ghrelin, in preterm newborns mechanical ventilated, to identify the correlation with BPD development. Only few studies performed EBC on intubated preterm newborns to identify others markers of lung injury.

EBC collection in first day of life showed no leptin and ghrelin concentration neither in BPD and nor in control group. Significant difference ($p=0.002$) in gestational age of controls compared to BPD was found, and significant difference ($p=0.005$) in the body weight of control group compared with BPD. An improved understanding of markers in BPD requires animal model, and the expression of leptin and ghrelin and their receptor were assessed in lung tissue of murine model of BPD obtained exposing newborns Wistar rats at high oxygen concentration for 14 days (PN 14), and to achieve severe BPD, prolonging the exposition for 28 days (PN28). PN14 and PN28 exposition showed a suffering look: shaggy hair, and general growth slowed if related with healthy controls. Architectural changes at PN14 confirms impaired alveolar lung development, and a reduction in the number and development of alveoli. Additionally, increased interstitial thickness, and epithelial cells with low cilia number, compared with control were found, and a continued exposure (PN28) showed an inflammatory and pulmonary edema increase. In pups statistically significant lowest values of body weight were found

in BPD group at PN14 ($p=0.002$) and at PN28 ($p=0.012$) compared with controls in air room. Different dimensions of trachea were found in two groups of treatment at PN14: BPD presented high trachea caliber ($p=0.008$) if related to control. In lung tissue no significant changes expression of leptin and ghrelin and their receptors in control group and BPD group were observed for both exposure periods. In conclusion, it's possible that leptin and ghrelin may not be predictive markers of lung injury: probably we have collected EBC too prior with respect the possible BPD development, but is essential to standardize EBC collection, with specific attention in hardware. Another explanation is that these markers are not involved in BPD lung injury as well as chronic lung disease such as asthma and COPD. Specific role and tissue expression of leptin and ghrelin remain to be clarify, for this reason is necessary to further investigate and focus the attention on metabolic issue with other approaches like proteomic and metabolomics, to better understand and identify this disease that nowadays presents high incidence.

SOMMARIO

La broncodisplasia (BPD) è una patologia cronica che colpisce i neonati pretermine e già dalla sua prima descrizione nel 1967, è stata associata ad un arresto dello sviluppo polmonare che necessita di ossigenoterapia. Il prelievo sanguigno risulta oggi giorno il metodo clinico meno invasivo per valutare il rischio di sviluppare BPD, tuttavia le metodiche di elezione prevedono il dosaggio di *markers* ottenuti attraverso procedure invasive quali l'aspirazione endotracheale ed il lavaggio bronchiale. Negli ultimi anni è stata posta particolare attenzione su una nuova metodica non invasiva che prevede la raccolta del condensato dell'aria esalata (EBC dall'acronimo Exhaled Breath Condensate). In un campione di EBC è possibile rilevare numerose molecole che permettono di valutare le condizioni respiratorie del soggetto. Tra i molteplici *markers* respiratori presenti, la leptina è un ormone recentemente associato a patologie croniche come l'asma e la BPCO, anche se tuttavia non è stata ancora indagata la possibile correlazione con la BPD. In questo studio sono stati raccolti, durante il primo giorno di vita, campioni di EBC in soggetti prematuri sottoposti a ventilazione meccanica per valutare i livelli di leptina e della sua antagonista metabolica, la grelina. Sono emerse differenze significative ($p=0.002$) nell'età gestazionale del gruppo di soggetti che hanno sviluppato BPD, rispetto ai soggetti controllo. Inoltre, è stato osservato come il peso corporeo alla nascita è nettamente superiore nei soggetti sani rispetto ai soggetti con BPD ($p=0.005$). Il dosaggio dei putativi *markers* nel campione di EBC non ha rivelato la presenza di leptina e di grelina, e questo esito potrebbe essere riconducibile al fatto che il campione sia stato raccolto troppo precocemente rispetto allo sviluppo della patologia. Un'altra supposizione prende in considerazione la mancanza di linee guida sulla corretta modalità di raccolta in pazienti intubati, causa di un probabile deterioramento del campione stesso. È stato quindi creato un modello animale di BPD esponendo ratti Wistar neonati ad alte concentrazioni di ossigeno per 14 e 28 giorni (rispettivamente PN14 e PN28). Al termine dell'esposizione sono stati prelevati i tessuti polmonari per una valutazione dell'espressione di leptina e grelina, ma anche in questo caso non sono emerse differenze significative nel gruppo BPD rispetto agli animali cresciuti in normossia, indipendentemente dalla durata

dell'esposizione. Tuttavia sono state riscontrate sostanziali differenze nel peso e nello sviluppo dell'architettura bronchiale, con particolare sofferenza nel gruppo con BPD comparato con gli animali controllo dove è emerso un calibro tracheale nettamente superiore come segno di danno respiratorio. Questi risultati suggeriscono come probabilmente leptina grelina non siano direttamente coinvolte nel danno polmonare presente nella broncodisplasia, e pertanto non possono essere considerati dei marcatori precoci di patologia. Tuttavia, questo studio ha gettato le basi per ulteriori approfondimenti nell'ambito della standardizzazione della raccolta del campione di EBC per evidenziare i più idonei *timepoint* di campionamento, e preservare il possibile deterioramento del campione raccolto. Sarà comunque necessario approfondire il putativo ruolo di leptina e grelina, indagando anche sugli aspetti di metabolomica e proteomica, con l'obiettivo di analizzare il rischio di sviluppo di una patologia cronica che tutt'oggi presenta ancora un'elevata incidenza.

INTRODUCTION

Bronchopulmonary Dysplasia

Bronchopulmonary dysplasia (BPD) is a chronic inflammatory lung disease associated with arrested pulmonary development and a need for supplemental oxygen. Todays the survival of preterm newborns is increasing, but infants frequently suffer chronic respiratory disorders, especially if had developed this disease. BPD was first described in 1967 by Northway and colleagues in a group of preterm newborns, defining developed chronic respiratory failure as sequelae of hyaline membrane disease/surfactant deficiency/respiratory distress syndrome (RDS). The results are based upon a large group of preterm infants with pulmonary changes resulting from mechanical ventilation and oxygen supplementation to treat hypoxemia (Northway *et al.* 1967). Few years later, it was highlighted how the treatment with oxygen increases production of cytotoxic oxygen free radicals in mice knock-out of antioxidant mechanisms, and the exposition to high levels of oxygen showed lung injury observable in morphometric analysis (Crapo *et al.* 1978) (Bonikos *et al.*1976). From the late 1980s the National Heart Lung and Blood Institute (NLHBI) further describe the disease, and suggested to focus new clinical researches in this disease (Report of NIH, 1979). Other studies in 1985 described the pathophysiology of this disease and it was a lunch pad to apply the research in animal models (O'Brodovic *et al.* 1985): extremely preterm newborn baboons with BPD, exposed at high concentration of oxygen in mechanical ventilation, showed a significant decrease in the number of alveoli and enlargement of airspaces that caused a reduction in the total lung internal surface (Coalson *et al.* 1995) (Coalson *et al.* 1999). Several studies explained the long-term consequences of BPD, concentrating the aim on the incidence of airway hyper-reactivity and then intense lung inflammation as disruption of normal pulmonary structures and lung fibrosis (Baraldi and Filippone, 2007), conditions that increase susceptibility to infection and altered respiratory function. Additionally, lung performance is decreased in infants with BPD (Bryan *et al.* 1973), because lung compliance is reduced and bronchial tree presents high airways resistance due at reduction of parenchymal elasticity

(Goldman *et al.* 1983). Although the morphologic process of lung development has been well described, it's necessary to introduce the timing and ageing in lung growth, to better understand the onset of BPD in immature lung. It is well known the prenatal period consisting in embryonic stage (1-7 week) and a fetal period, divided in 3 stages: pseudo-ghiandolar stage (5-17 weeks), canalicular stage (16-26 weeks) and saccular stage (24-38 weeks). The saccular period represents the timing for the formation of the alveoli and go on in postnatal time when the alveolar stage starts from 36 weeks and go on up to 2 years of age. This follows the micro vascular maturation and the late alveolarization (up to 5 years of age) (Burri, 2006). Term birth, corresponds with the alveolar phase when alveolar sacs evolve by alveolar ducts, conversely in preterm infants who develop BPD, are in the late canalicular or early saccular stage of lung development. This period is characterized by cells differentiation (pneumocytis type I and type II) and growth of the primitive alveoli and alveolar-capillary barrier (Balany and Bhandari, 2015). In these conditions the normal lung advance programming is blocked, increasing the risk of occurrence of BPD. In human, in the 4th week of gestation the sketch of separation of trachea and esophagus is observed (Fig.1) (Herriges and Morrisey, 2014).

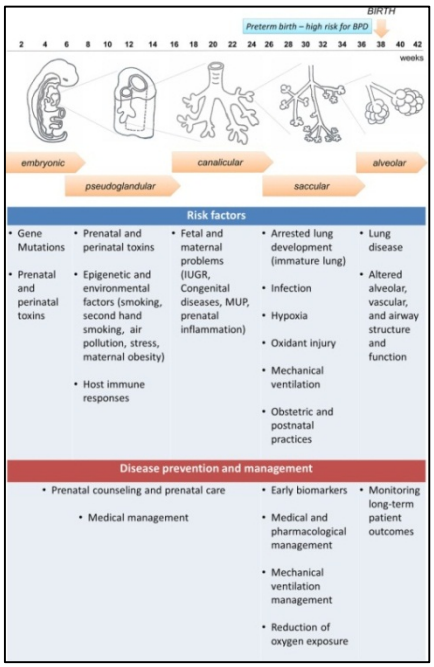

Fig.1. Human lung development and BPD. (from Rivera *et al.* 2016)

Next phase is the formation of the trachea (Rawlins, 2011) that will generate the bronchial branch and forms all the ramifications that lead to the evolution of capillary network, the final large surface area and a thin capillary barrier dedicated to gas exchange. This lung evolution allows to transport air from external ambient, into the bloodstream, and exhaled out the carbon dioxide. Gas exchange process takes place in alveolar-capillary barrier, a thin line composed by alveolar-vascular epithelial cells in close association, when the deoxygenated blood through the lung to be oxygenated (Nardiello *et al.* 2016). Preterm infants begin early pulmonary gas exchange with an inefficient alveolar and capillary network, and most severely affected patients remain symptomatic even in adult age. These infants, in the first years have a low quality of life because present recurrent infections with altered pulmonary function (McEvoy *et al.* 2014). The risk of infection increases if infants are affected by BPD, indeed is documented that 73% of these infants required at least one admission to hospital, and the 27% needed more than three hospitalizations (Greenough *et al.* 2001). In addition, they have a high risk of mortality after birth, and high risk to develop visual and ocular motility dysfunction (Chau *et al.* 2013) characterized by delayed visual maturation and subnormal visual acuity, but also visual field defects, and visual perceptual-cognitive problems. Infant with BPD have a high predisposition to develop pulmonary hypertension (Koroglu *et al.* 2013), and altered gastrointestinal motility (Broussard, 1995), although the most important disorder during the first two years of life of patients with BPD (Lamarche *et al.* 2004) is respiratory exacerbation caused by viral infections. Genetic factors added to environmental interactions (Fig.2) leading to the multifactorial origins of this disease that in humans is the earliest onset and probably lasts the longest, as showed in a recent study by Carraro and colleagues (Carraro *et al.* 2015) when are describe alteration of the lipid metabolomic profile in exhaled breath condensates (EBC) of adolescents with history of BPD, compared to controls.

