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Onset of anemia in pulmonary diseases: role of hypoxia, oxidative stress, iron metabolism and hematopoietic regulators

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TESI DI DOTTORATO

Onset of anemia in pulmonary diseases: role of hypoxia, oxidative stress, iron metabolism and hematopoietic regulators

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Riassunto

INTRODUZIONE: L'anemia è una comorbilità importante nei pazienti affetti da ipossia grave, ad esempio per la sindrome da distress respiratorio acuto (ARDS), una condizione acuta caratterizzata da insorgenza improvvisa di grave ipossiemia e una grave manifestazione di COVID19. L'infiammazione aumenta l'epcidina, che interferisce con i fisiologici meccanismi di compensazione ematopoietica dell'ipossia e aumenta lo stress ossidativo indotto dall'ipossia.

OBIETTIVI:

- Indagare l'eziologia e la progressione dell'anemia nei pazienti ARDS, attraverso biomarcatori di infiammazione, metabolismo del ferro e stress ossidativo.
- Identificare gli effetti dell'aumento dell'epcidina e gli effetti potenzialmente protettivi dell'eritropoietina (EPO) nel metabolismo energetico delle cellule di polmone e sistema nervoso.

METODI: Abbiamo raccolto campioni di sangue in tre time points da 20 pazienti ARDS: alla diagnosi (T0), quando l'emoglobina (Hb) era inferiore di 3 g/dl a T0 o <8 g/dL (T1) e due giorni dopo T1 (T2). Venti pazienti con broncopneumopatia cronica ostruttiva ambulatoriale (BPCO) sono stati reclutati come esempio di ipossia cronica non anemica e dieci volontari non ipossici come gruppo di controllo. Sono stati valutati parametri del metabolismo del ferro e di stress ossidativo. Abbiamo poi riprodotto in vitro alcune condizioni osservate nei pazienti: ipossia e infiammazione in cellule polmonari A549, ipossiche e trattate con lipopolisaccaride (LPS) e danni mitocondriali in cellule neuronali SHSY5Y. L'effetto del trattamento con epcidina e dell'EPO sul metabolismo energetico di tali modelli cellulari è stato valutato con lo strumento XF24 Seahorse.

RISULTATI- Nei pazienti con ARDS, l'Hb scende di valore a distanza di circa 7-10 giorni da T0. Abbiamo osservato che in ARDS aumentano l'interleuchina 6, l'epcidina e la malondialdeide (MDA), rispetto sia alla BPCO che ai controlli. La barriera antiossidante (FRAP test) si indebolisce da T0 a T1. I valori ARDS COVID19 sono simili a quelli non COVID ARDS, ad eccezione di epcidina e ferro libero (NTBI) più elevati. I valori dell'EPO rimangono invariati nonostante l'ipossia. Nelle cellule SHSY5Y in cui abbiamo indotto un danno a livello mitocondriale, l'EPO ha parzialmente ridotto i danni e aumentato la glicolisi. Nelle cellule A549 in condizioni ipossiche, l'LPS ha diminuito la vitalità e, in combinazione con l'epcidina, ridotto la respirazione mitocondriale e aumentato la glicolisi.

CONCLUSIONI: L'ARDS è caratterizzata dal rilascio sistemico di mediatori infiammatori e da stress ossidativo, il che interferisce con l'adattamento ematopoietico all'ipossia. Lo stress ossidativo e l'infiammazione portano a un aumento dell'epcidina, che si traduce in uno sbilancio del metabolismo del ferro, in anemia e in danni mitocondriali.

Abstract

BACKGROUND: In patients affected by severe hypoxia, such as those with Acute Respiratory Distress Syndrome (ARDS), an acute condition characterized by sudden onset of severe hypoxemia and a severe manifestation of COVID19, anemia is a common complication. It remains to be established weather iron metabolism, especially hepcidin, and oxidative stress are involved in negatively interfering with physiological hematopoietic compensatory mechanisms to hypoxia.

AIMS:

- To investigate etiology and progression of anemia in ARDS patients, through biomarkers of inflammation, iron metabolism and oxidative stress.
- To identify the effects of increased hepcidin and the potentially protective effects of erythropoietin (EPO) in the energetic metabolism of lung and brain cells.

METHODS: We collected blood samples at three timepoints from 20 ARDS patients: at the diagnosis (T0), when hemoglobin (Hb) was 3 g/dl less than T0 or <8 g/dL (T1), and two days after T1 (T2). Twenty ambulatory chronic obstructive pulmonary disease (COPD) patients were recruited to compare with non-anemic chronic hypoxia and ten non-hypoxic volunteers represented the control group. We reproduced hypoxia and inflammation in hypoxic lung A549 cells treated with lipopolysaccharide (LPS), and mitochondrial damage in neural SHSY5Y cells. The effect of hepcidin treatment and EPO on energetic metabolism was evaluated by measuring oxygen consumption and extracellular acidification rate (Seahorse technology).

RESULTS: In ARDS patients, [Hb] decreased within 7-10 days after T0. We observed that ARDS induced higher IL6, hepcidin and malondialdehyde (MDA) with respect to both COPD and controls. The antioxidant barrier (ferric reducing antioxidant power test, FRAP) was weakened from T0 to T1. COVID19 ARDS values were similar to non-COVID ARDS, except for higher hepcidin and non-transferrin-bound iron (NTBI). EPO values were unchanged despite hypoxia. In hypoxic A549 cells, LPS decreased viability and, in combination with hepcidin, decreased mitochondrial respiration and increased the glycolytic rate. In SHSY5Y challenged cells, EPO partially rescued mitochondrial damage and increased glycolysis.

CONCLUSIONS: ARDS is characterized by systemic release of inflammatory mediators, which negatively interfere with the hematopoietic adaptation to hypoxia. Oxidative stress and inflammation increase hepcidin, which induces a dysregulation in iron metabolism, with anemia and mitochondrial damage.

List of abbreviations

APACHE IV score	Acute Physiology And Chronic Health Evaluation Score
ARDS	Acute Respiratory Distress Syndrome
BE	Base Excess
CaO_2	Arterial Oxygen Content
Cer	Ceramide
COPD	Chronic Obstructive Pulmonary Disease
COVID19	Coronavirus Disease 2019
ctrl	Controls
ECAR	Extracellular Acidification Rate
EPO	Erythropoietin
FiO_2	Fraction Of Inspired Oxygen
Fpn	Ferroportin
FRAP	Ferric Reducing Antioxidant Power
GSH	Glutathione (Reduced Form)
GSSG	Glutathione (Oxidized Form)
Hb	Hemoglobin
Hep	Hepcidin
HIFs	Hypoxia Inducible Factors
IL-6	Interleukin 6
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MPP+	1-Methyl-4-Phenylpyridinium Iodide
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NT	Non-Treated
NTBI	Non-Transferrin-Bound Iron
O_2	Oxygen
OCR	Oxygen Consumption Rate
PaO_2	Arterial Partial Pressure Of Oxygen
PCO_2	Partial Pressure Of Carbon Dioxide
PEEP	Positive End-Expiratory Pressure
RBC	Red Blood Cells
ROS	Reactive Oxygen Species
S1P	Sphingosine-1-Phosphate
SOFA score	Sequential Organ Failure Assessment
SpO_2	Peripheral Saturation Of Oxygen
sTfR	Soluble Transferrin Receptor
WBC	White Blood Cells

Research integrity

The author of this thesis declares under her own responsibility that this research was performed in accordance with the Research Integrity rules, as stated in the European Code of Conduct for Research Integrity (edition 2018):

•The author understands and maintains the expected standards of rigor and integrity relevant to the research.

• The author understands and complies with ethical, legal and professional frameworks, obligations and standards as required by statutory and regulatory authorities, and by employers, funders and other relevant stakeholders.

• This research was designed, conducted, and reported in ways that embed integrity and ethical practice throughout.

• This research is subject to appropriate and active consideration of ethical issues

• The authors collaborated to maintain a research environment that encourages research integrity

• The author declares no conflicts of interest.

1 Introduction

1.1 Pathophysiology of hypoxia

Hypoxia, or lack of oxygen (O_2), is characterized in aerobic vertebrates by a decrease in the arterial partial pressure of O_2 (PaO_2) and a consequent decreased supply of O_2 to the tissues. In humans, this condition can occur both in physiological conditions (subjects exposed to minor atmospheric pressures, at high altitudes) and because of respiratory pathologies, such as acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD). Alveolar hypoxia is a strong inducer of pulmonary inflammation. It is well recognized that by generating reactive O_2 species (ROS) and hypoxia promotes nuclear factor kappa B (NFκB) activation, which upregulates several pro-inflammatory cytokines, such as IL-1, IL-6, and TNFα [1].

1.1.1 High altitude

At high altitude, partial pressures of O_2 are decreased because of the decreased atmospheric pressure. Atmospheric pressure and inspired O_2 pressure fall with altitude to be 50% of the sea level value at 5500 m and only 30% of the sea level value at 8900 m (the height of the summit of Everest). For this reason, the physiological adaptation to high altitude is very often considered as a good model to study hypoxia in humans [2]. In experimental models, a fraction of inspired O_2 (FiO_2) of 14% is used as a model of hypobaric hypoxia, to reproduce the conditions of an altitude of 3500 m above sea level [3]. In healthy subjects exposed for 10 months to 3900 m altitude, the average arterial blood O_2 saturation was 92%, and the average hemoglobin (Hb) was 19.5 g/L [4]. The primary response to high altitude hypoxia is a ventilatory response: hyperventilation occurs within minutes after exposure to hypoxia and is followed by hypercapnic ventilatory depression approximately 10 to 30 minutes after exposure [5, 6]. In about 8–10 days from the beginning of the exposure to decreased oxygen, in healthy subjects a greater number of erythrocytes will be produced to compensate for reduced O_2 availability [7]. Such increase can be beneficial for athlete training, but can be temporary, depending on individual factors [8]. Intermittent hypoxia was studied as potentially beneficial, as it increases antioxidant and protective mechanisms such as erythropoietin (EPO) production [9].

1.1.2 Acute Respiratory Distress Syndrome

Acute respiratory distress syndrome (ARDS) is an acute condition characterized by sudden onset of severe hypoxemia without evidence of heart failure nor volume overload [10, 11]. It involves about 10% of Intensive Care Unit (ICU) patients with hospital mortality in the range of 34.9- 46.1% [12]. The main causes are pneumonia or severe flu, sepsis, a severe chest injury, accidentally inhaling vomit, smoke or toxic chemicals, acute pancreatitis, an adverse reaction to a blood transfusion (Transfusion-Related Acute Lung Injury or TRALI). ARDS is, in several patients, associated with low Hb which may require transfusions [13]. It was suggested that iron in the blood components elevate iron levels in the recipient, which causes damage to the cells through sudden changes of iron homeostasis[14].