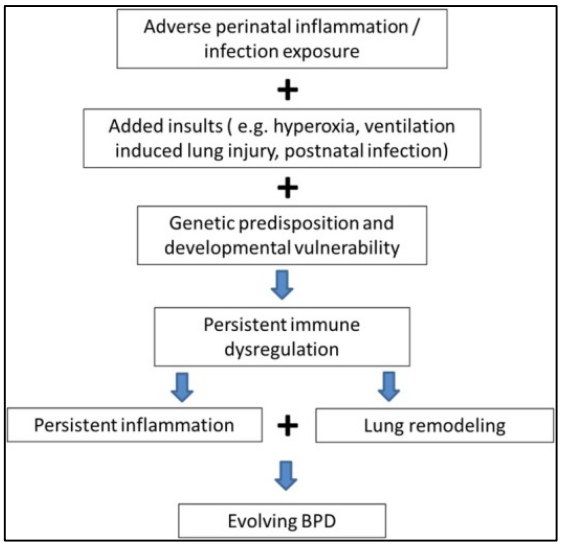

Fig.2. Genetic and environmental factors and inflammation leading to the development of BPD. (from Balany and Bhandari, 2015)

Medical advances in the care of premature infants such as use of prenatal corticosteroids and postnatal surfactant treatment, the avoid intubation but use of oxygen supplementation by continuous positive airway pressure (CPAP) (Van Marter *et al.* 2000), and use of heated high-flow nasal cannula (HHFNC) (Collins *et al.* 2013), permit relatively consistent survival of preterm infants born in the late canalicular, and early saccular stage of lung development. In preterm non-human primates was observed that nasal CPAP improve the alveolar formation and alveolar capillary growth, if compared with mechanical ventilation (Thomson *et al.* 2004). Additionally, use of nasal low pressure high frequency ventilation (nasal LPHFV) shows the same improve in lung of preterm lambs and preserves alveolar architecture (Reyburn *et al.* 2008). Exposing to high tidal volumes increases risk factor for developing BPD in premature infants, indeed in mechanical ventilation, lungs present injury such as volutrauma, barotrauma, or atelectrauma (Hillman *et al.* 2011), and increase the release of pro-inflammatory cytokines like IL-6, IL-8, and TNFα, as well as reduced production of anti-inflammatory cytokines like IL-10 (Carvalho *et al.* 2013). Also the aggressive treatment of symptomatic ductus arteriosus, and new insights of nutrition have showed a improve of outcomes. For example, nutrition both during hospitalization and at discharge is an important step for the correct management of health: in fact

rapid weight gain and crossing of centiles is not recommend, because increase the risk of developing hypertension, and risk to become insulin resistant (Lapillonne *et al.* 2013), especially in newborn with low weight for gestational age. According to nutrition, is necessary to considered that while a newborn with BPD receiving supplementary oxygen in delivery room, his antioxidant protection is low, then is more exposed to oxidative stress (Solberg *et al.* 2012), therefore the future perspectives suggest supplementation with dietary intake, or nutraceutical dietary supplement such as (curcuma, resveratrol and vitamin D) (Tenero *et al.* 2016), to reduce the risk to development inflammatory disorders, as has been observed in other lung disease such as asthma (Perrone *et al.* 2012). Since the classical characterization of BPD, defined as persistent oxygen requirement at 36 weeks' gestational age, respiratory distress such as retractions, adventitious breath sound and tachypnea but also a changes in lung development confirmed by radiograph test, there have been many steps forward in neonatal medicine. The new knowledge on management of BPD lead to define this disease with a different grade of severity. In 1998 Husain and colleagues (Husain *et al.* 1998) described a "new version of BPD" highlighting the pathology found autopsy of newborn with BPD, and the term "new BPD" has been proposed a few years later by Jobe and Bancalari (Jobe and Bancalari, 2001). In the old BPD was present a large parenchymal fibrosis, inflammation and airway injury, instead in the new BPD is present a low level of fibrosis and a more uniform inflation. Moreover, in the new BPD smooth muscle not presents hypertrophy, no metaplasia in epithelial tissue, and minimal to moderate (but diffuse) alveolar septal fibrosis. In this tissue, alveolar development is arrested and this causing compromised lung elastic tissue maturation and perturbation; in new BPD we find an arrest of lung development instead that iatrogenic injury. Nevertheless, injuries occurring in the canalicular and saccular stage of bronchial development, compromise the vascularization and alveolarization and then make pulmonary gas exchange with an inefficient alveolar and capillary network. In spite of many improve in neonatal medicine since the first description of this disease, the incidence of BPD has nowadays not decline (Balany and Bhandari 2015), but to improve the management of this disease in 2000 a consensus categorized BPD in preterm newborn based on need

of oxygen supplemental: “none” (oxygen needed up to 28 days), “mild” (need 28 days of oxygen therapy, but not at 36 weeks gestational age), “moderate” (need up to 30% oxygen at 36 weeks gestational age), “severe” (need for more 30% oxygen, with or without positive pressure ventilation or continuous positive pressure at 36 weeks gestational age) (Bancalari, 2001). During postnatal life the lung development is characterized by an increase of vascularization and develop of alveoli, important in the normal evolution of respiratory system (Baldwin, 1996) (Gebb and Shannon, 2000). This growth is mediated in pathway regulated by the presence of specific signal peptides, cytokine and growth factors (Mustonen and Alitalo, 1995), and lung injury after the birth compromises the normal evolution, in fact in the lung of rats was observed that the inhibition of angiogenesis decreases alveolarization (Jakkula *et al.* 2000). BPD has been linked to the development of an inflammatory response that can occur in absence of clinical infection. Interactions occurs between proteins in the develop of lung, and with chronic lung disease of preterm birth, increase levels of pro-inflammatory cytokines express after alveolar injury, and characterize BPD pulmonary inflammation. In fact, common pro-inflammatory cytokines are the most extensively studied in this category of disease, because are important biomarkers for the prediction of adverse pulmonary outcomes in preterm infants. Several studies in infants showed that the bronchoalveolar lavage fluid obtained from newborn, presents pro-inflammatory cytokines (IL-1β, IL-6) (Kotecha *et al.* 1996) released in response to infection, but the same results was described also in conditions of specific inflammation. These proteins induce the release of inflammatory mediators and modify the adhesion molecules on endothelial cells by up regulation. For example, in tracheal aspirates of preterm newborn IL-1β induces airway epithelial cell IL-8 expression via an NF-kB dependent pathway (Shimotake *et al.* 2004) and IL-1β/Il-6 ratios are associated with risk of colonization with Ureaplasma urealyticum in respiratory tract (Patterson *et al.* 1998). High leukotriene levels are present in lung of newborn with BPD (Cook *et al.* 1996), and also in early phases of an inflammatory response, high presence of TNF-α in tracheobronchial aspirate correlates with the presence of BPD compared with health infants (Jònsson *et al.* 1997). Adhesion molecules such as ICAM-1

(Kojima *et al.* 1996) (Kotecha *et al.* 1995) firm adhesion and migration of neutrophils, and L-selectin involved in the rolling of neutrophils increased concentrations on day 7 and on day 10 in infants with BPD (Kotecha *et al.* 1998). Moreover, the inducible PGHS-2 isoform of the enzyme involved in the production of prostaglandins, (autocrine and paracrine lipid mediators), are implicated in the lung's response to inflammation given that pro-inflammatory cytokine, and other isoform constitutively expressed, PGHS-1, present in cells to regulate the homeostasis. PGE2 is the PGHS-2 metabolite give from fibroblasts and vascular endothelial cells, that are stimulated by IL-1β and TNFα and the presence of this substance suggests specific role in fetal inflammation (Westover *et al.* 2012). Moreover, few mature cellular elements such as granulocyte and macrophage are present in alveolar washes of preterm newborn, and the presence of this cells increase after oxygen supplementation. For example, in lamb underwent to mechanical oxygen ventilation, this cellular type can be considering as possible early markers of lung injury oxygen induced (Carlton *et al.* 1985), and if applied in preterm newborn by measurements of reduction in circulating granulocytes, can predicts the risk to develop BPD (Ferreira *et al.* 2000). In Tab.1 are visible the expression of all markers nowadays known, and involve in this multifactorial disease.

Mediators of inflammation	Role	Expression in BPD
Inflammatory cytokines		
Interleukins: anti-inflammatory		
IL-10	Suppresses inflammatory response by inhibiting NF-κB	↓/↔
IL-4, IL-13	Suppresses inflammation by inhibiting pro-inflammatory cytokine prod.	↔
Interleukins: pro-inflammatory		
IL-1, IL-6	Acute phase inflammatory response	↑
IL-8 (CXCL-8)	Main chemoattractant for neutrophils	↑
CC chemokines		
Monocyte chemoattractant protein (MCP)-1, 1α, 1β, 2, 3	Recruit inflammatory cells to area of injury	↑
Macrophage migration inhibitory factor (MIF)	Upstream regulator of innate immune response	↓
Tumor necrosis factor alpha (TNF-α)	Enhances expression of other pro-inflammatory cytokines	↑
Transforming growth factor-beta 1 (TGF-β1)	Pro-inflammatory	↑
Matrix proteins		
Matrix metalloproteinase-8	Disordered pulmonary remodeling after inflammation	↑
Matrix metalloproteinase-9	Pro-inflammatory, interferon-gamma (IFN-γ) signaling	↑
Growth factors		
Endothelin-1	Pro-inflammatory	↑
Vascular endothelial growth factor	Pro-inflammatory	↑/↓
Connective tissue growth factor (CTGF)	Pro-inflammatory	↑
Bombesin-like peptide (BLP)	Increases mast cells in the lung	↑
Breast regression protein-39 (human analog is YKL-40)	Anti-inflammatory	↓
Pulmonary hepatocyte growth factor (HGF)	Alveolar septation, repair	↓
Keratinocyte growth factor (KGF)	Regulates proliferation of alveolar epithelial cells	↓
Miscellaneous		
Interferon-inducible protein 9 (IP-9 – also known as CXCL11)	Pro-inflammatory, IFN-γ signaling	↑
Cyclooxygenase-2 (Cox-2)	Pro-inflammatory, IFN-γ signaling	↑
CCAAT/enhancer-binding protein (C/EBP)	Pro-inflammatory, IFN-γ signaling	↑
Endoglin	Pro-inflammatory	↑
Periostin	Pro-inflammatory	↑
Clara cell secretory protein	Modulates acute pulmonary inflammation	↓
Parathyroid hormone-related protein (PTHrP)	Alveolar growth	↓
Angiopoietin-2	Pro-inflammatory	↑
Lactoferrin	Anti-inflammatory	↓

Tab.1. Markers of inflammation: function in health and level in BPD. Symbols ↑ indicates increase, ↓ indicates decrease and ↔ indicates no change. (from Balany and Bhandary 2015).