The severity of the hypoxemia defines the severity of the ARDS:

- Mild ARDS – The PaO_2/FiO_2 is >200 mmHg, but ≤300 mmHg, on ventilator settings that include positive end-expiratory pressure (PEEP) or continuous positive airway pressure (CPAP) ≥5 cm H_2O.
- Moderate ARDS – The PaO_2/FiO_2 is >100 mmHg, but ≤200 mmHg, on ventilator settings that include PEEP ≥5 cm H_2O.
- Severe ARDS – The PaO_2/FiO_2 is ≤100 mmHg on ventilator settings that include PEEP ≥5 cm H_2O.

An impairment in the lung endothelium function and increased ROS production are some of the main characteristics of ARDS. Despite the identification of microparticles as markers of endothelium dysfunction in case of acute lung injury [15], lower levels of circulating microparticles early after ICU admission were associated with the development of ARDS in patients with acute lung injury [16].

The treatment requires O_2 as one of the administered drugs [17, 18]. The effects of O_2 therapy in preventing mortality in ARDS have been recently observed in two studies, which found no specific effects of normobaric hyperoxic therapy (conservative, in which O_2 is administered in order to avoid too much hypoxia vs liberal, in which O_2 is administered to reach normoxia) [19, 20].

In survived patients, ARDS may result in long term (at least 2 years) neurocognitive morbidity and decreased quality of life[21].

1.1.3 Coronavirus Disease 2019

Coronavirus Disease 2019 (COVID-19) was declared as global pandemic by WHO on March 12th. COVID19 patients are often admitted to ICU because of severe hypoxia, which remembers ARDS [22]. Decreased circulating Hb may represent a pivotal comorbidity [23], because the SARS-CoV-2 viral proteins and surface glycoproteins may bind the heme group of the Hb β-chains favouring iron and porphyrin release [24]. This chain of events represents a further challenge to health in COVID19 through the action of SARS-CoV-2 in hurdling blood oxygenation in the alveoli and in reducing the O_2 delivery to tissues, thereby exacerbating systemic hypoxia and creating a vicious cycle. An additional mechanism underlying the harmful effect of hypoxia, aggressive ROS formed through the Fenton reaction, where free iron plays a key role, and the depression of endogenous antioxidants, further increases the oxidative stress [25].

It is worthwhile to note that despite initial similarities between ARDS and the severe respiratory presentation of COVID19, new evidence is supporting the idea that COVID19 is a separate entity from the typical ARDS, as shown by different lung compliance and stronger endothelial inflammation [22] .

1.1.4 Chronic Obstructive Pulmonary Disease

Chronic Obstructive Pulmonary Disease (COPD) is an important cause of mortality and morbidity [26]. It is a chronic lung disease characterized by persistent respiratory symptoms and airflow limitation, due to small airway obstruction and parenchymal destruction. Smoke habit is an important risk factor of this disease, but not all the smokers develop it [27]. Around the world, there

is large variability in the prevalence of COPD, with 10–95% under-diagnosis and 5–60% over-diagnosis [28], which makes the investigation of new biomarkers important in order to identify it.

O_2 inhalation at low flow is commonly used as a treatment in the most severe cases of COPD. Endothelial disfunction, one of the features of COPD, which contributes to the higher cardiovascular mortality and morbidity [29], is improved when O_2 is administered [30].

The physiological hematological adaptation to hypoxia is observed only in a limited fraction of COPD patients: 40-50% of COPD patients instead develop iron deficiency, with anemia, a predictive risk factor of worse outcome [31, 32].

1.2 Responses to hypoxia

1.2.1 Hypoxia inducible factors

Cellular adaptive response to hypoxia are mainly orchestrated by the activation of transcription factors called hypoxia inducible factors (HIFs) [33]. HIFs are heterodimeric proteins composed of an O_2-regulated HIF-1α or HIF-2α and a constitutively expressed HIF-1β subunit. Under hypoxic conditions, HIF-α proteins are stabilized for heterodimerization with HIF-β and bind to hypoxia response elements, in the promoter regions of specific genes. The fine regulation of HIF-1α is caused by HIF prolyl-hydroxylases (PHDs) that start the ubiquitination process that leads to HIF-1α proteasome degradation. O_2 acts as co-substrate of PHD enzymes. Since PHD enzymes rely on iron molecules to perform oxygen-dependent HIF1-α hydroxylation, iron chelators can produce HIF1-α stabilization [34].

1.2.2 Erythropoietin

One of the genes induced by HIF2-α [35] is located in chromosome 7 and codifies for erythropoietin (EPO), a glycoprotein cytokine, and one of the main responsible for the regulation of erythropoiesis. This protein is mainly secreted by the kidneys to maintain tissue O_2 homeostasis in response to both anemia and hypoxia. The generation of oxidative stress in these situations may play an additional pivotal role in HIF2-α stabilization and hence EPO production. Both EPO and its receptor were later detected in the brain, where they are upregulated by injury conditions [36]. HIF-2-α contains an iron-responsive element in its 5' untranslated region. Under iron-deficient conditions, when Hb synthesis is decreased, this RNA structure inhibits HIF2-α translation. These mechanisms ensure that EPO synthesis is adjusted to iron availability[7].

1.2.3 Iron metabolism

Since iron is an essential component of Hb, the hematopoietic response to hypoxia also increases the need for iron. In fact, hypoxic pulmonary vasocostriction and the potential consequent hypoxic pulmonary hypertension may be reduced by iron supplementation and exacerbated in case of iron deficiency [30, 37]. To optimize iron use for hematopoiesis, the ferroportin (Fpn) must be augmented. Fpn is the transmembrane protein that allows the release of iron from the cells, mediating its intestinal absorption and subsequent release into the circulation, bound to its transporter, the transferrin (figure 1). Fpn degradation is mediated by the regulating peptide hepcidin (Hep). Hep decrease, in healthy subjects, improved the compensatory response to hypoxia,

as well as to iron deficiency [7]. Hep downregulation, also mediated by HIFs [38], depletes enterocytes of iron, stabilizing HIF-2α [39]. A decrease in Hep was observed in healthy subjects exposed to high altitude hypoxia, both after acute (hours) and chronic (weeks) exposure [40, 41]. Such decrease occurs quickly and follows the release of factors from the activated erythropoietic bone marrow[42]. However, in the clinical setting, Hep is not yet considered among the routine parameters for the assessment of iron metabolism, although some studies are considering its decrease as a factor suggestive of the need for iron-based intravenous therapy [43]. The Hep values described in literature are very variable and denote a marked difference between healthy subjects [44] or anemic subjects hospitalized in Intensive Care Unit (ICU), in which high values are observed [45]. In another group of ICU patients, very low Hep concentrations (< 20 ng/l) at ICU discharge were suggested as one of the criteria to define iron deficiency, which was associated with higher one-year mortality[46].

About 30% of plasma transferrin is saturated with iron. In case of systemic iron overload, when transferrin saturation to rise above 60%, non-transferrin-bound iron (NTBI) accumulates. The transferrin receptor mediates the collection of iron by cells. The dosage of the soluble serum transferrin receptor (sTfR) is equivalent to an indirect determination of the transferrin receptor present on cell surfaces. As the expression of TfR increases in the presence of an iron deficiency and most TfR is expressed in the progenitor cells of the erythroid line, the level of sTfR in the serum may reflect both the cellular requirement (mainly of the erythroid line) of iron, and the size of the pool of erythroid progenitors, and therefore the rate of erythropoiesis. A positive correlation between EPO and sTfR was also observed especially in anemic subjects [47].

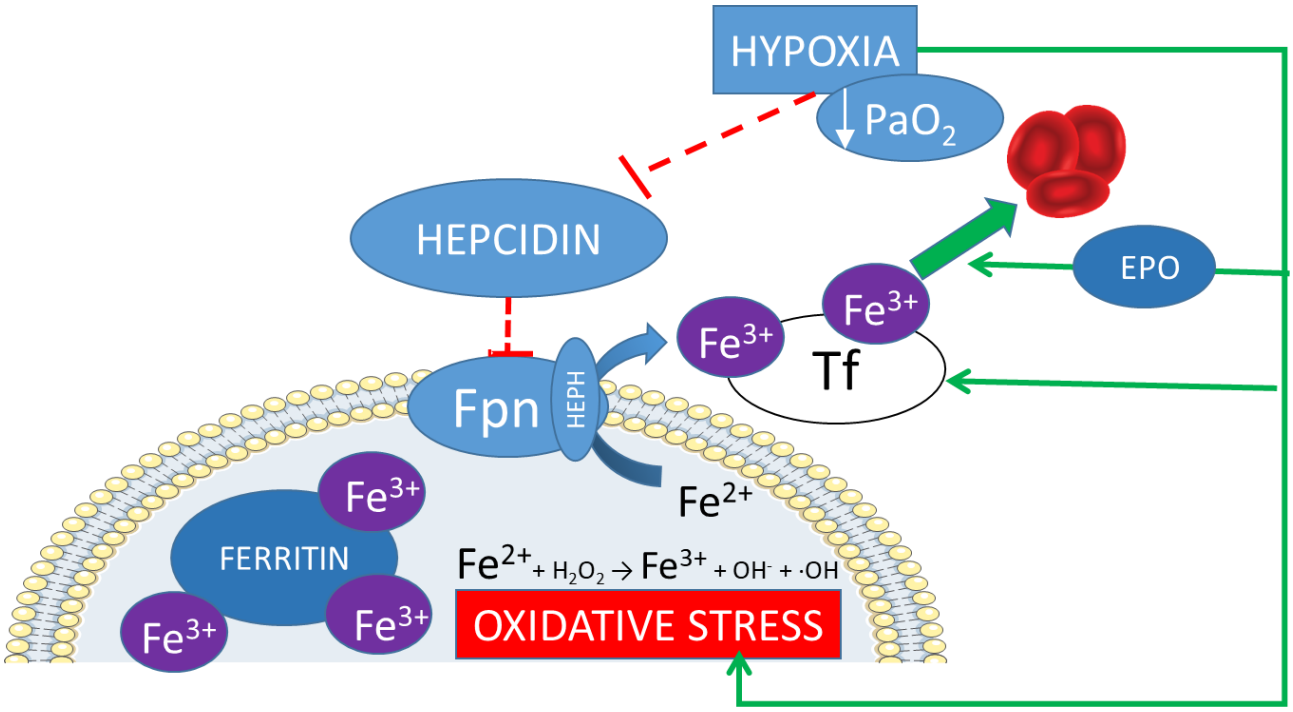

Figure 1 A correct adaptation to hypoxia results in the inhibition of the regulator peptide Hep. *Hep main action is the reduction of the outflow of the intracellular ferrous iron (Fe^{2+}), which is mediated by ferroportin (Fpn).*

1.2.4 Oxidative stress and antioxidant response

Excess iron acts as a Fenton reagent in the Habel-Weiss reactions, that results is the formation of Reactive Oxygen Species (ROS). ROS, such as superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (HO•), are radical and non-radical O_2 species formed in the partial reduction of the O_2 molecule (figure 2). Catalyzed by iron, $O_2^{\bullet -}$ reacts with H_2O_2 to produce O_2, HO• and hydroxide anion.

$$\text{First step: } Fe^{3+} + O_2^{\bullet -} \rightarrow Fe^{2+} + O_2$$

$$\text{Second step (Fenton's reaction): } Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH- + HO\bullet$$

$$\text{Sum Reaction: } O_2^{\bullet -} + H_2O_2 \rightarrow HO\bullet + OH- + O_2$$

This reaction is physiologically used, for example, by macrophages for the phagocytosis process.[48]

Under physiologic conditions, 2% of electrons involved in the mitochondrial respiratory chain leak react with molecular O_2 to produce such species. ROS generation, which is mainly dependent on complexes I and III, is highly dependent on metabolic conditions and on the intra-mitochondrial balance between oxidative and antioxidant factors [49]. A damage in the mitochondrial respiratory chain, as it occurs during hypoxia, can be a source of ROS formation. ROS upregulate the expression of pro-inflammatory, amplifying the tissue damage and pulmonary edema in ARDS [50].

1.2.4.1 Glutathione and Glutathionyl Hb

Reduced glutathione (GSH) can function directly as an antioxidant, by scavenging a variety of radical species, as well as participating in the reactions of glutathione peroxidase, which catalyzes the oxidation of glutathione at the expense of hydroperoxides, as hydrogen peroxide or lipid hydroperoxides. In response to excess ROS production, antioxidant tasks are pursued by enzymes as catalases, glutathione peroxidases, thioredoxins, and peroxyredoxins. These enzyme use electron donors, to avoid the intermediate formation of HO• [51]. The cell organelles peroxisomes have an important role in reducing intracellular H_2O_2, to avoid further formation of free radicals. The enzyme glutathione peroxidase on the peroxisome wall catalyzes the oxidation of the reduced form of the tripeptide thiol glutathione (GSH) to its oxidized form (GSSG):

$$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$$

Under conditions of pro- and anti-oxidant balance, up to 98% of the total glutathione pool occurs in its reduced form (GSH) [52]. The GSH/GSSG ratio is therefore used as a measure of the oxidative stress and its compensation. The red blood cell (RBC) glutathione levels in the population varies within a factor of six (0.6–3.6 mmol/L) and the strength of its antioxidant barrier shows a similar distribution, suggesting that glutathione homeostasis is under individual genetic control [53]. A decrease in glutathione firstly results in defects in cellular iron homeostasis [54].

Glutathionyl Hb is a minor modified form of Hb that contains a glutathione unit covalently linked to reactive cysteine -SH position. Its formation is reversible, but not transient, and it constitutes a variable fraction of the intraerythrocytic glutathione pool [55]. It was found elevated in iron deficiency anemia [56] and in several other pathological conditions where oxidative stress is not

coped by antioxidant defences [55]. It also shows the effect of oxidative stress associated to tobacco smoking, and was suggested as useful to assess the extent of damage caused by the harmful effects of tobacco addiction and to monitor the health improvement associated to smoke cessation interventions [57].

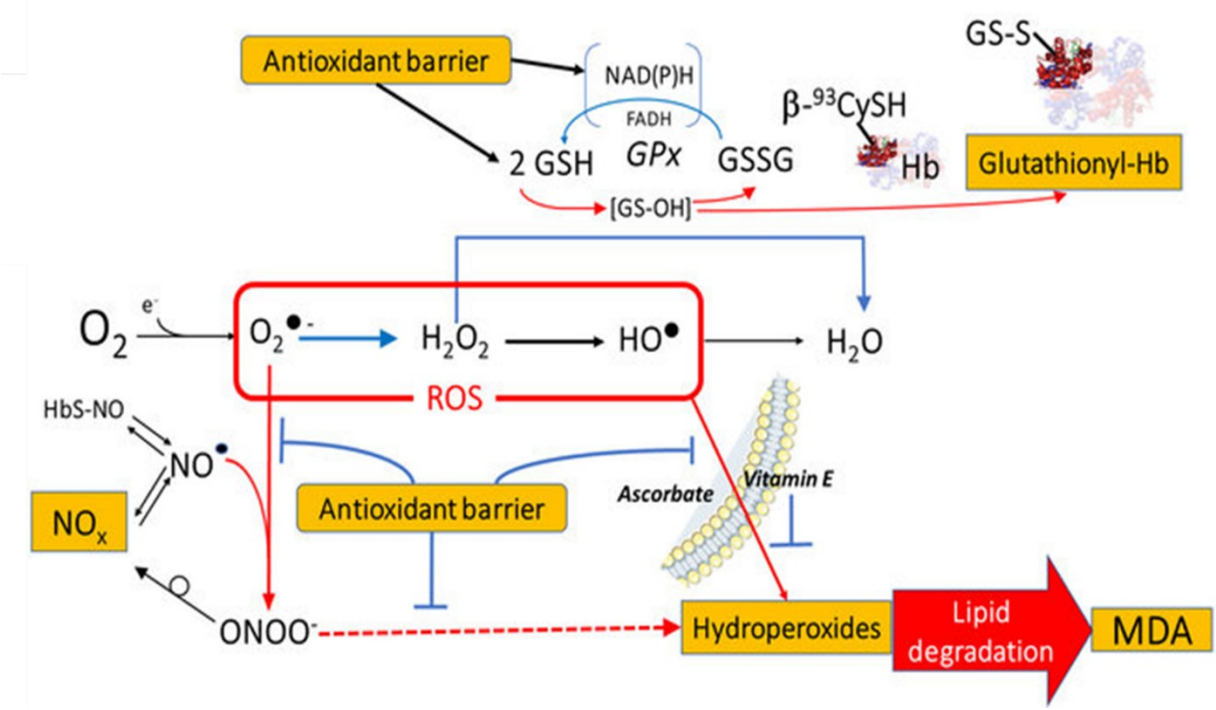

Figure 2: Some mechanisms for the production of ROS *and the biomarkers used to evaluate its effects. Orange squares: oxidative stress markers. Blue arrows: reduction processes. Red arrows: oxidation processes. Adapted from [58]*

1.2.4.2 Coenzyme Q10

Coenzyme Q, or ubiquinone, is an important coenzyme of the mitochondrial respiratory chain. The mitochondrial complex I transfers electrons from NADH to ubiquinone, with the transportation of 4 electrons through the inner mitochondrial membrane to participate in the synthesis of adenosine triphosphate (ATP). Decreased plasma concentrations of Q10 coenzyme were detected in several cardiovascular diseases [59]. In mice, coenzyme Q administration protects against sepsis-induced acute lung injury [60]. Ubiquinol, the reduced form of Coenzyme Q10, stops the initial process of lipid peroxidation. For this reason, coenzyme Q10 is also considered as one of the main antioxidants on mitochondrial and cell membranes [61].

1.2.4.3 Vitamin E

Vitamin E (α tocopherol) is a lipid-soluble vitamin that is found within most cell membranes, where it serves as a "chain breaking" antioxidant to halt the progression of lipid peroxidation, which proceeds as a chain reaction. Vitamin E donates an electron to a free radical intermediate in lipid peroxidation and becomes an innocuous free radical. Its concentration is decreased in ARDS patients[62]. Coenzyme Q10 is able to regenerate the oxidized form of vitamin E [59].

1.2.5 Lipid peroxidation and sphingolipid metabolism

Lipids, which often contain conjugated double bonds, are exposed to peroxidation when in contact with ROS. Arachidonic acid, with four double bonds, is the most easily oxidized amongst fatty acids.

This oxidation is a chain reaction, which can affect several molecules of lipids at the same time [63]. Lipids are the main components of cellular membranes. As phospholipids act a major role in preserving membrane architecture, their peroxidation causes changes in fluidity that can influence receptor function and trigger inflammatory or apoptotic pathways [64]. The inner mitochondrial membrane structure has a peculiar shape, with cristae that are essential for the efficiency of oxidative phosphorylation. This structure is also deeply affected by glycerophospholipids, such as the anionic cardiolipin, a molecule very susceptible to oxidation, which can be the cause of an energy impairment in the cell, and trigger necrosis or apoptosis. This mechanism has been hypothesized to be one of the reasons for cellular aging [65]. A major product of membrane peroxidation is malondialdehyde (MDA), which can be easily measured in plasma [66].

Lipids are modulated by diseases and recognized as therapeutic targets. Lipidomics is the most powerful tool to approach the study of lipids-related diseases. The increasing popularity of the lipidomics approach is strictly connected to the progress in the related analytical techniques, especially mass spectrometry.

Sphingolipids are a minor class of lipids of all mammal cells, composed by a hydrophilic head group protruding into the extracellular environment and a hydrophobic moiety, ceramide (Cer), located into the membrane bilayer [67]. Iron toxicity correlates with increased synthesis of sphingolipids in several eukaryotic organisms, as well as in mammalian cell lines [68]. Cer can be cleaved by a ceramidase to produce sphingosine, which can be phosphorylated by two enzymes for the synthesis of sphingosine 1 phosphate (S1P). Human healthy volunteers exposed to high altitude hypoxia (5260 m for up to 16 days) showed a time-dependent increase in plasma levels of S1P concurrently with elevated Hb capacity to release O_2 [69]. Exogenous S1P has been proved effective as a potential preconditioning agent favouring adaptation to hypobaric hypoxia in *in vivo* models of different pathologies, such as respiratory, cardiovascular, cerebral [70]. S1P pre-treatment facilitates hypoxia adaptation in healthy rats exposed to high altitude hypoxia for 6 hours. This beneficial effects of S1P rely on the enhanced blood O_2 carrying potential, mediated by HIF-1α stabilization and consequent dependent transcription of adaptive gene expression [71].