The effect of severity in BPD is also associated at gender and racial differences, in fact neonatal lung diseases may have a genetic background: for example, the production of hydrophilic surfactant proteins, also known as “collectins” that have the ability to bind and improve the control of a big group of pathogens and allergens, is influenced by polymorphism of intron 4 of *SP-B* (Surfactant Protein B), and specific alleles of the SP-A are associate with respiratory distress syndrome. Moreover, other alleles and genotypes of SP-A and SP-D (Silveyra and Floros, 2012) associate with severe respiratory infections in early infancy, and dominant mutations of SP-C are associate with different manifestations of chronic lung disease (Hallman and Haataja, 2003) (Marttila *et al.* 2003). In addition, growth factors, hormones, and other molecules that control lung homoeostasis

may influence recovery from inflammatory injury and for this reason is treated with nitric oxide (Truog *et al.* 2007), a lung protective inhaled therapy that reduce the pulmonary vascular resistance and improve oxygenation (Soll, 2012). Clinical intervention with corticosteroid therapy is applied for preventing respiratory disease syndrome (RDS) and in last years was the most effective therapy (Roberts and Dalziel, 2006) to prevent preterm delivery as shown by Liggins in a trial in fetal lumb in 1969: "*It is suggested that this may be the result of accelerated appearance of surfactant activity*" (Liggins, 1969). In a clinical trial was described as prenatal treatments with corticosteroids reduced the of 17% the incidence of develop RDS, improving neonatal health outcomes (Crowther *et al.* 2015). Nevertheless, there are controversy in the antenatal use of corticosteroids (Wapner and Jobe, 2011), and in animal models (Jobe *et al.* 2003) is described as the result after treatment is not always detectable, and sometimes can presents adverse events. In addiction, recent guidelines recommend to treat, with antenatal corticosteroid therapy between 24 up to 34 weeks of gestational age, women at high risk to preterm delivery (Moss *et al.* 2002) (Practice Bulletin n.159, 2016) although the correct use of corticosteroids in pregnancy is unclear (Romeiko *et al.* 2014) (Doyle *et al.* 2014). There are several animal experiments conducted on fetal sheep to investigate the frequency and timing of treatment, and the optimal dose (Jobe *et al.* 2007) (Jobe *et al.* 2009). However, the treatment is only a help for the infant, because the therapy with corticosteroids can prevents the develop of RDS in the 30% of preterm newborn, but have no effect if BPD has already been diagnosed (Roberts and Dalziel, 2006). BPD is frustrating for neonatologist affecting up 30% of very low birth weight infant. Nevertheless, all the mechanisms associated with the disease pathogenesis are nowadays unclear, because this chronic lung disease is multifactorial (Bhandari, 2014). Today BPD is diagnosed according to the criteria of the National Institute of Health when the infant required >21% oxygen for more than 28 days. (Ehrenkranz *et al.* 2005).

Use of animal models

Over time the survival rate of patients with BPD is increased, and autopsy lung tissue, necessary to explore the pathogenesis, to study new treatments, or new intervention strategy, it's difficult to find. For this reason, many studies are addressed on animal models of BPD, a field in constantly expansion because the characterization of pathogenic pathways to identify the disease mechanisms (Hilgendorff *et al.* 2014) in preterm newborn, and identify in-vivo pulmonary property are nowadays not completely clear. Several studies are needed with specifics animal models of BPD in rats (O'Reilly and Thèbaud, 2014) mice (Berger and Bhandari, 2014), in rabbits (Manzano *et al.* 2014) (D'Angio and Ryan, 2014), pre-term lambs (Albertine, 2014) and pigs (Caminita *et al.* 2015) (Arrindell *et al.* 2015), and finally in non-human primates (baboons) (Yoder and Coalson, 2014). In -vitro protocols, and microscopic imaging analysis are in progress, and this technology improvement, allows to better explore the lung development (Nardiello *et al.* 2016).

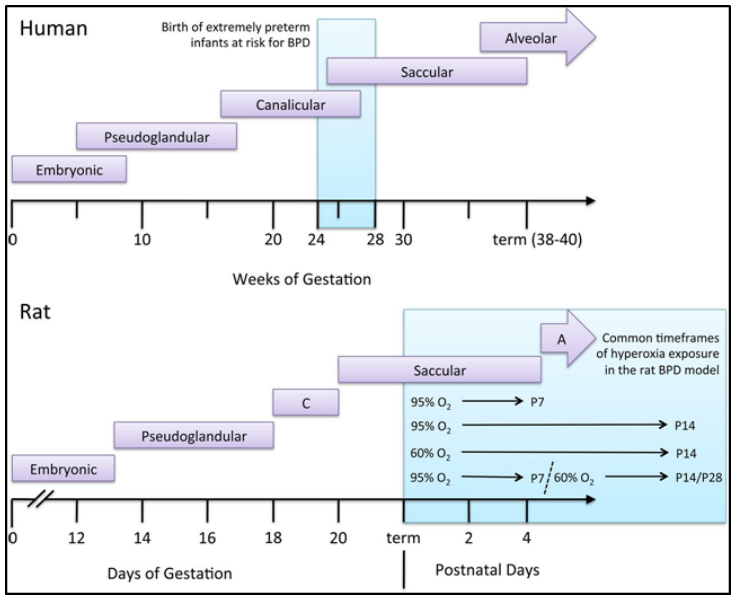

Fig.3. Stages and gestational ages of lung development in humans and rats. **C** stands for canalicular, **A** stands for alveolar and **P** stands for postnatal day. (from O'Reilly and, Thébaud, 2014)

To better understand the develop of BPD it's Necessary to remember That there are some small differences between the lung development in animals and humans. In fact, while in sheep, baboons and humans, the saccular stage occurs in utero, in rodent models it begins at embryonic day 18 and the transition to the alveolar stage occurs at postnatal day 5, with total development at 30days after birth (Joshi and Kotecha, 2007). Therefore, study of the rat lung has provided to insight in the postnatal development stages (Kuffman *et al.* 1974) (Burri *et al.* 1974) (Kramer *et al.* 2007) (Fig.3). Another advantage on the using rats, is the duration of gestation (21-22 days), and the short estrus (4-5 days), but also post-partum estrus, consent to researcher to plan by date accurately by means of single mating. Moreover, a large litter size (11 in average, but depends on the strain) consents to have a consistent number of available pups (Burri, 1974). In animal models, is simple obtaining a relatively high quantity of blood sample, and after sacrifice is also simple collect the different organs. Also in human there are many advantages of using blood, first of all the presence of reference values normalization, although in preterm newborn sampling is a common problem, especially if are very low birth weight. To overcome this problem, the most promising biomarkers to be used in future studies, are probably non-protein molecules such as microRNAs (De Paepe *et al.* 2008) and end tidal carbon monoxide (Zhang *et al.* 2014), or biomarkers like cytokines and other molecules not only be derived from the bloodstream.

EBC (exhaled breath condensate)

Bronchoalveolar lavage technique (BAL) and direct aspiration of lung epithelial lining fluid in preterm newborns with BPD assess markers of lung disease, but this sampling is invasiveness and potential to cause transient inflammation. Additionally, BAL is difficult to repeat many times in near future, because increase risk of impairment of gas exchange and pneumonitis in previously compromised tissue (Effros *et al.* 2002). Non-invasive means of sampling the airway-lining fluid of the lungs have been purposed in last years and exhaled breath condensate (EBC) is a new technique by which pulmonary specimens are obtained to assess inflammatory markers of respiratory disease. The study of lung

inflammation with non-invasively procedures is better, and exhaled breath air contains aerosols that can be collected and analyzed for study characteristics capable to describe pathologic processes in the lung. This approach is based on previous studies carried out in the 70s afterwards summarized by Manolis in 1983 (Manolis, 1983) when described the amount of substances present in the exhaled air of human breath. In the recents years several works described the correlation between exhaled breath condensate and disease such as asthma (Corradi *et al.* 2007) (Baraldi and Carraro, 2006) and others lung disease, and for this reason sampling of exhaled breath condensate has been purposed as new approach for assessing biomarkers. As described by Hunt (Hunt, 2002), EBC contains different classes of aerosols and vapors, also non-volatile compounds such as lipids, ions, oxidation products, cytokines, adenosine, serotonin, histamine, acetylcholine, and surfactant. Volatile organic compounds such as ammonia, hydrogen peroxide and ethanol are also possible to find out. Aerosols are released during the normal inspiratory and expiratory phase in tidal breathing, and particular interest is due EBC gives a substrate by which inflammatory and biochemical components of lung disease can be noninvasively evaluated. EBC collection is obtained by cooling exhaled breath and condensing the sample into test tube kept under zero Celsius degrees (Hunt, 2002). Several studies on EBC biomarkers were conducted to assess the relationship between different classes of compounds and different respiratory disease such as asthma, COPD, gastroesophageal reflux disease (GERD), cystic fibrosis, lung cancer and many others respiratory syndrome (Kononikhin *et al.* 2106). Although in adults were described guidelines on EBC collection, but a standard protocol for sampling collection in mechanical ventilated patients is nowadays missing (Muller *et al.* 2006). Sample collection in mechanically ventilated patients is based on principles of condensation, but is influenced by several factors. A correct sampling requires low cooling temperatures and adequate sampling times (usually several minutes). Moreover, the sample can be contaminated and for this reason that storage and analysis should occur promptly at -80° C to prevent sample degradation. Based on this protocol we can safely collect the sample also in intubated patients underwent to

mechanical ventilation without conditioning the clinical practice: procedure is possible by placing the EBC device in-line with the circuit of the ventilator.

In this study we collect EBC to assess a new putative marker of lung disease expressed in lung tissue of animal model of BPD. In ventilated infants the lack of collection methods, compared to others collaborative subjects (Baraldi *et al.* 2003) affected by asthma, is critical for early identification of preterm infants at greatest risk for BPD. In fact, in ventilated preterm newborns, due of this absence on the management of EBC collection, is difficult obtain a significant marker to relate with animal models. In the last few years a precious addition to clinical investigation, especially for metabolic changes that occur in different diseases, was performed. These studies are also useful for following the efficacy of therapy (Lal *et al.* 2015) and identification of specific biomarkers is also suitable for earlier diagnosis of BPD (Fig.4) and in the clinical management permits, to start specific treatments just after birth.

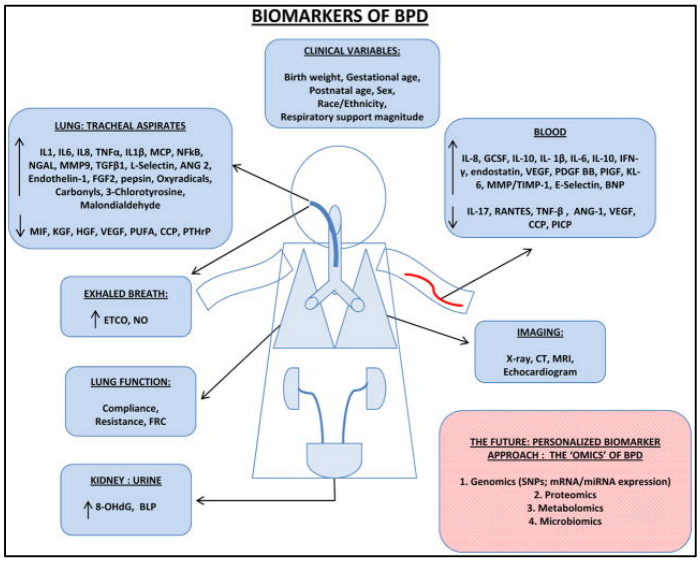

Fig.4. Markers of BPD.