1.3 Anemia of inflammation and iron deficiency

Anemia of critical illness develops within days in patients who are hospitalized in intensive care units with infections, sepsis or other inflammatory conditions[72, 73]. ARDS is, in several patients, associated with low Hb despite persisting hypoxia, down to severe anemia (<8 g/dl Hb), which correlates with worse prognosis[13, 74]. Frequent among ICU patients [75], this anemia may be due to inflammation. Anemic ICU patients, among which patients with ARDS, have higher values of Hep than healthy subjects [45]. Inflammation, particularly through the pro-inflammatory cytokine interleukin 6 (IL-6), is a cause of increased Hep production [76], which may therefore interfere with the previously described hematopoietic compensation mechanism (figure 3). Increased production of leukocytes and decreased circulating iron diminish the number of erythroid precursors, among which reticulocytes, and macrophage activation shortens the red blood cells lifespan[77]. In anemic patients, the sTFR/log ferritin index is used to distinguish iron deficiency anemia from anemia of chronic diseases [78]. According to some authors, in an inflammatory state, the increased levels of Hep may be beneficial to protect the host from free iron-induced damage with various acute

diseases, by inducing also intracellular ferritin expression [79]. Among patients with sepsis, non-survivors have lower iron, EPO, and sTfR/log ferritin, but higher Hep, ferritin and IL-6 than survivors on days 1, 3 and 7 of ICU admission. EPO, Hep, ferritin, IL-6 and sTfR/log ferritin positively correlate with 28-day mortality [80].

Traditional treatment of anemia of inflammation aims at reverting/controlling the underlying disease, whenever possible. Pathophysiology-based treatments are limited to EPO-like compounds and iron. Hep inhibitors were recently proposed to induce hematopoiesis expansion, together with HIF stabilizers [81, 82]. This approach is widely used in patients with chronic kidney disease, low-risk myelodysplastic syndromes, and cancer undergoing chemotherapy, but not as a first line in ARDS.

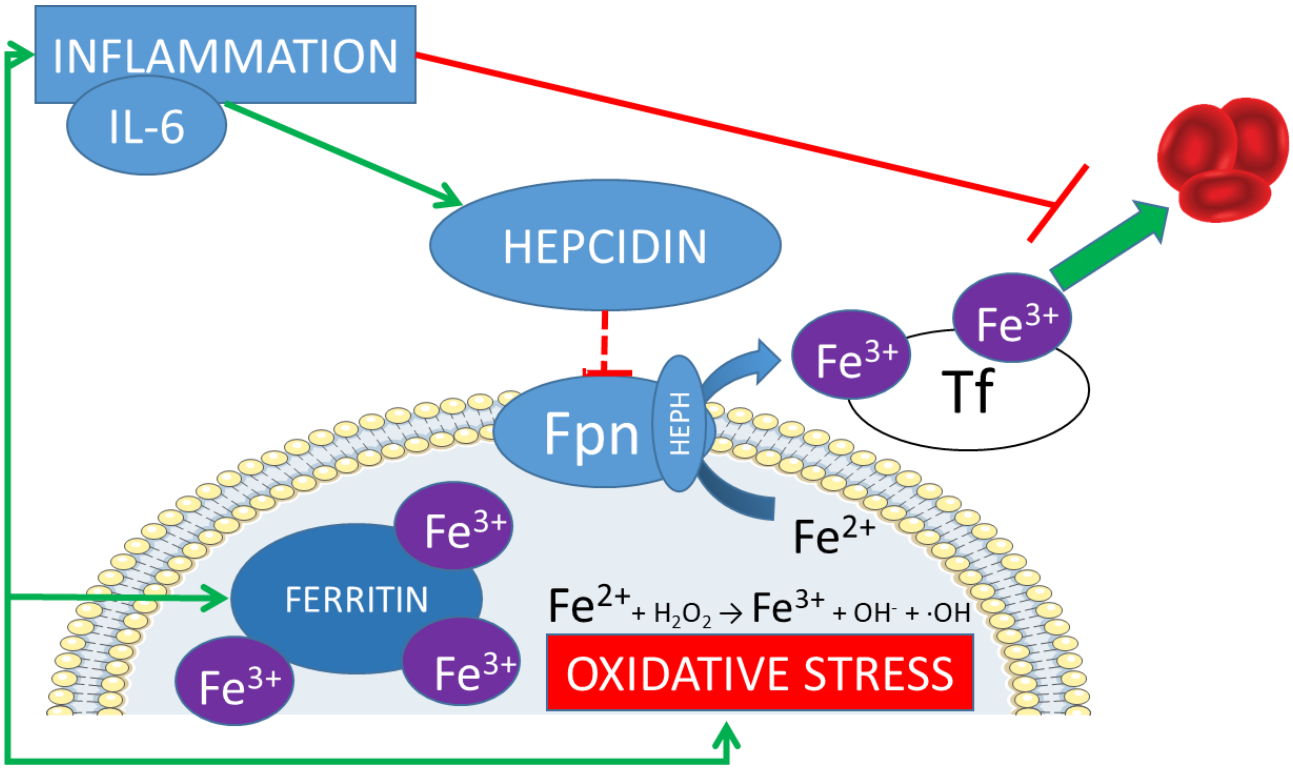

Figure 3: Inflammation induces an increase in Hep, which blocks such adaptation. *Both inflammation and hypoxia are sources of oxidative stress. An excess of intracellular iron can be a further source of oxidative stress, through the Fenton reaction (at the bottom).Modified from [83]*

1.4 Non hematopoietic roles of hematopoiesis regulators

1.4.1 Antioxidant and neuroprotective properties of erythropoietin

Numerous cell, animal and human studies have shown that the administration of recombinant human EPO might be effective against hypoxic, ischemic, and traumatic brain injury, as well as chronic and progressive degenerative diseases [84-87]. An increase in BCL2 gene expression, and a decrease of ROS induced by EPO ligand against oxidative stress activates a pathway through Nrf1 and a group of vital genes to preserve cell survival, prevent apoptotic signals and promote the inflammation decrease [88]. Many pieces of evidence suggest also that the neuroprotective effects of EPO are related to increased resistance to oxidative stress and stabilization of the redox equilibrium, for example through the JAK/STAT pathway and the up-regulation of anti-apoptotic

genes, as observed in vitro and ex-vivo [88, 89]. Pleiotropic positive effects of EPO administration in case of acute lung injury was recently observed in animal models. In fact, the suppression of the inflammasome by EPO contributes to the attenuation of Acute Lung Injury in mice models [90].

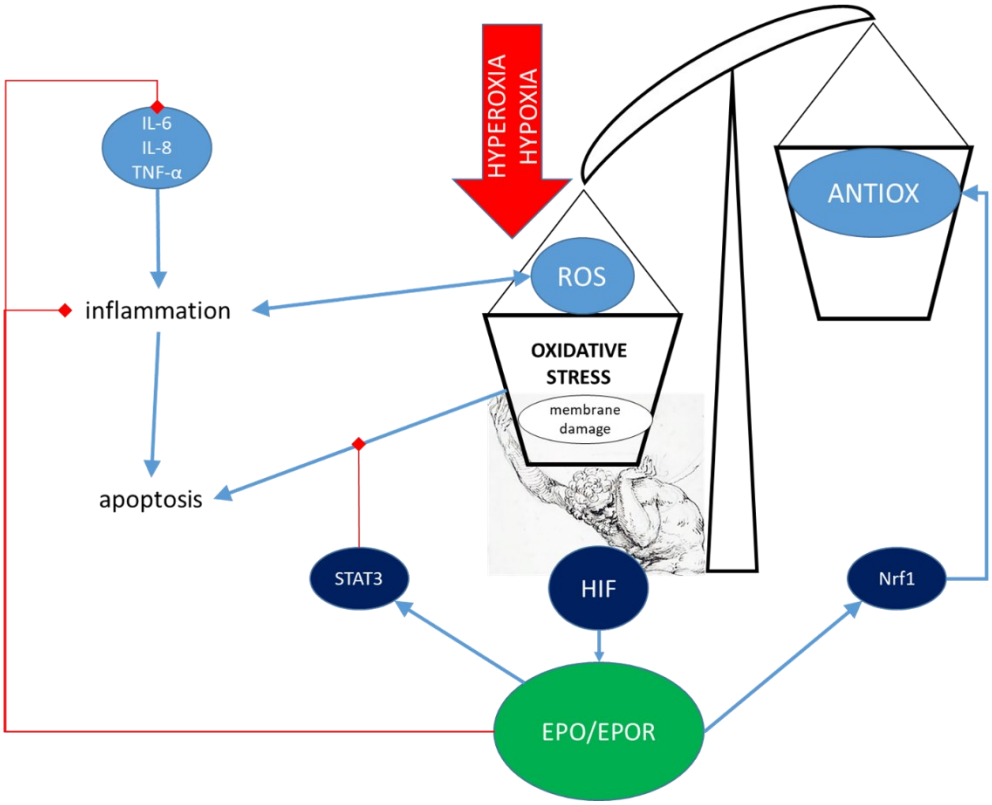

Figure 4: Role of the EPO/EPOR pathway in opposing the oxidative stress induced by hypoxia. *Hypoxia is a source of oxidative stress and activate the HIF pathway, which leads to increased EPO expression and EPO/EPOR interaction, which activates the antiapoptotic STAT3 pathway, and increases the antioxidant enzymes expression through the Nrf1 pathway. Blue arrows: activation. Red lines: inhibition. Adapted from [91]*

1.4.2 Non hematopoietic role of hepcidin

Hep is mainly produced by the liver, but in the past years there has also been growing attention on the role of Hep produced by other organs [92]. Hep acts on iron homeostasis in the central nervous system, where it plays a key role in regulating the transport of iron across the brain blood barrier [93]. It is variously distributed in several brain areas and can be induced in astrocytes by lipopolysaccharide (LPS) administration or by intracranial hemorrhage. Astrocytes and microglia are important regulators of neuronal iron metabolism and neuronal survival [94]. Hep and Hep-to-ferritin ratio were decreased in astrocytes after co-administration of iron and α-synuclein (inflammation inducer in astrocytes) [95].

1.5 Effects of hypoxia and iron on cell bioenergetics

1.5.1 Cell models of hypoxia, ARDS and oxidative stress

A549 cells, as an in vitro model of Type II alveolar cells, have been widely used in literature to study the response to inflammation in ARDS, when treated with LPS [96, 97]. Because of their anatomical location, alveolar type II cells are normally exposed to the environmental fraction of O_2 and

therefore normally more oxygenated than the rest of the body cells (the equivalent of about 13% O_2 [98]. A decrease in such fraction of O_2 is another feature of ARDS which deserves investigation. Such decrease can be recreated in a hypoxic chamber (figure 5), through the increase in nitrogen concentration in order to get a fraction of O_2 lower than the room air.