Nowadays the prediction of BPD is evaluated with new method such as metabolomic (Baraldi *et al.* 2016), a new scientific approach that detects changing in metabolic profiles, leading to identify patients with more precision than clinical

tools available. Among models prediction of the risk to develop BPD there is a poor discriminative ability (Lal and Ambalavanan, 2015) and for this reason is necessary focus on new biomarkers development and at same time standardization of sample collection, for early correct finding and treatment of this disease.

Leptin

In 2011 was well described by Banfo and colleague that among 5-10% of pregnancy can present fetal growth restriction with trouble to achieve the genetically determined development (Bamfo and Odibo, 2011). Is well known how in preterm newborn exist a fetal immaturity gas exchange network, and a failure of the surfactant production, conditions to expose to developing respiratory distress syndrome, pneumonia, asphyxia and BPD (Bose *et al.* 2009). Despite in last year there are improve of medical knowledge and a more target use of prenatal corticosteroids and postnatal surfactant treatment, but also the avoid the mechanical ventilation, many countries have reported an increase of preterm birth rates over the past 20 years, and this general trend was recently confirmed by a WHO global survey (Zeitlin *et al.* 2013). The mechanism of lung development is regulated by several interactions mediated by a bi-directional signal that involve molecules and their receptors can have too a putative role for the treatment of fetal growth restriction. For example, in the group of non-traditional cytokines there are molecules with double role: show cytokine functions in modulating the immune response, but present also hormone characteristics, especially during metabolic processes (Ouchi *et al.* 2011). Among these molecules Leptin, a 16-kDa peptide product of the *ob* gene, is a cytokine-like hormone primarily secreted from mature adipose tissue and, in physiological conditions, its circulating levels correlate positively with white adipose tissue mass. This adipokine links nutritional status with neuroendocrine and immune functions and is expressed in fetal lung tissue, but high level expression of leptin receptors in lung and isolated fetal type II cells was found. In recent years, in in-vitro studies have been demonstrated how leptin is secreted by lipofibroblasts and its release stimulates type II cells to produce the surfactant. The same stimuli were highlighted in fetal

rat type II cells, and adult human airway epithelial cells (Torday *et al.* 2002). Leptin is also expressed (Cohen *et al.* 1996) from many other tissue, mammary epithelium (Handy *et al.* 2010), placenta (Leroy *et al.* 1996), gastric fundus, liver and muscle, and plays a specific role in the inflammation and immune response (Otero *et al.* 2005), because differentially increases production of TH1 cytokines such as IL-2, interferon-γ, TNF-α. It was also observed increasing of vascular endothelial growth factor (VEGF) release by airway smooth muscle, that promotes sub epithelial neovascularization and vascular permeability (Shin *et al.* 2008). Leptin acts a pro-fibrogenic effect in primary human lung fibroblasts augmenting the transcriptional activity of TGF-β_1 via suppression of the antifibrotic activity of PPARγ, and this observation is confirmed with animal model of leptin-deficient mice: these animals were resistant to the development of airways lung injury (Jain *et al.* 2011). Leptin is also involved in the regulation mechanisms of the T cell proliferation and activation, natural killer cell function (Matarese *et al.* 2005), angiogenesis promotion and monocytes and macrophages system recruitment, playing significant role in airway inflammation. The diseases involved in these processes are acute lung injury (Ubags *et al.* 2014), but also chronic disease COPD (Chronic Obstructive Pulmonary Disease) (Kim, 2014) and asthma (Vernooy *et al.* 2013) (Ali Assad and Sood, 2012). Leptin presents a pleitropic effect (Paraskevas *et al.* 2006) contributing to control of the body weight by influencing appetite and energy expenditure, and acting on the hunger centre of the hypothalamus and brown adipose tissue (Fig.5) (Mancuso *et al.* 1985) (Campfield *et al.* 1995). Focusing the attention on other role of leptin is it's possible describe its action as a lypostate: when the amount of fats stored in the adipocytes increases, leptin is released into the bloodstream, a negative feedback signal for the hypothalamus. With this message of metabolic information is highlights that energy reserves are sufficient, and hypothalamus releases anorexigenics peptide and suppressing orexigenic factors (de Luis *et al.* 2009). Studies in humans and in animal models have been demonstrated that low levels of leptin are associated with obesity (Fig.5) (Montague *et al.* 1997) (O'Donnell *et al.* 2000) (Wabitsch *et al.* 2015). For this reasons it has been hypothesized a possible leptin resistance condition in obese patients because high plasma leptin

concentrations in obese with normal weight subjects were found (Auwerx and Staels, 1998). Leptin exerts its biological actions by binding to its receptor (Ob-R), a member of class I cytokine receptor superfamily, presents in six isoforms (Matarese *et al.* 2005): a soluble isoform, with short cytoplasmic domains. Other isoforms, the long isoforms, are found in almost tissues and which seems to be the only isoform capable of transducing the leptin signal in many tissues including brain, placenta, hematopoietic cells, liver, heart and lung (Bruno *et al.* 2005). Leptin binds to short and long forms of its receptors, which are generated by alternative splicing of leptin receptor gene (Lee *et al.* 1996). This link transmits extracellular signals through the janus kinase (JAK) and signal transducer, and activator of transcription (STAT) signaling pathway (Fruhbeck, 2006). Most immune cell types express Ob-R at their surface, which suggests a role for leptin in immune responses. In the last years, many studies have showed the potential role of leptin in lung development and remodeling (Vernooy *et al.* 2013), suggesting an evident role of this hormone in pulmonary homeostasis. For example, animal models show how leptin contributes to the regulation of respiratory lung function, acting as a stimulant factor of ventilation. In an obese mouse model (*ob/ob mouse*) respiratory abnormalities including tachypnea, decrease lung compliance and aberrant respiratory muscle are common and prolonged treatment with leptin attenuates respiratory abnormalities, suggesting a role for leptin also as a neuro-humoral modulator of central respiration (Tankersley *et al.* 1998). Several studies have been conducted regarding the leptin impact on inflammatory lung disease, and reported a relationship between serum leptin levels and the presence of chronic lung disease like asthma. Serum leptin levels in asthmatic children, especially males, were higher than healthy control, despite no difference in BMI (body mass index) (Guler *et al.* 2004). Based on the association between leptin and systemic inflammation, it has been hypothesized also a potential link between obesity and chronic lung disease such as asthma (Antonopoulou *et al.* 2008). In human, obese subjects showed high leptin levels in bronchoalveolar lavage (BAL) compared to lean subjects and these results are supported by a positive correlation with BMI, lung function, TNF-α in BAL, nitrates, 8-isoprostanes. Moreover, it's possible observe values increasing if

subjects were affected by asthma (Lessard *et al.* 2011) (Holguin *et al.* 2011) (Lugogo *et al.* 2012). In a recent paper (submitted to reviewers) we demonstrate a statistically significant difference between serum levels of leptin in obese children with asthma compared with controls non-obese and not affected by asthma (healthy children). The same results were found in the leptin levels in exhaled breath condensate (EBC). These data suggest how leptin can be a new marker of lung inflammation to evaluate the outcome of chronic disease. Currently while many investigators reported a possible link between several diseases in the lung and systemic levels of leptin, describing leptin and its receptor, the relationship with infants affected by BPD has not yet been evaluated. A putative link between BPD and serum leptin remains nowadays unclear because no data are available on the potential presence of leptin in epithelial lung fluid. Further studies are needed to found the relationship in systemic inflammation of the lung and evaluate as specific target the BPD.

Ghrelin

Among non-traditional cytokine it's possible citing ghrelin, a unique 28 amino acid peptide purified form the stomach of rat (Kojima *et al.* 1999) that acts as endogenous ligand for the growth hormone secretagogue receptor (GHS-R) and has a strong effect on GH (growth hormone) regulation (Sun *et al.* 2004). Ghrelin is mainly produced by a subset of stomach cells and by the hypothalamus, pituitary, and other tissues, and after transcription is necessary a post translational modification consisting in the acylation of the hydroxyl group of the serine 3 in the endoplasmic reticulum. This peptide is major express in digestive system, but low levels were found in nervous system, in the pituitary but also in other tissue (Gnanapavan *et al.* 2002), including lung (Ghelardoni *et al.* 2006). Subsequent studies have demonstrated the wide distribution of ghrelin and its receptor, and this proposes potentially exhibit of multiple biological activities such as myocardial injury, neurogenesis, bone metabolism, reproductive function, memory and sleep (Nerula and de Boisblanc, 2015). Moreover, has been found that ghrelin acts in modulation of glucose and lipid metabolism, appetite control,

and food intake (Mokrosiński and Holst, 2010) and has been hypothesized a promising therapeutic target for cognitive dysfunction, but also metabolic syndrome and such as obesity and type 2 diabetes mellitus (Cong *et al.* 2010). Most of the research focuses on the role of ghrelin in food intake and its related endocrine functions. However, given the wide distribution of GHS-R on several immune cell subsets, it was evaluated the effect on the immunological system (Dixit *et al.* 2004) (Dixit and Taub *et al.* 2005). There is a specific relation between immunological system and food intake: the cellular adaptive response is dependent from energy supply in the cells. For example, an event like the loss of appetite can be a predictive cause of illness or general injury (Kelley *et al.* 2003), and is well known how plasma ghrelin concentration increase before (Cummings *et al.* 2001) and decrease after (Ghelardoni *et al.* 2006) every meal (Fig.5). The central nervous system controls food intake and energy homeostasis by releasing inflammatory cytokine (Hart,1988). Ghrelin inhibits the production of inflammatory cytokines in BALB/C mice after intraperitoneal injection of LPS endotoxin shock (Dixit *et al.* 2004).

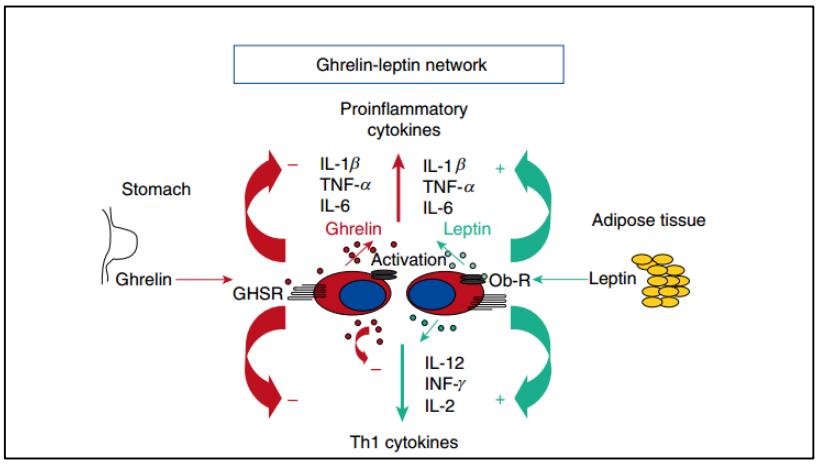

Fig. 5. Ghrelin and leptin exert mutually antagonist regulatory effects on metabolism and inflammation. (from Narula and de Boisblanc, 2005)

Moreover, during inflammation ghrelin and its receptor are present in neutrophils, lymphocytes and macrophages and their expression is regulated during acute and chronic injury. The influence on the immune system it was recently demonstrated in vivo and in vitro (Li *et al.* 2015). Ghrelin can also antagonize leptin, a pro

inflammatory protein that promote the cytokine production, in direction of pro-inflammatory pathway. These findings suggest a specific role in the homeostasis regulation as well as in the setting of immune system (Fig.5) (Narula and de Boisblanc, 2015). Several research conducted in animal models have demonstrated the protective effect of ghrelin on acute lung injury on improving pulmonary pathological damage, permeability, mechanics and gas exchange (Li *et al.* 2016). Over the last few years have been conducted clinical trials (Miki *et al.* 2012) in witch was administered ghrelin in human to assessed the effect in chronic lung injury like COPD and bronchiectasis (Kodama *et al.* 2008) (Matsumoto *et al.* 2015). In infants, especially in the newborns, the role of ghrelin has been evaluated to study the nutritional status (Andreas *et al.* 2016), the oxidative stress (Luo *et al.* 2015), but no studies assessed the possible relation between ghrelin and the lung injury presents in preterm infants and in infants with BPD.

Aim

The aim of this research is the evaluation of level of two markers in the airways. In particular, to avoid further stress in the management of care, or going against the clinical practice and ethical principle, this research has focused on the evaluation of leptin in airways with non-invasive technique. Among the sampling techniques the aim of the present study is evaluate the levels of leptin and ghrelin in EBC of preterm newborn underwent to mechanical ventilation. The hyperoxia-induced neonatal lung injury model is widely used as murine model of BPD to assess pathological hallmarks and tissue injury. In this study the tissue expression of leptin and ghrelin was not assessed in human, because is no ethical perform a biopsy in children without needed clinical, and so we have evaluated the expression of these markers in a lung tissue of animal model of BPD exposed to high oxygen concentration for 14 days and 28 days.

METHODS

Study design

Preterm newborn

After birth, preterm newborns were underwent to mechanical ventilation and oxygen supplementation, according to medical vital parameters and only for the clinical stabilization. Exhaled breath condensate was collected, after parents had signed the informed consent, and however without interpose in any way with clinical practice and on child health.

Animal model

At the birth Wistar pups and mother were exposed to continuous 60% of oxygen concentration for 14 postnatal days (PN14), to induce BPD according to O'Reilly (O'Reilly and Thébaud, 2014). The other group was exposed for 28 days (PN28) to continuous 60% of oxygen concentration. The control group was maintained in room air concentration during all experiments (Fig. 6).

<table>
<tr><th></th><th>Birth</th><th>PN14</th><th>PN28</th></tr>
<tr><td>Control group</td><td colspan="2">room air</td><td rowspan="2"></td></tr>
<tr><td>BPD group</td><td colspan="2">60% O_2</td></tr>
<tr><td>Control group</td><td colspan="3">room air</td></tr>
<tr><td>BPD group</td><td colspan="3">60% O_2</td></tr>
</table>

Fig. 6. Treatment schedule of controls and BPD.

Sampling of EBC in preterm newborn

This clinical study was designed and shall be implemented and reported in accordance with the Guidelines for Good Clinical Practice, an international ethical and scientific quality standard for designing, conducting, recording, and reporting studies that involve the participation of human subjects. Were applied local regulations (including European Directive), and the ethical principles laid down in the Declaration of Helsinki. Participation of patients is based on the Italian regulations and local Ethic Committee requirements. This study is approved by local Independent Ethical Committee with number CESC37. We have enrolled 14 mechanically ventilated patients (8 with BPD and 6 with acute respiratory distress syndrome) admitted to Neonatal Intensive Care Unit of Azienda Ospedaliera Universitaria Integrata, University of Verona, Italy. The diagnosis of BPD was defined as oxygen dependence for at least 28 postnatal days for infants 32 weeks or greater postmenstrual age or oxygen dependence at 36 weeks postmenstrual age for infants born before 32 weeks according to Jobe (Jobe and Bancalari, 2001). Control group of healthy newborns was not included because, to collect EBC sample with the suggested method, is necessary mechanical ventilation.

Inclusion criteria

- Gestational age <33 weeks, or weight at birth ≤1500g need for mechanical ventilation.
- Signed the informed consent from parents.

Exclusion criteria

- Subjects with major surgery just after the birth.
- Subjects with major congenital anomalies (e.g. arteriovenous shunt), congenital heart disease or known cytogenetic disorders (e.g. Down's syndrome). Open ductus arteriosus and open foramen ovale are not exclusionary anomalies.
- Subject has known or suspected immunodeficiency, such as known human immunodeficiency virus (HIV) infection.
- Subject has suspected hepatitis B or C infection.

- The subject's legally acceptable representative, i.e., parents or legal guardian/caregiver, is not able to communicate reliably with the clinicians involved in this study.
- Withdrawal of informed consent by parents.

Newborns group were comparable for gender, body weight at birth and ethnicity. Patient's characteristics are shown in Tab.2.

Exhaled breath condensate was collected in the supplemental oxygen ventilator circuit after clinical stabilization. To assess the reproducibility of the EBC the sample was collected for 3 hours just after positioning the ventilation circuit. The volume collected is depending by the flow stream, and after collection all samples were stored in polypropylene (Eppendorf) tubes at -80°C.In this time were collected 3 different samples of EBC to assess the reproducibility. All neonates were mechanically ventilated with Babylog 8000Plus Ventilator (Drager Williamson Ct. Louisville, Kentucky 40223 USA) and were monitored in continuous oxygen saturation, respiratory rate, tidal volume, heart rate, and electrocardiography (data not cited). The circuit used was RT225 (Fisher & Paikel Healthcare Ltd, New Zeland). Sample integrity was guaranteed by maintaining of EBC cooling trap in ice bath, applied to the container dedicated to EBC collection as illustrated by Hunt (Hunt, 2002).

Leptin measurement

The Enzyme Linked Immuno Sorbent Assay (ELISA) kit (BioVendor Human Leptin ELISA) was used to measure leptin concentrations in EBC sample. The lower detection limit of the assay was 1 ng/ml and the sensitivity was 0.2 ng/ml. This technique involves the use of an indicator molecule coupled covalently with an enzyme, which converts the colorless substrate into a colored product, so that it can be detected by a spectrophotometer. The scheme is the following: use of a polystyrene plate with 96 wells coated with the primary antibody monoclonal anti human leptin; construction of a standard curve using serial dilutions of a known

concentration of the antigen and the specimens were added of condensate and serum concentration in unknown; addition of secondary antibody conjugated to peroxidase, and finally addition of the TMB detector substrate (3,3', 5,5'-tetramethylbenzidine). The substrate in the presence of HRP complex (horseradish peroxidase complex) develops a blue color. The reaction was quenched adding acid solution. The intensity of staining is proportional to the amount of specific antibody present in the sample. The plate is then read in a spectrophotometer at λ=450nm.

Ghrelin measurements

A commercial ELISA kit (EZGRT-89K; Millipore) was used to measure ghrelin concentrations in EBC sample. The lower detection limit of the assay was 165.62 pg/ml and the scheme work is the same as previously described for leptin detection. The absorbance was read in a spectrophotometer at wavelength 450 and 590 nm.

Animal model

The study was approved from Technical Ethical Committee of the University of Verona for experimental animal. Female rats were purchased from Charles River Laboratories Italia s.r.l, Calco, Lecco, Italy. Animals were acclimatized to the departmental animal facilities for at least 1 week before to program the mating. They were kept in individual cages with sawdust and free access to food and water; a temperature-controlled environment (21–23 °C) and humidity of 50% on a 12 hours' light–dark cycle with light ON at 8:00AM. All animals were handled in accordance with the Helsinki Declaration and recommendation for animal experimentation established of the Italian Public Health Authorities. The study was conducted on Wistar female rats weighing 250 g and pregnancies were dated accurately by means of single mating. At the term of gestation Wistar females and pups were divided in 2 groups: control group placed in acrylic chambers (Tecniplast mod 1290D, Buguggiate, VA, Italy) in room air, and BPD group

according with O'Reilly (O'Reilly and Thébaud, 2014). The BPD group (exposed) was placed in acrylic chambers (Tecniplast mod 1290D, Buguggiate, VA, Italy) contained in the hyperoxic setup (BioSpherix, Animal Chamber A-30274-P, Redfield NY). Except for the concentration of oxygen created by introducing a continuous 60% oxygen flow constantly monitored (BioSperix, OxiCycler model A84XOV, Redfield, NY), in the BPD group the ambient conditions are the same than control as previously described. To reduce the possible confounders in both groups, confinement was briefly stopped every 6 days just the necessary time for changing of cage with partial new sawdust, and restored food and water. The "gentle handling" procedure is based on a direct interaction with the experimenter, who keeps the animal under control and provided to change the cages. All efforts were made to minimize animal suffering and to keep the lowest number of animals used. The study was conducted on Wistar rats in hyperoxic chamber (n=14) and in room air (n=10). In the end of experiment all Wistar pups were anesthetized with an overdose of isoflurane. Body weight was recorded at the time of the death. Thorax was opened and lungs were removed *in toto*, then fixed in buffered formalin and embedded in paraffin, then sectioned using a microtome at 5μm and stained, for histological evaluation with hematoxylin and eosin (H&E).

Histological examination

The chest of the rats was opened and trachea, bronchial tree and lungs were carefully removed *in toto* and fixed in 4% paraformaldehyde, then dehydrated in rising gradient ethanol, vitrified in xylene and embedded in paraffin. The paraffin-embedded sections (5μm-thick) were stained with H&E. Morphological changes were evaluated (×40 magnification) using an optical microscope Olympus BX51 integrated with digital camera (JVC CCDKY-F58) equipped with the image analysis digital system Image-Pro Plus 7.0 (Media Cybernetics, Silver Spring, MD, USA).

Immunohistochemistry (Merigo *et al*. 2016)

In this study were selected the following primary antibodies: rabbit anti-ghrelin (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA, cat #H-031-31); rabbit anti-ghrelin receptor (-GRLN-R Abcam, Cambridge, UK, cat #ab85104); rabbit anti-leptin (Abcam, Cambridge UK, ab3583); rabbit anti-leptin receptor (Abcam, Cambridge, UK, ab5593). Peroxidase-immunohistochemistry was performed as described by Merigo *et al* 2011. Sections were treated with blocking solution (0.3% Triton X-100, 1% bovine serum albumin (BSA), and 1% swine serum 0.1M phosphate buffered saline (PBS) for 1 hour. Previously the endogenous peroxidase was quenched for 20 min in a peroxidase blocking solution (DAKO, Milan, Italy), then washing in PBS buffer pH 7.5. Sections were incubated overnight with primary antibodies diluted in blocking solution, then washed and reacted for 1h with diluted 1:400 biotinylated swine anti-rabbit immunoglobulins (DAKO). The immunoreaction was detected using a Vectastain ELITE ABC kit (Vector, Burlingame, CA, USA) and then visualized with 3.3-diaminobenzidine tetrahydrochloride (DAKO) for 5-10 min. Moreover, control sections were prepared by pre-absorbing the primary antibodies with the corresponding peptide (5 µg/1 mL of antibody; Ghrelin peptide, Phoenix Pharmaceuticals Inc.; Ghrelin Receptor peptide, Abcam) or by omitting the primary antibody. Sections were observed using an optical microscope Olympus BX51 integrated with digital camera (JVC CCDKY-F58) equipped with the image analysis digital system Image-Pro Plus 7.0 (Media Cybernetics, Silver Spring, MD, USA).