***Figure 5: Hypoxic chamber, a way to test the effects of hypoxia in cell models**, Creteil, Universitè Paris Est*

SH-SY5Y cells, a neuroblastoma a cell line, is commonly used to model ischemic and hypoxic damage in the central nervous system. The role of hypoxia induced oxidative stress in inducing brain damage is often investigated in such model [99].

1.5.2 Mitochondrial damage and oxidative phosphorylation

With prolonged exposure to hypoxia at high altitude (>5500 m), mitochondrial volume density decreases and electron transport chain complexes are downregulated [100]. Excessive demand for ATP or cell damage can result in increased ROS leakage by complexes I, III and IV and consume the antioxidant defences. This can lead to rupture and release of mitochondrial components including mitochondrial DNA, which can induce an inflammatory response. Increases in mitochondrial ROS can alter the signalling of redox sensitive transcription factors such as HIF-1α, which can further alter the metabolic state of the cell [101]. Therefore, ROS generated by mitochondria have very broad implications on cellular homeostasis. Mitochondrial respiratory complex I and III have been shown to be the major site of mitochondrial ROS production, which contribute to HIF-1α protein stabilization during hypoxia [102].

The entity of mitochondrial activity and the glycolytic pathway can be estimated in experimental conditions and with specific equipment (for example a Seahorse machine, see Materials and Methods) as O_2 consumption rate (OCR) and Extracellular Acidification rate (ECAR), respectively (figure 6). With this and other methods, 1-methyl-4-phenylpyridinium iodide (MPP+), a neurotoxin

which is usually used to reproduce in vitro the oxidative damage in models of Parkinson disease, was observed to exert mitochondrial toxicity in SH-SY5Y cells, by targeting complex I [103].

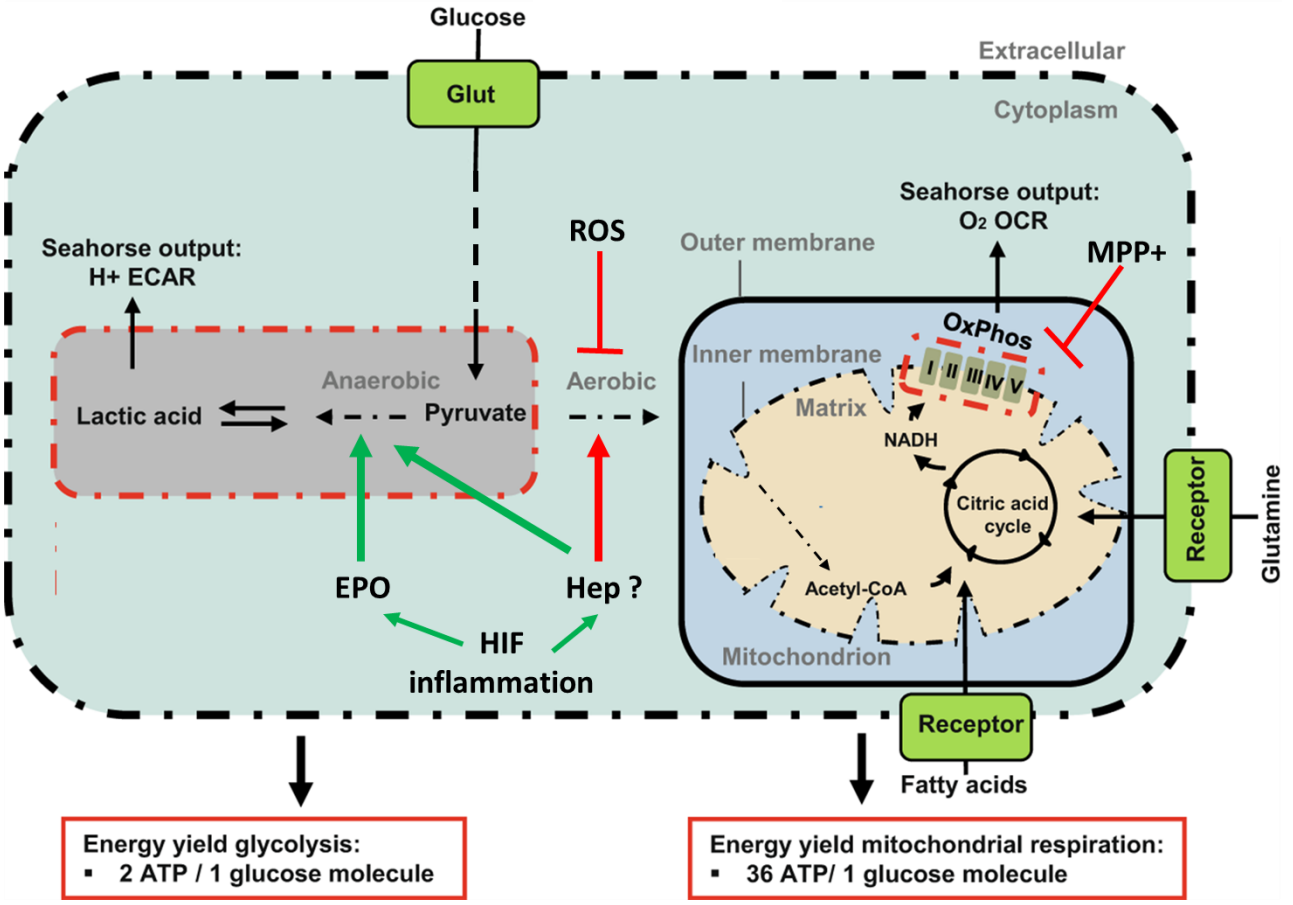

Figure 6: Potential effects of EPO, Hep and MPP+ on glycolysis and mitochondrial respiration, the two main energy-yielding pathways. *Glucose is converted into pyruvate in the glycolytic pathway. The fate of pyruvate is dependent on many factors among which O_2 availability. Modified from [104]*

1.5.3 ATP production through glycolysis

With the loss of mitochondrial density and enzymatic activity, anaerobic glycolysis makes a greater contribution to ATP demand (figure 6). This should be happening especially at extreme altitude, particularly during exercise. The genes for many glycolytic enzymes contain hypoxia-response elements in their promoter regions [33]. In hypoxic cells, the inhibition of pyruvate dehydrogenase empowers the expulsion of lactate from the cell [100]. The increase in circulating lactate induces a decrease of blood pH, which may greatly alter O_2 transport. Such increase is transiently reduced even after exercise in healthy subjects after 4–6 weeks of altitude exposure, a phenomenon known as "lactate paradox"[105]. In acclimatized subjects, in fact blood lactate levels are decreased, despite the lack of O_2. This may happen especially in the muscle through metabolic factors that, by enhancing oxidative phosphorylation by tighter ATP demand-supply coupling, and maximizing the yield of ATP per mole of fuel and per mole of O_2, later reduce the size of anaerobic glycolysis [6, 105]. Likewise, intra erythrocyte S1P enhances the glycolytic metabolic fluxes leading to the generation of more 2,3-biphosphoglicerate that in turn promotes O_2 release to protect against tissue hypoxia [106].

2 Aims

The main objective of this PhD thesis is the identification of iron handling and oxidative stress markers useful to investigate etiology and progression of anemia in patients exposed to acute (ARDS) or chronic (COPD) hypoxia, with healthy subjects as control, to address adequate therapeutic targets. To clarify the mechanisms of the increase or decrease of such markers and their potentiality as therapeutic targets, our observational study in blood samples from human subjects was complemented with two in vitro models.

The final goal of this thesis is to assess the role of Hep in downregulating hematopoiesis in hypoxic patients and explore the metabolic effects of potentially protective hematopoietic factors such as EPO.

The aims of this study are:

In patients:

- To characterize the role of iron handling parameters in the hematological compensation to acute (ARDS) and chronic (COPD) pathological hypoxia.
- To determine a panel of secondary endpoints (biomarkers of *ox-redox balance)* that integrate iron metabolism assay in defining the inflammatory and oxidative state of the patient, and help in validating interventions aimed at restoring iron metabolism and counteracting the onset of anemia in ARDS patients.

In vitro:

- To characterize non hematopoietic and potentially protective roles of EPO, as one of the proteins induced by physiological response to hypoxia
- To identify the effects of increased Hep, in the energetic metabolism of lung cells, in order to establish if such increase is a further source of mitochondrial and metabolic stress.

3 Materials and methods

3.1 Patients and controls: study design

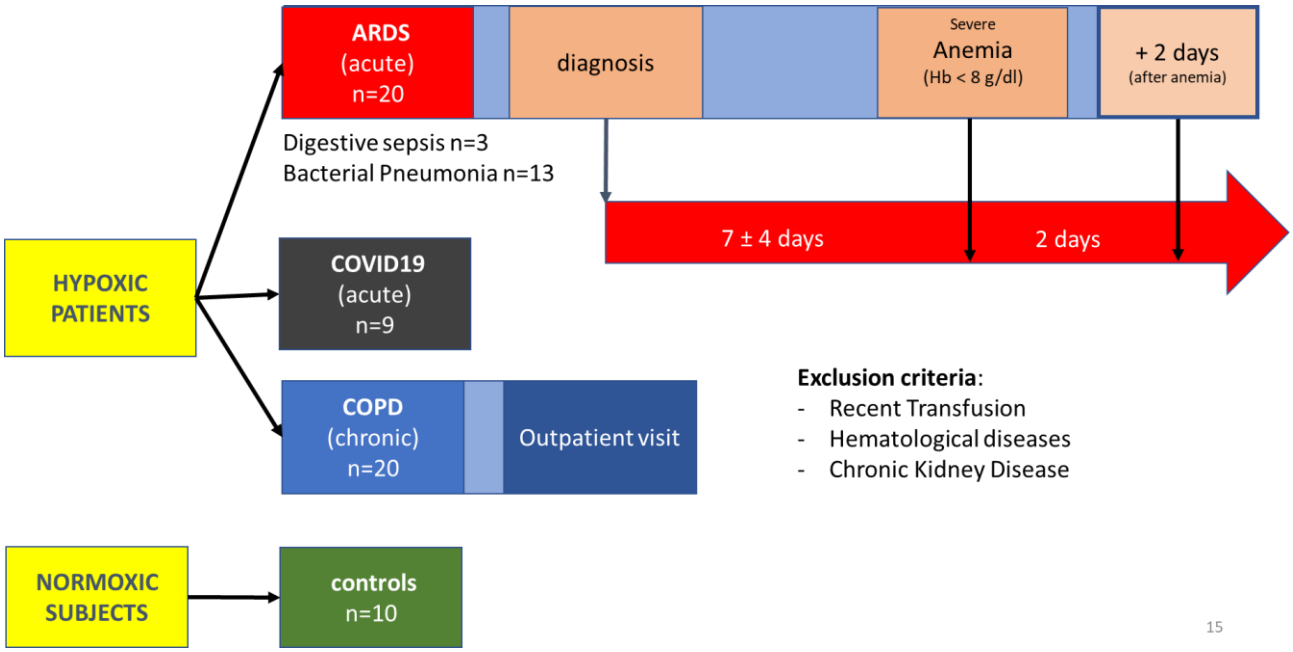

Figure 7: Observational study design: characteristics and numerosity of the recruited patients. *Clinical data and blood samples were collected from ARDS and COVID19 patients at the diagnosis, and from COPD patients during an outpatient visit. The value of Hb in ARDS patients was controlled through the hospitalization and clinical data and blood samples were collected again at the onset of anemia and 2 days later.*

3.1.1 Ethical committee

The Milan Area 1 Ethics Committee has approved the execution of this study on patients and healthy subjects (prot. 5098/2020, 2019/ST/144).

3.1.2 Acute Respiratory Distress Syndrome patients

This observational longitudinal study involved ARDS patients, recruited on their first admission to the ICU, with an established ARDS diagnosis. Blood samples were collected from patients at three timepoints according to literature [107]: at the ARDS diagnosis; at the anemia onset (when Hb will be 3 g/dl less than at the diagnosis or <8 g/dL whichever occurs first) two days after anemia onset (figure 7). Exclusion criteria include age<18y, chronic kidney disease, hematological disorders, recent transfusions before the ARDS diagnosis.

3.1.3 Chronic Obstructive Pulmonary Disease patients

Stable hypoxic COPD patients were recruited in the Pneumology Ambulatory, during routine check-ups. They were selected for the study if the execution of an arterial blood gas analysis was part of the visit to check hypoxia (e.g. peripheral saturation of O_2 <94%).

3.1.4 Healthy normoxic volunteers

Age matched volunteers were recruited as controls. Exclusion criteria for volunteers were respiratory diseases, recent transfusions, chronic kidney disease.

3.1.5 COVID19 patients

The COVID19 pandemic struck Italy during the recruitment of ARDS patients for this study. As most COVID19 Intensive Care Unit patients were responding to the Berlin criteria for ARDS, they were also included in the study at the diagnosis. The later time points were not collected because of the lockdown, which imposed to close the university laboratories where samples are processed and analyzed.

3.2 Clinical parameters

3.2.1.1 Blood gas analysis

The blood gas analyzer (Siemens RAPIDPoint 405) is used in the Emergency Department and in the Intensive Care Unit to quickly assess a panel of parameters. With a small amount of blood (< 1 ml), the machine gives an estimated value of a wide range of important parameters. Among those, we considered the following:

- pH
- PaO_2, mmHg
- PCO_2, mmHg
- Base Excess (BE), mmol/l
- Lactate, mM
- Hb concentration, g/dl

Several oxygenation parameters were considered to assess hypoxia, such as peripheral O_2 saturation (SpO_2) PaO_2, fraction of inspired O_2 (FiO_2), and the arterial O_2 content (CaO_2), calculated with the following formula:

$$CaO_2 = (1.34 \times [Hb] \times SpO_2) + (0.003 \times PaO_2)$$

3.2.1.2 APACHE IV and SOFA score

The Acute Physiology And Chronic Health Evaluation (APACHE) score is one of the most widely used score in ICU to quantify the severity of the illness of the patients. It represents a useful tool to compare populations of patients in clinical studies. The APACHE II score was published in 1985 and the APACHE IV, the latest version, published in 2006 [108]. As ARDS occurs in patients with various disease etiologies, predicting outcome after ARDS onset is difficult, but can commonly assessed through APACHE IV [109]. This score combinates clinical and biochemical parameters such as: age, body temperature, blood gas analysis data, sodium, glycemia, serum bilirubin, urea, albumin, hematocrit, white blood cells and cardiovascular and neurological status. For this study, these data were collected from the ARDS patients at all three time points and inserted in the following online platform for the calculation: https://intensivecarenetwork.com/Calculators/Files/Apache4.html.

The Sequential Organ Failure Assessment (SOFA) score is a scoring system to determine the extent of a septic patient's organ function or rate of failure according to respiratory, cardiovascular (with adjustments for noradrenaline and dopamine adminstration), hepatic, coagulation, renal, and neurological systems [110].

3.3 Blood samples gathering and storage

12 ml of venous peripheral blood were gathered from each of the subjects in two separate vacutainer tubes: one with EDTA and one with serum separating gel. Full blood was centrifuged for 15 min at 3000 rpm within 30 min from the collection. Serum, plasma and red blood cells were aliquoted and stored at -20°C for the biochemical assays.

3.4 Serum Biomarkers

3.4.1.1 Erythropoiesis and iron metabolism

3.4.1.1.1 Hepcidin

Hep was measured through an EIA kit (DRG, Marburg-Germany). The average reference value is about 20 ng/mL [44].

3.4.1.1.2 Soluble Transferrin receptor

The dosage of the sTfR was performed through and enzyme-linked immunosorbent assay (sTfR Human ELISA, Biovendor, Brno, Czech Republic). Each sample was assessed in duplicates.

3.4.1.1.3 Erythropoietin

Erythropoietin (EPO) was measured according to the manufacturer's instructions, through Human EPO/Erythropoietin ELISA Kit (Sigma Aldrich). Each sample was assessed in duplicate.

3.4.1.1.4 NTBI

NTBI was assayed using the high performance liquid chromatography (HPLC) analysis method proposed by Porter et coll [111], in the laboratory of Internal Medicine and Rare Diseases, Policlinico hospital of Milan.

Centricon 30 microtubes with 30 kDa cut-off MW ultrafiltration membranes (Amicon Corporation, Lexington, Massachusetts), previously washed with 10 mM nitrile triacetic acid (NTA) and pre-purified dH2O, were used for sample preparation purified, to minimize iron contamination. A 50µlof 800 mM NTA (pH 7.0) was added to 450µlof serum to remove and bind low molecular weight iron not specifically bound to proteins, thus creating a Fe-NTA complex. After incubation for 20 minutes at room temperature, the sample was ultrafiltered. After centrifugation for 30 min at 12000 rpm, the ultrafiltrate was used for analysis in HPLC. Given the variable, despite minimal, content of iron in the NTA solution, a blank (450uL dH2O + 50uL 800mM NTA) was prepared, which was subtracted from that of the sample to obtain the real concentration. This procedure involves the detection of negative values (<0 µM) in normal subjects; water itself contains small amounts of iron which is not bound by transferrin, while in serum samples, transferrin, which is not completely saturated, is capable of detaching iron from the Fe-NTA complex [112].

The HPLC analysis was carried out under isocratic conditions at a flow of 1.5 ml/min with a C18 4 µm, 3.9x150 mm inverse phase column (Waters, Milford, MA, USA). The mobile phase consists of 20% acetonitrile and 80% of 5mM $NaHPO_4$ / Na_2PO_4 buffer, pH 7.0 containing 3mM CP22 (3-hydroxy-1-propyl-2-methyl-pyridin-4-one). The CP22 chromogen has a high affinity for binding with iron and converts the iron-NTA complex into the colored complex 3-iron-(CP22). Monitoring of the elution profile was conducted with a spectrophotometer at a wavelength of 450 nm. Under these

conditions the retention time of the NTBI is 4.6 minutes. The amount of NTBI in the biological samples was determined by comparison with a standard curve prepared in NTA 80 mM, by serial dilutions, starting from a stock 1 mg / mL of $FeCl_2$ (17.8 mM) by atomic absorption spectrophotometry (1-5 -7.5-10 µM). The HPLC system used includes Perkin Elmer Series 200 IC titanium pumps, with pipes in completely inert material (peek ®) to avoid iron contamination. Elution profile monitoring was done with Perkin Elmer 785A UV / Vis detector. For this procedure, each swab was prepared using Milli-Q dH2O (Millipore, Bedford, MA, USA) and all manipulations were performed with disposable sterile materials always to avoid contamination.

3.4.1.2 Oxidative stress

3.4.1.2.1 Ferric reducing antioxidant power

The Ferric Reducing Antioxidant Power (FRAP) analysis is an assay for the measurement of antioxidant capacity ferric salt Fe(III)(2,4,6-tripyridyl-*s*-triazine)$_2$Cl$_3$ as the desired oxidant. The reaction is carried out at an acidic pH (3.6) to maintain solubility of the iron involved. 5 microliters of plasma were added to 300µlof a freshly prepared FRAP solution [113] in a 96 well plate and the absorbance measured at 593 nm. After 5 min of incubation at 37°C against a blank of FRAP solution. The standard curve may be done in several ways. When the curve is created with iron, one FRAP unit is defined by convention as the reduction of 1 mol of Fe^{3+} to Fe^{2+}. In this study, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (TROLOX), a vitamin E soluble analog, was used for the standard curve, therefore the results are expressed as mmol eq of Trolox. Reference values are about 1.5 mmol eq Trolox/L [113]. All the reagents for this assay were acquired from Sigma Aldrich. The assay was performed in duplicates for each serum sample and repeated if the intra-assay coefficient of variation (CV%) was higher than 10%.

3.4.1.2.2 Malondialdehyde

Lipid peroxidation, a measure of consequence of oxidative stress, was measured through BIOXYTECH LPO-586 Colorimetric Assay For Lipid Peroxidation (Oxys Research). Lipid peroxides are unstable and form a complex series of compounds, among which reactive carbonyl compounds. Polyunsaturated fatty acid peroxides generate MDA and 4-hydroxyalkenals upon decomposition. Measurement of MDA is used as an indicator of lipid peroxidation. The LPO-586 method is designed to assay MDA in hydrochloric acid. It is based on the reaction of, N-methyl-2-phenylindole (R1), a chromogenic reagent, with MDA and 4-hydroxyalkenals at 45°C. This reaction results in a stable chromophore with absorbance at 586 nm. The assay was performed in duplicates, according to the manufacturer-s instructions.

3.4.1.2.3 dROMs

This method measures reactive O_2 metabolites (ROMs), a class of compounds generated by the interaction of ROS with organic molecules. They are mainly hydroperoxides and, in presence of iron released from plasma proteins by an acidic buffer, they generate alkoxyl and peroxyl radicals through the Fenton's reaction. These radicals, exposed to an alkyl-substituted aromatic amine (which is solubilized in a chromogenic mixture) will oxidize it, giving the mixture of sample and regents a pink-colored mixture. The d-ROM assay reagents were purchased from Diacron Inc

(Grosseto, Italy). The method, designed to be performed in 2 ml cuvettes, was adapted to be performed in 96 wells plates. This adaptation allowed us to spare reagents and perform each assay in duplicate.