RESULTS

Statistical analysis

All data were expressed as median and IQR (interquartile range). Comparisons between 2 groups analysis were made using Mann-Whitney U test for non-parametric test. Statistical analysis was carried out GraphPad Prism v5.00 for Windows (GraphPad Software, San Diego, CA, USA). In all cases was considered significant a value of $p<0.05$.

Preterm newborn

Characteristics of patients

The characteristics of patients are showed in Tab.2. All 14 patients had been underwent to mechanical ventilation after birth and all had received the treatment with surfactant. All moms were nonsmokers, and passive tobacco smoke non-exposed. The difference in mode of delivery was no significant as well as other birth characteristics reported in Tab.2.

	Non-BPD	BPD	p-value
Number of patients	6	8	/
Gender			/
male	2	4	/
female	4	4	/
Mode of delivery			/
cesarean section	2	4	/
vaginal	4	4	/
Maternal pren. corticosteroids	3	2	/
Maternal antibiotic exposure	1	1	/
Surfactant treatment	6	8	/
*Apgar at 1 min.	5.5 (3.5-6)	4 (3-6)	n.s.
*Apgar at 5 min.	7.5 (6.3-8)	8 (6-8)	n.s.
*Gestational age, weeks	29.4 (28.3-30.9)	24.8 (24.6-26.6)	0.002
*Body weight at birth, g	1275 (1000-1646)	755.0 (700-840)	0.005

Tab.2. Demographical and clinical data on newborns. *Data are expressed as median and (range).

As can be seen in Tab.2 a significant difference (p=0.002) was found in gestational age. In particular, in the control group the median of weeks was 29.4 (IQR 28.3-30.9) if compared with BPD group that shows a median of 24.8 (IQR 24.6-26.6) weeks of gestational age. At birth a significant difference (p=0.005) in body weight in control group 1275g (IQR 1000-1646g) compared with BPD group 755.0g (IQR 700-840g) was found. BPD group is classified according to Jobe (Jobe and Bancalari, 2001): after 28 days of oxygen dependence for at least 28 postnatal days for infants 32 weeks or greater postmenstrual age, or oxygen dependence at 36 weeks postmenstrual age for infants born before 32 weeks. For this study, body weights were not registered longitudinally at the same time point for all patients during the admission to intensive care, and for this reason we prefer to don't cite this data at discharge and when oxygen treatment was stopped.

EBC leptin and ghrelin

Pulmonary levels of leptin in EBC of mechanical ventilated patients were not found with ELISA commercial kit previously described. Three test were repeated in three different back-up samples stored at -80°C. In EBC was assessed the levels of ghrelin with ELISA kit previously described. After three repeated test on back-up (stored at -80°C) were found no ghrelin concentration EBC sample. Levels of leptin and ghrelin were not found neither BPD group and nor in controls group.

Animal model of BPD

Mortality index

The study was conducted on Wistar rats in hyperoxic chamber (n=14) and in room air (n=10). 71.4% of neonatal rats exposed to hyperoxia survive, with the majority of deaths occurring between 2 and 8 days after birth. These finding have been that group continuously expose to 60% of oxygen presents a high rate of mortality at time PN14 compared with control keep in room air, from 14 pups (100%) to 10 pups (71.4%). Prolonged neonatal exposure to high oxygen concentration presents equal index of mortality at PN28 (Fig.7).

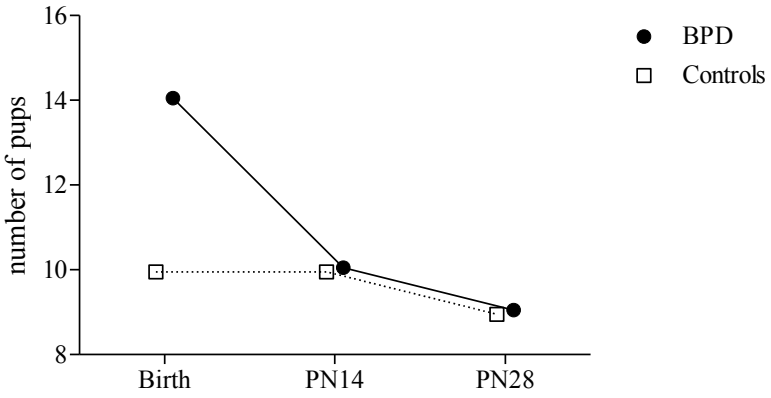

Fig.7. Mortality index in animal model of BPD.

Body weight

Maternal body weight was assessed at the time of the mating and at spontaneous delivery. Moreover, the weight was assessed after 14 and 28 days of exposition at high concentration of oxygen and was observed a body weight loss, suggesting a massive exposition at oxidative stress. No difference in body weight was observed in maternal body of control group. In the litter, statistically significant lowest values of body weight were found in BPD group (19.53g; IQR 18.63-21.89) after 14 days of exposure of 60% oxygen concentration if compare with controls in air room (28.02g; IQR 25.75-29.45) p=0.002 (Fig.8a).

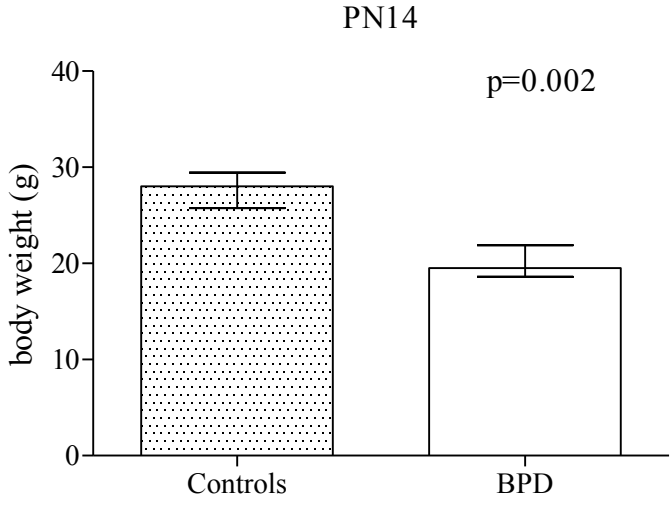

Fig.8a. Body weight in animal model of BPD compared with controls at PN14.

A statistically significant difference was found in BPD group exposed at 60% oxygen for 28 days compared with control group. In particular, BPD group shows a body weight 61.53g (40.95-80.26) compared with group kept in air room 128.6g (123.1-131.9) p=0.012 (Fig.8b).

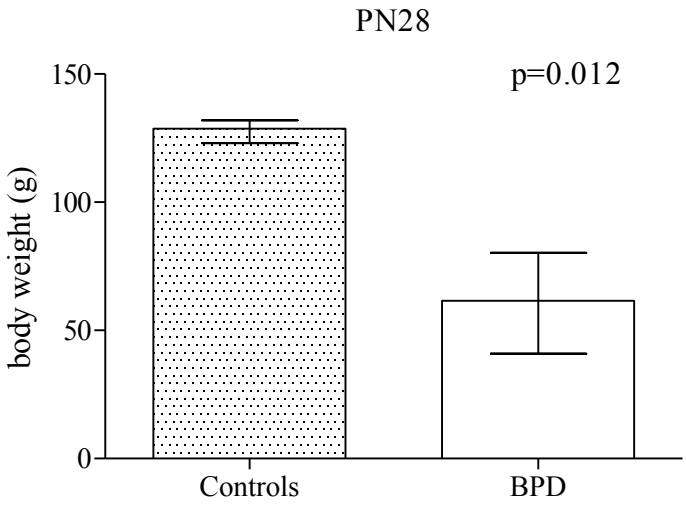

Fig.8b. Body weight in animal model of BPD compared with controls at PN28.

Body characteristics

At PN14 hyperoxic rats showed a suffering look, with shaggy hair, and general growth slowed if compared with healthy controls kept in air room (Fig.9). Critical differences in motor activity and neurological status were not found in two groups.

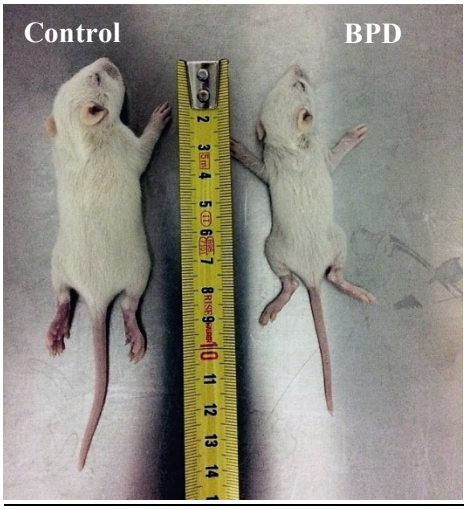

Fig.9a. General growth in healthy control and BPD model at PN14.

Same results in body development were found at PN28. In particular, control group presents a normal shiny hair, without sign of suffering. Conversely, the hyperoxic group presented a hispid and rugged hair (Fig.9b).

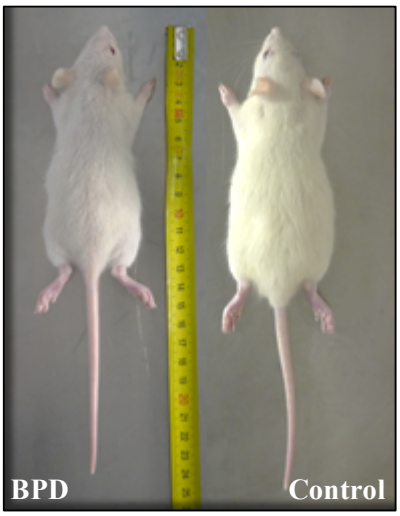

Fig.9b. General growth in healthy control and BPD model at PN28.

All animals presented a normal development of whiskers and no other morphological abnormalities were found.

Histology and immunohistochemistry

Different dimensions of trachea were found in two group of treatment during tissue collection. H&E stained tissue section were used to evaluate the dimensions of trachea in two groups. In particular, the caliber in the control group presents normal cavity with a thin trachea wall compared with BPD model, where is showed a large lumen and a thick wall. As can be seen, tracheal mucosa presents normal tract but also an increased thickness of *lamina propria* (Fig.10).

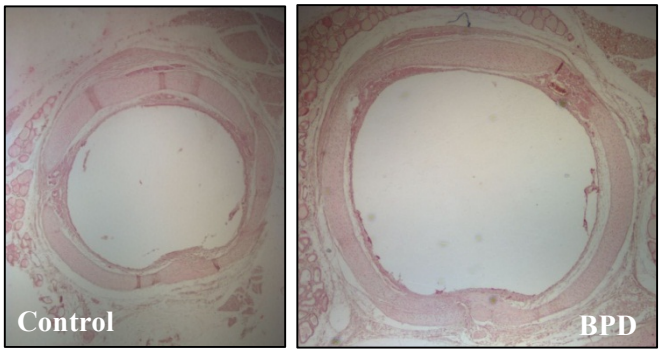

Fig.10. Section of trachea stained with H&E in Wistar control group (room air) and Wistar BPD group at PN14; (40X).

In control group at PN14 we found a significant difference in median values of trachea caliber. For each rat in the experiment we performed 5 measures on trachea caliber. Control group presented value of 0.75mm (IQR.0.70-0.76), instead in the BPD group presented a median value of 1.67mm (IQR 1.52-1.74) with significant difference (p=0.008) (Fig.11).

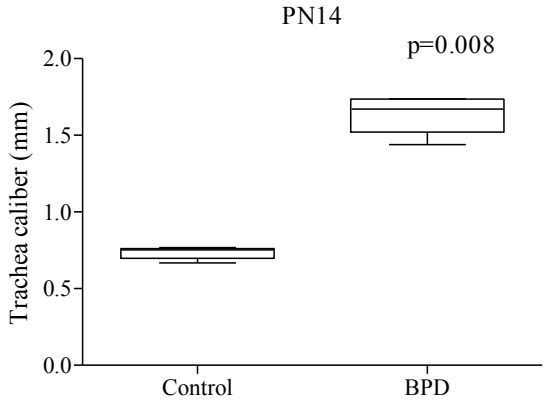

Fig.11. Caliber of trachea Wistar control group (room air) and Wistar BPD group at PN14;

Architectural changes at PN14 shows impaired alveolar lung development. In particular, a reduction in the number of alveoli and a simplification of structure in acinar development were found. Specimens stained with H&E show an increased interstitial thickness, and epithelial cells reported a low number of cilia if compared with control (data no showed). A continued exposure to high oxygen concentration (PN28) shows increasing in inflammatory and pulmonary edema response.

Leptin and ghrelin expression

Leptin expression was observed in control and exposed groups to compare the results. The experiment was conducted as described by Merigo (Merigo *et al.* 2016). In this case we don't apply a co-localization technique using immunofluorescence. In BPD rats' leptin immunoreactivity was similar than control rats (Fig.12a). Leptin receptor expression were observed mainly in in apical surface of cell but a minimal expression was found also in the cytoplasmic vesicles (Fig.12b) but there are no significant differences between control group and BPD group. Moreover, with the continuous exposition to high oxygen concentration in the BPD group at PN28 can be seen the worsening in the tissue architecture, but difference in leptin expression, in comparison two groups, were the same those observed at PN14 (Fig.12c).

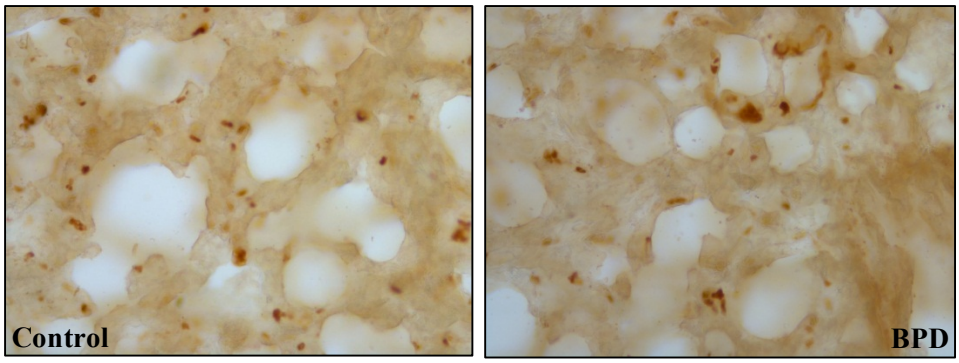

Fig.12a. Leptin expression in free floating sections at PN28 (20x).

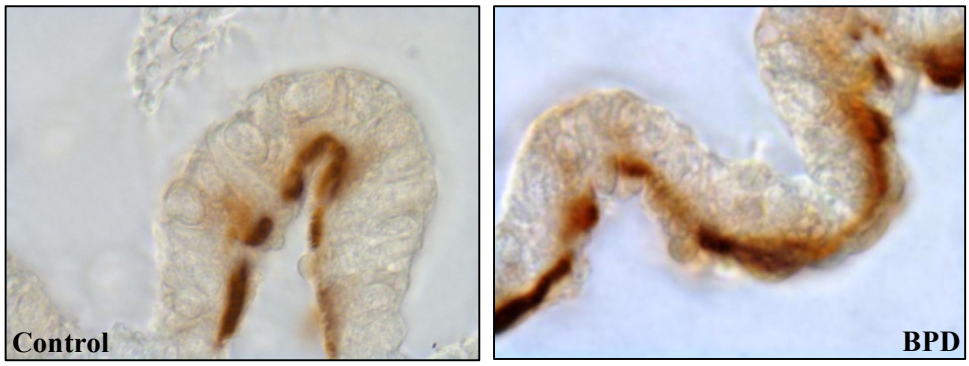

Fig.12c. Leptin receptor expression at PN28 in control group and in BPD group (100x). There are no different expressions in two group. Apical expression and minimal cytoplasmic expression in lung tissue was found.

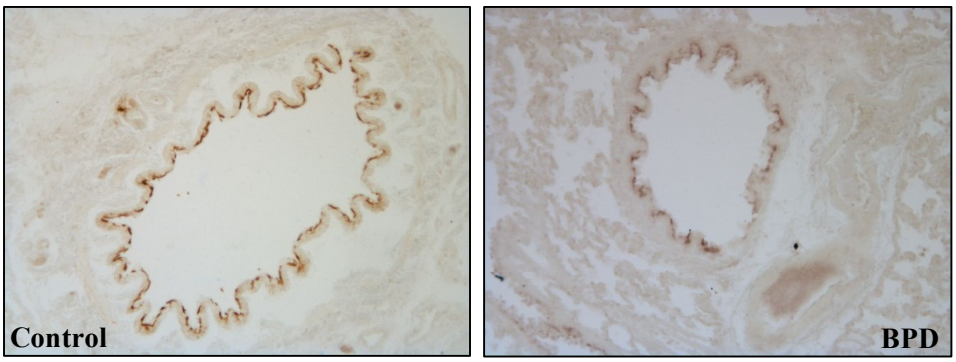

Fig.12b. Leptin receptor expression at PN28 in control group and in BPD group (10x).

Ghrelin expression was observed in control and exposed groups to compare the results. The experiment was conducted as described by Merigo (Merigo *et al.* 2016). Also in this case we don't apply the immunofluorescence technique to

assess the co-localization. Lung section of BPD rats show ghrelin immunoreactivity similar than control rats (Fig.13a and b) in time point PN14 but also in PN28. Ghrelin receptor shows the same expression comparing control group and BPD group (data no showed).

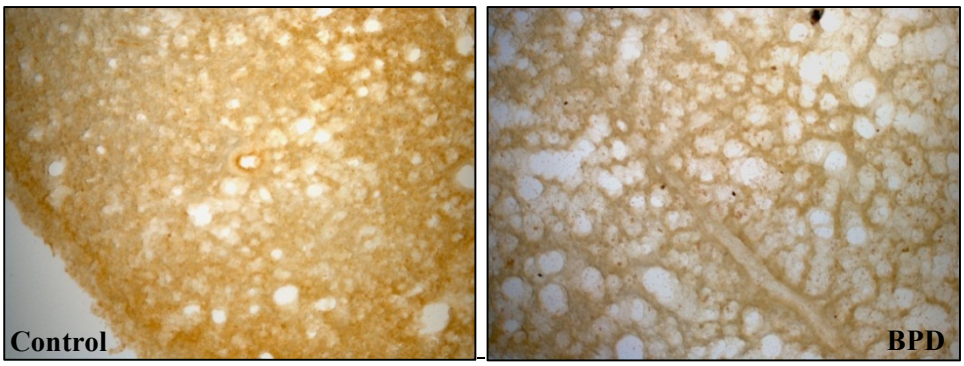

Fig.13a. Ghrelin expression in lung of control group and BPD group (Free floating 10X

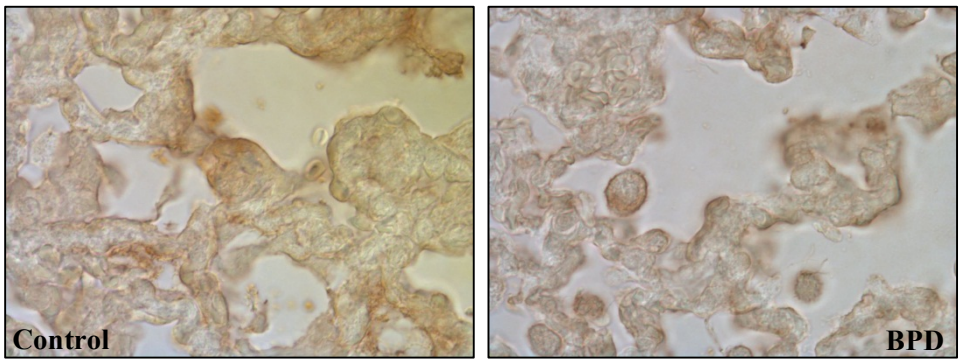

Fig.13b. Ghrelin expression in lung of control group and BPD group (Free floating 60X).

DISCUSSION

Bronchopulmonary dysplasia is the most common chronic lung disease in infants associated with arrested pulmonary development. Nowadays BPD remains the most common complication of preterm newborn with important impact on mortality and morbidity. These infants are the principal division of intensive care units, and an important target is the early diagnosis and precise clinical management strategies to improve outcome of disease. BPD was first described in 1967 by Northway and colleagues in a group of preterm infants resulting from high oxygen concentration and mechanical ventilation (Northway *et al.* 1967). In particular, postmortem acute lung injury, edema, extensive airway epithelial metaplasia, pulmonary fibrosis, and airway and vascular smooth muscle hyperplasia were found. Oxygen therapy plays an important role in modern neonatal intensive care, improving survival of preterm newborn with respiratory disorders. Although mechanical ventilation and oxygen supplementation found large use, they induce lung injury with a worsening of outcome, increasing the risk to develop a neonatal lung disease, like BPD. Improve medical care of premature infants with new management, such as the use of prenatal corticosteroids and postnatal surfactant replacement, the gentle ventilation modalities by CPAP and HHFNC, but also improved nutrition, now permit significant transition from “old BPD” described in 1967 to “new BPD” (Jobe and Bancalari, 2001) (Husain *et al.* 1998). New BPD was described in 2001 and compared to the old BPD presents less prominent inflammation, no hypertrophy in smooth muscle, no metaplasia in epithelial tissue, and minimal to moderate (but diffuse) alveolar septal fibrosis occurs mostly in extremely and/or in very low birth weight. Despite of many improve in neonatal medicine since the first description, incidence of BPD not decline showing high impact on 28 weeks of gestational age newborns (Balany and Bancalari, 2015). Infants with BPD present a high risk to develop respiratory infection with repercussion on pulmonary function that requires prolonged and recurrent hospitalization in the first years of life (McEvoy *et al.* 2014). In the lung development, the saccular period represents the timing for the formation of alveoli and go on in postnatal time from 36weeks of gestational age to 2 years of age, characterized by an increase of micro vascular