Before carrying out the test, a control serum, with known d-ROMs concentration, was reconstituted from a lyophilized calibrator. A control high and control low serum, with known values, were also reconstituted to be assayed on the same plate as the samples.

Two reagents (R1 and R2) were mixed (1:100) to get a working solution. 303 µL of this mixture were added to 3 µL of sample/control serum/distilled water, in a 96 well plate.

After a 90 min incubation at 37 °C, photometric reading was performed by assessing the absorbance at 546 nm of each solution (EnSight™ Multimode Plate Reader, Perkin-Elmer, Monza, Italy). The distilled water (blank) absorbance value was subtracted from every absorbance value. To obtain the result, expressed in Carratelli Units (CARR U), this formula was applied to all the absorbance values (knowing that the calibrator absorbance value matches to a value of 300 CARR U).

$$\text{CARR U} = \frac{\text{Abs sample}}{\text{Abs calibrator}} \times [\text{calibrator}]$$

3.4.1.2.4 Total glutathione assay

Total glutathione in RBC was assessed by using the Glutathione Colorimetric Detection Kit (Invitrogen). For deproteinization, 250µlof RBC were added to 1 ml of ice cold 5% Aqueous 5-sulfo-salicylic acid dihydrate (SSA, Sigma-Aldrich). then incubated for 10 minutes at 4°C. Samples were then centrifuged at 14,000 rpm for 10 minutes at 4°C and the supernatant was collected for the measurement, according to the manufacturer's instructions. The assay was performed in duplicates and the colorimetric reaction was read at 405 nm in an Ensight™ (Perkin-Elmer, Monza, Italy) spectrophotometer.

3.4.1.2.5 Glutathionyl Hb

Glutathionyl Hb (HbSSG) was measured in cold-water hemolyzates of thawed aliquots of RBC by Matrix-Assisted Laser-Desorption in a Time-of-Flight mass spectrometer (MALDI-ToF), essentially improving a published method [55]. To improve reproducibility of sample measurement, all samples were run in quadruplicate depositions, obtained from the same amount of Hb loading. The Hb concentration of the individual 1:100 hemolizates (150 µl in a 96-well polystyrene plate) was measured at 420 nm in an EnSight™ (Perkin-Elmer, Monza, Italy) spectrophotometer, and compared to that of standard human Hb (Sigma-Aldrich, Milano, Italy).

According to their individual values (20-50 µmol/l), samples were diluted to a 10 µmol/l concentration. For the MALDI analysis, 10µl of the sample was mixed to an equal volume of freshly prepared sinapinic acid matrix (Sigma-Aldrich, MALDI-grade brand, 30 mg/mL in 50% v/v acetonitrile – 0.1% trifluoroacetic acid). Four one-microliter aliquots were manually spotted in adjacent circular wells of a stainless-steel plate, air-dried at room temperature and loaded into the Bruker Autoflex III mass spectrometer for measurement.

3.4.1.3 Inflammatory markers

3.4.1.3.1 IL-6

Interleukin 6 (IL-6) is released by activated macrophages and T cells. It is known as an acute phase response marker [114] and has already been used in several contexts as an early infection marker. It was assessed through IL6 ELISA kit (IBL international).

3.5 Metabolomics and lipidomics

New markers suitable for further investigations can be detected by using metabolomics assays, through an untargeted approach based on mass spectrometry methods. For untargeted approach, a minimal sample preparation should be employed to avoid loss of metabolites from the biological samples. The increasing popularity of the lipidomics approach is strictly connected to the progress in the related analytical techniques, especially mass spectrometry. This large-scale technique can cover the whole human lipidome, comprising from 10 to 100-thousand different chemical entities in a complex biological system [115].

3.5.1 Lipidomics

For lipid extraction, 100 µL of 1:1 diluted serum sample were mixed with 850 µL of a methanol/chloroform 1:1 solution, then sonicated for 30 min. The organic phase was evaporated under a stream of nitrogen. The residues were dissolved in 100 µL of 2:1 isopropanol/acetonitrile solution, centrifuged for 10 min at 13400 RPM, and withdrawn in a glass vial, as described by Dei Cas et al, 2020 [115].

3.5.2 Metabolomics

Plasma (50 µl) were diluted with water (50 µl) and added with 400 µL of a methanol/ehtanol mixture (1:1 v/v), then extracted with an oscillator thermo-mixer (30 min 5°C, 1000 RPM). Then it was centrifuged for 10 min at 13400 RPM, the protein debris were discharged and the clean supernatant was evaporated under a stream of nitrogen. The residues were dissolved in 50 µL of water and withdrawn in a vial.

3.5.2.1 LC-MS/MS untargeted method

The LC-MS/MS consisted of a Shimadzu UPLC coupled with a Triple TOF 6600 Sciex (Sciex, ON, CA) equipped with Turbo Spray IonDrive. All samples were analysed in duplicate in both positive and negative mode with electrospray ionization. The instrument parameters were: CUR 35, GS1 40, GS2 40, capillary voltage 5,5 kV and source temperature 500 °C. Spectra were contemporarily acquired by both full mass scan from 50-800 *m/z* (100 ms accumulation time) and data-dependent acquisition from 40-800 *m/z* (40 ms accumulation time, top-20 spectra *per* cycle 0.8 s).

Declustering potential (DP) was fixed to 60 eV and collision energy (CE) was 30 with a collision energy spread of 15. Chromatographic separation was achieved on an Acquity HSS T3 column 1.7 µm 2.1 x 50 mm (Waters, MA, USA) equipped with pre-column using as mobile phase A water and as mobile phase B methanol both containing 0.1% of formic acid. The flow rate was 400 µL/min and the column temperature was 40°C. The elution gradient (%B) was set as below: 0-2.0 min (1%), 2.0-6.0 min (1-25%), 6.0-10.0 min (25-80%), 10.0-12.0 min (80-90%), 12.0-21.0 min (90-99%), 21.0-23.0 min

(99-99%), 23.0-23.2 min (99-1%) held until 30 min. 5 µL of clear supernatant were directly injected in LC-MS/MS.

3.5.2.2 Metabolomics data processing

The spectra deconvolution, peak alignment and sample normalization were attained using MS-DIAL (ver. 3.4). Data raw files (.wiff) were converted into ABF format to perform retention time correction, peak alignment and identification. Identification was achieved matching molecular and MS/MS experimental spectra with (1) Fiehn Hilic library (.msp) or (2) an in-house built library taking also into consideration retention times. MS and MS/MS tolerance for peak profile was set to 0.01 and 0.05 Da, respectively. Data were then filtered for blank samples signals with a fold-change >10. Intensities of metabolites were normalized by Lowless algorithm and those which presented a CV% superior than 30% in the pool sample were excluded.

3.6 In vitro: non hematopoietic antioxidant role of EPO in brain

3.6.1 SH-SY5Y cells

The SH-SY5Y cells are widely used for the study of neurodegenerative diseases [116]. SH-SY5Y cells were grown in DMEM-F12 medium supplemented with 10% Fetal Bovine Serum (Euroclone, Pero, Milano, Italy), 1% glutamine (Gibco, ThermoFisher Scientific, Waltham, MA USA) and 1% penicillin/streptomycin (Gibco, ThermoFisher Scientific). Cells were split every 4-5 days after trypsinization (trypsin-EDTA 1X 0,05% ThermoFisher Scientific). Cells were subjected to 3 experimental conditions: non-treated (NT), treated with 500 µM MPP+ (MPP+), and treated with 500 µM MPP^++4U/mL EPO (MPP^++ EPO). MPP^+ iodide (Sigma-Aldrich) was freshly weighed for each experiment, dissolved in phosphate buffered saline (PBS), diluted in the appropriate medium and administered for 24 or 48 h.

3.6.2 Assessment of cellular respiration and glycolysis and Mitostress

The acute effect of MPP+ and the potential beneficial action of EPO on SH-SY5Y cell metabolism was assessed with the Seahorse Bioscience XF24 Extracellular Flux Analyzer (XF24, Agilent). The XF24 measures O_2 consumption (OCR) and extracellular acidification rate (ECAR, an index of glycolysis) in real time in viable adherent cells, therefore allowing the assessment of metabolic function during different conditions. SH-SY5Y cells (passages 16-21) were seeded in low glucose DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at a density of 80000 cells/well 24 hours before the assay as previously described [103]. The day of the assay, the growth medium was replaced with the XF assay medium (5.5 mM glucose, 1 mM pyruvic acid, 1 mM L-glutamine) according to manufacturers' instructions. After three basal measures, MPP+ was injected at a final concentration of 500 µM. EPO (4, 10 and 40 U/ml) was injected immediately after MPP and OCR and ECAR were measured for 200 minutes. In a separate experiment, OCR and ECAR were measured 24 h after treatment of cells with MPP+ 500 µM in the presence of 4 U/ml EPO. A Mitostress assay was performed to assess mitochondrial function. The test consists of the sequential injection of the following modulators of the mitochondrial respiratory chain: oligomycin (1 µg/ml), an inhibitor of ATP synthase and ATP linked mitochondrial respiration, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP, 0.63 µM), an uncoupler of mitochondrial respiration, and rotenone/antimycin A (R/AA, 0.5 µM), inhibitors of complex I and complex III, respectively, that

cause a complete impairment of mitochondrial function. This test allows the quantification of different parameters, including basal respiration, ATP-linked respiration, proton leak, maximal respiratory capacity and non-mitochondrial respiration [117].

3.6.3 ATP assay

ATP levels were measured in SH-SY5Y cells 4 and 24 h after treatment with 500 µM MPP+ in the presence or absence of 4 or 10 U/ml EPO using the ATPlite Luminescence Assay System (PerkinElmer). Cells were seeded the day before the assay in a 96 well plate (80000 cells/well) and the assay was performed according to manufacturer's instructions.

3.7 In vitro: model of hypoxia, inflammation and hepcidin overload

3.7.1 A549 cells

A549 cells, thawed at passage 3, were cultured in Ham's F-12K (Kaighn's) medium (ThermoFisher) with 10% FBS and 1% Penicillin/streptomycin, in a 37°C, 5%CO_2, 21% O_2 incubator. Cells were split 1:4-1:6 twice a week when at 80% confluence. Cells at passage 8-13 were used for experiments. LPS (eBioscience™ Lipopolysaccharide (LPS) Solution (500X, 2.5 mg/ml)) was acquired from ThermoFisher. Hep-25 (human) trifluoroacetate salt (500 ug, Sigma Aldrich) was solubilized in 1 ml of PBS and stored at -20°C. The final concentration of 50 ng/ml Hep was chosen to reproduce the average values observed in ARDS patients.