maturation and late develop of alveoli (up to 5 years of age) important for the ultimate the evolution of respiratory system (Burri, 2006). In term birth children the alveolar stage is normally developed, but in preterm infants, in the late canalicular or early saccular stage, the degree of lung immaturity increasing the risk of occurrence of BPD. Diagnostic biomarkers for predicting early BPD risk and standardized clinical research is needed, to better characterize the long term outcomes, and risk to develop other chronic lung disease such as asthma and COPD. Is well known the quantification of airway inflammation, including assessment of biomarkers with BAL and tracheal lavage, but these procedures are invasive, not suitable for routine use in clinical research, and potential cause of transient inflammation. Non-invasive technique based on evaluation of markers of lung inflammation or predictive of lung injury are significant for neonatologists during management of preterm infant in intensive care. In recent years EBC technique, based on cooling and condensing of exhaled breath air into test tube, was well described and has found a large use because non-invasive and safe. Moreover, the sample collection is very easy to perform even in children, offering various biomarkers to evaluate the lung inflammation. Accordingly, several studies have assessed respiratory lung disease looking for EBC biomarker such as, isprostane, leukotrienes, MMP, GSH, ADMA and others. Nevertheless, only few studies assessed EBC to evaluate the risk to develop BPD, especially from mechanical ventilated preterm newborn. For example, it has been used EBC to test levels of hydrogen peroxide and the levels of GSH, suggesting as these molecules may be potential parameters to define chronic lung disease (Kononikhin *et al.* 2016). Among markers of inflammation in EBC the leptin, a hormone expressed in a variety of tissues (Cohen *et al.* 1996) such as hypothalamus, placenta, and lung, and is implicated in energy homeostasis with neuroendocrine and immune functions, was found. In our experience (submitted to reviewers) asthmatic children present high level of EBC leptin compared with healthy controls, and this data confirm how leptin is a marker of lung inflammation. Also several studies have been conducted on leptin existence in inflammatory lung disease, and reported a relationship between serum leptin levels and the presence of chronic lung disease like asthma (Antonopoulou *et al.*

2008) (Ubags *et al.* 2014). BPD has been linked to the development of an inflammatory response that can occur in absence of clinical infection: an increase of pro-inflammatory cytokines values was found in tracheobronchial aspirates from premature infants TNFα, IL-8, IL-1β and IL-6, have been shown to correlate with increased risk of BPD (Kotecha *et al.* 1996) (Jònsson *et al.* 1997). Various studies mentioned the role of leptin in the inflammation, for example acute lung injury like pneumonia (Ubags *et al*. 2014) or chronic lung inflammation such as COPD (Kim, 2014) and asthma (Vernooy *et al.* 2013) but there is no studies to evaluate the levels of leptin in BPD, in particular the expression in epithelial lung fluid. Among non-traditional cytokine it's possible cite ghrelin, a leptin antagonist involved in energy homeostasis, mainly produced by stomach cells, and secondly by hypothalamus, pituitary, and other tissues. This peptide is major express in digestive system, but low levels were found in nervous system, pituitary but also in lung (Ghelardoni *et al.* 2006). Studies about ghrelin tested the role on metabolism and food intake (Mokrosiński and Holst, 2010) and role in inflammation (Dixit *et al.* 2004) but there are no studies about the putative link with BPD. The finest non-invasive and first-step-method to test the presence of a molecule in lung is the EBC collection. To the best of knowledge, the main limitation in the analysis of EBC is the missing of standardized protocols to support validation studies (Hovàrt *et al.* 2005). EBC is obtaining by cooling exhaled air into a system of sample tube maintained at low temperature, below water freezing point. In our study, just after mechanical ventilation the sample collection was kept with the procedure described by Hunt in 2002 (Hunt, 2002), and the sample condensing into test tube. Correct preservation of sample integrity depends on the maintaining of constant temperature, avoiding thermal excursion. In our ventilated circuit, the tube begins from medical ventilator, but its length was too much extensive if compared with a exhaled kit for sample collection, recommend in guidelines for collaborative patients (Hovàrt *et al.* 2005). Tubes of ventilates newborns can be considered as high death space where the condensates compounds can partially destroy, leading to lack of sample quality. Probably for this reason in this procedure applied to children underwent to mechanical ventilation, we not found levels of leptin and ghrelin in EBC sample. Moreover, to

avoid a possible sample bias the EBC collection started just after clinical stabilization and the sample was collected only in the first day of intubation. The collection after several days of intubation can't be scheduled, because clinical conditions lead to extubate infants in different time point, with results difficult to compare. In literature is well described how these infants show a premature lung development, but according to our time point, the lung injury is probably not enough markedly expressed to be evaluated with presence of leptin and ghrelin in condensate of epithelial lung fluid. On other hand, it is possible that leptin and ghrelin may not be predictive markers of lung injury. This supposition is supported by fact that we have collected the sample too prior with respect the possible development of BPD. This finding suggest how improve sample collection, avoiding every possible confounder involved in physical, but also molecular integrity of each sample. Moreover, it's necessary to better define the time point setting and research best period, during the mechanical ventilation, where is better collect the sample. Nevertheless, a non-invasive approach well integrated in clinical practice was showed efficacy to collect sample of EBC in ventilated infants, because clinical parameters were unchanged during the sample collection, and not influenced negatively the prognosis of clinical stabilization after preterm birth. As described before, EBC should be considered bio-fluid of great interest for research in clinical analysis, but there is a need in the standardization of EBC collection used for evaluating mechanical ventilated infants, because this field is still relatively limited for practical applications. Moreover, the present approach has demonstrated how collect EBC from ventilated infants and children is possible and safe, but also is needed commercially available hardware to collect the sample and preserve it from degradation. Self-made collectors can't afford to establish standards procedures and doesn't allow data comparison from different sampling, because doesn't give setting up the correct temperature and duration of collection. Failure presence of leptin and ghrelin in EBC, as previously described, suggests they probably aren't early markers of BPD and for this reason we assessed the expression of leptin and ghrelin in the lung of animal model of BPD. In literature are showed several studies on BPD, first with neonatal mice (Bonikos *et al.* 1976) and studies that use

rat BPD employed high oxygen concentration (60%) (O'Reilly and Thébaud 2014). Hyperoxia induced neonatal lung injury model has found a large use in animal model of BPD because it is easy program the birth, they have a large pups size in only 21 days of gestation. Regarding the lung, most rapid rate of alveolar development occurs between 3 to 8, and is almost ended by the day 14. Although rats born in this particular stage of lung development, their lungs are functionally fully developed and no requires, as in human, specific intervention to stabilize clinical conditions. After birth and exposure at high levels of oxygen in this period produces anatomical and functional changes in the lung (Thurlbeck, 1975) (Meyrick and Reid, 1982). The induced lung injury is similar to those seen in preterm newborn that has developed BPD. Moreover, we have extended the period of hyperoxia demonstrating that the morphological changes in alveolarization occur in the first days of life and the outcome is designed in this period. Although it has been extended period of hyperoxia, the minimal expression of ghrelin and leptin in lung tissue level does not changed. Conversely to the sample collected in the first day of life in infants ventilated, in this experiment the BPD was induced, and according to several studies with hyperoxia treatment it's possible obtaining an animal with lung development very similar at human BPD. Nevertheless, a minimal expression of leptin and ghrelin was found in lung tissue of BPD and this data is no significantly different if compared with control group. The finding probably confirm also the negative answers obtained with ELISA assay. In fact, also in this measures, no levels of leptin and ghrelin in EBC were discovered. In our study the most impressive results of treatment with 60% of oxygen concentration was the body development, and this is partially in concordance with other studies reported by O'Reilly in a recent review (O'Reilly and Thébaud 2014). In fact, important differences on body weight in control and BPD group were found, but also an evident hair suffering. Hyperoxia group presented body characteristics worse than control group kept room air, and significant differences were found in trachea caliber of two groups. In particular, hyperoxia group presented a high caliber than control group suggesting how, for these animals, the treatment was weighty in respiratory conditions. In the current study we demonstrated that prolonged exposure to high oxygen concentration

(PN14) lead to lung injury very similar to those presents in infants with BPD, and moreover a continued exposure to high oxygen concentration (PN28) shows an increase in inflammatory and pulmonary edema response. In next future we will test our sample with a screening of proteomic and metabolomics features, and identify putative markers of early develop od BPD during the ventilation and oxygen support. Additionally, is necessary to focus and standardize the sample collection, with particular attention on the hardware, because methodology at the end of ventilated circuit is not able to reduce the risk of sample contamination or sample degradation. This is an important point and suggests that the temperature of sample tube and in the collect circuit are probably a key points where is required to refine the methods. Even if in the current study a specific role of leptin and ghrelin remain to be clearly established, and the expression of these two hormones in lung of animal model of BPD was not strongly expressed, is necessary to further investigate these two markers and probably focus the attention on metabolic part, that remains the main role of these two hormones. In a recent work Merigo and colleagues (Merigo *et al.* 2016) described how molecules implicated in glucose homeostasis are differently express in airways of rats. Next future could provide new details in airways of animal model of BPD, to better understand the complex functions and putative implication of energy homeostasis.

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LIST OF ABBREVIATIONS

ADMA	Asymmetric Dimetilarginine
BAL	Broncho Alveolar Lavage
BMI	Body Mass Index
BPD	Bronchopulmonary Dysplasia
BSA	Bovine Serum Albumin
COPD	Chronic Obstructive Pulmonary Disease
EBC	Exhaled Breath Condensate
ELISA	Enzime Linked Immuno Sorbent Assay
GERD	Gastro Esophageal Reflux Disease
GH	Growth Hormone
GHS-R	Growth Hormone Secretagogue Receptor
GSH	Glutathione
H&E	Hematoxylin and Eosin
HHFNC	Heated High-Flow Nasal Cannula
HIV	Human Immunodeficiency Virus
HRP	Horseradish Peroxidase
ICAM-1	Intercellular Adhesion Molecule 1
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-1β	Interleukin 1 β
IL-6	Interleukin 6
IL-8	Interleukin 8
Interferon- γ	Interferon gamma
JAK	Janus Kinase
MMP	Matrix Metallo Proteinase
NF-kB	Nuclear Factor Kappa B
PBS	Phosphate Buffered Saline
PGE	Prostaglandin
PGHS-1	Prostaglandin-endoperoxide synthase 1
PGHS-2	Prostaglandin-endoperoxide synthase 2
PN	Postnatal days
PPARγ	Peroxisome Proliferator-Activated Receptor gamma
RDS	Respiratory Disease Syndrome
SP-A	Surfactant Protein A

SP-B	Surfactant Protein B
SP-C	Surfactant Protein C
STAT	Signal Transducer and Activator of Transcription
TGF1β	Tissue Growing Factor beta
TH1	T Helper 1 cells
TH2	T Helper 2 cells
TMB	Tetramethylbenzidine
TNFα	Tumor Necrosis Factor alpha
VEGF	Vascular Endothelial Growing Factor