3.7.2 MTT assay

Cell viability 24 hours after treatment with LPS (1 or 5 ug/ml) and/or Hep (50 or 500 ug/ml) was assessed through MTT assay. Cells were seeded at a density of 20000 cells/well in a 96 well plate and treated 3 hours after seeding. To reproduce hypoxic conditions, a hypoxic chamber was used, set at 5% O_2. 24 hours after the treatment, a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well at a final concentration of 0.5 mg/ml. After 3 hours of incubation at 37°C, medium was discarded, and the formazan crystals were solubilized in Dimethyl sulfoxide (DMSO).

3.7.3 Assessment of cellular respiration and glycolytic function

The effect of LPS, Hep and hypoxia on A549 cell metabolism was assessed with the Seahorse Bioscience XF24 Extracellular Flux Analyzer (XF24, Agilent). The XF24 measures O_2 consumption (OCR) and extracellular acidification rate (ECAR, an index of glycolysis) in real time in viable adherent cells, therefore allowing the assessment of metabolic function during different conditions. A549 cells (passages 8-12) were seeded at a density of 20000 cells/well 27 hours before the assay. After 3 hours, cells were moved from 21% O_2 incubator to a 5% O_2 hypoxic chamber and treated with LPS 5ug/ml and/or Hep (50 ng/ml or 500 ng/ml). The day of the assay, the growth medium was replaced with the XF assay medium (7 mM glucose, 1 mM pyruvic acid, 2 mM L-glutamine) according to manufacturers' instructions.

3.8 Statistics

Data are reported as mean±SD. For the observational model, statistical tests were performed using ANOVA one-way test with Dunnett's unpaired post-test to compare ARDS time points, COPD and

controls and with Tukey's paired post-test to evaluate the ARDS patients through the three time points. Considering the expected intra-subject variability [44], each ARDS patient acted as a self-control to focus into the variations of Hep rather than its absolute value. For the in vitro models, data from independent replicates of same experiment were normalized by setting the average value of the non-treated (NT) as 100%. Results were analysed for significance through ANOVA one-way test (with Tukey's post-test). with the software Graphpad Prism.

4 Results

4.1 In patients: inflammation, anemia and oxidative stress

4.1.1 Patients characteristics

Among the recruited 20 non-COVID19 ARDS patients (8 females, 12 males, age: 63±19y), 17 had pneumonia as underlying cause of the respiratory distress, and 3 were originally hospitalized for gastrointestinal sepsis. Two patients were transfused between the *anemia* time point and the following *2 days*. Six patients died within 28 days from diagnosis. As shown in table 1, at the anemia onset timepoint mean arterial blood pressure and arterial pH increased and PCO_2 decreased, when compared to the day of the ARDS diagnosis.

Among the recruited 20 COPD patients (6 females, 14 males, age 74±9y), 8 were administered continuous home O_2 therapy (O_2 2 l/min), but blood gas analysis (table 2) was performed at 21% FiO_2.

The 10 healthy controls were 4 females, 6 males, aged 65±9y. Blood gas analysis was not performed in healthy subjects, as it is considered invasive.

Nine COVID19 ARDS patients (1 female,8 males, age 67±7y) were also recruited at the ARDS diagnosis during this study and later considered as a separate group. Seven of them died during ICU hospitalization.

***Table 1: main clinical parameters of the ARDS patients**. Data expressed as mean±SD. *p<0.05, Dunnett's range test*

Parameter	ARDS diagnosis	Anemia onset	+2 days
Age,years	63±17		
Temperature,°C	36.7±0.8	36.5±0.8	36.8±0.7
Mean arterial pressure, mmHg	81.6±11	86±8*	81±23
Heart rate, beats per min	88±20	88±17	90±20
Respiratory rate, breathes per min	17±2	20±2	20±7
Central venous pressure, cmH_20	10±3	12±3	10±2
FiO_2, %	54±18	45±12	40±13
PaO_2, mmHg	74±12	77±17	78±9
PCO_2, mmHg	46±6	38.±7*	39±4*
BE, mmol/L	2.9±3.2	4.7±3.2	3.9±4.3
Lactate dehydrogenase mU/ml	472±199	399±161	373±166*
Lactate, mmol/l	1.2±0.3	1.2±0.3	1.1±0.3
Arterial ph	7.40±0.06	7.45±0.04*	7.45±0.06*
Creatinine, mg/dl	0.9±0.5	1.0±0.8	1.0±0.9
Bilirubin, mg/dl	1.7±2.42	1.0±1.5	1.0±1.17
Albumin, g/dl	2.5±0.4	2.6±0.3	2.6±0.4
Urea, mmol/l	4.7±3.0	5.9±4.4	6.7±5.4
sTfR/log ferritin	0.97±0.19	0.87±0.20	0.93±0.09
Tidal Volume, ml	470+50	460±90	500±180
Total PEEP, cmH_2O	10±2	8±3	9±2
SOFA score	8±3	6±4	6±4
APACHE IV score	63±10		

***Table 2: Main arterial blood gas data of the hypoxic patients in this study**. Blood gas analysis was not performed in healthy subjects, as it is considered invasive.*

Parameter	COPD	ARDS at the diagnosis	COVID19
PaO_2, mmHg	67±9	74±12	75±11
PCO_2, mmHg	43±6	46±6	48±10
BE, mmol/L	2.0±3.5	2.9±3.2	1.0±3.2
Arterial pH	7.41±0.04	7.40±0.06	7.40±0.07
Lactate,mmol/l	1.0±0.4	1.2±0.3	1.0±0.4

4.1.2 Hypoxia and inflammation

In ARDS patients, Hb decreased within 7-10 days after diagnosis and was lower than in the COPD patients at all timepoints (figures 8 and 9, panel A). Despite a stable or slightly increasing PaO_2/FiO_2, the CaO_2, lower than reference values, decreased at the anemia onset (panels B and E). The PaO_2/FiO_2 ratio is used as measure of the hypoxemia and as a definition of ARDS. In ARDS patients,

it is always lower than 300. As shown in figure 9, in COPD patients PaO_2/FiO_2 was higher than ARDS, but below the healthy range (>400). Inflammatory markers, such as C Reactive protein (CRP), IL-6 and white blood cells (WBC) were also higher than reference values in ARDS patients. COVID19 patients had approximately same values as ARDS patients, except for lower white blood cells (WBC) (figure 9, panel C).

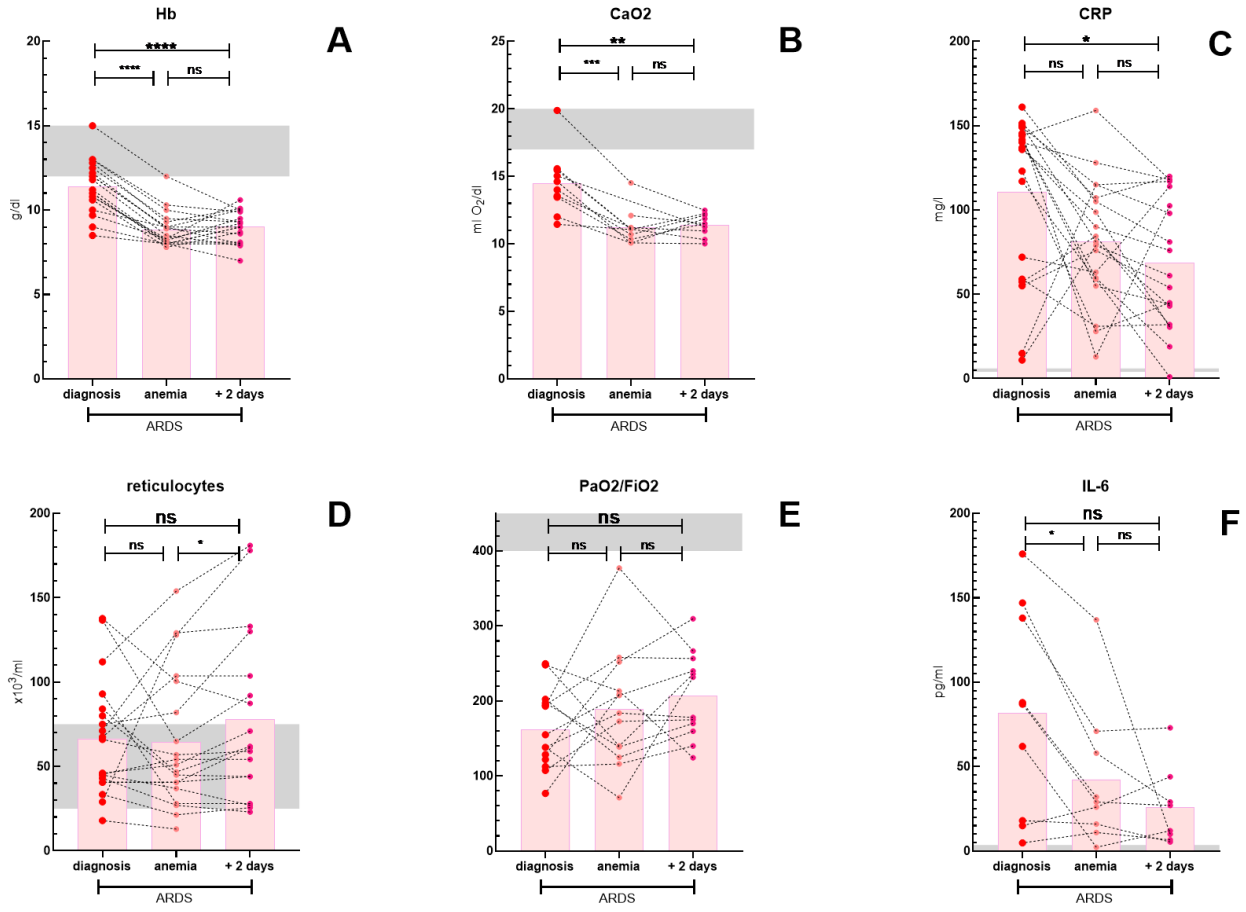

Figure 8: Anemia, hypoxia and inflammation in ARDS patients. **p<0.05; **p<0.01; ***p<0.005, Dunnett's test. Grey areas delimit the reference healthy values per each parameter.*

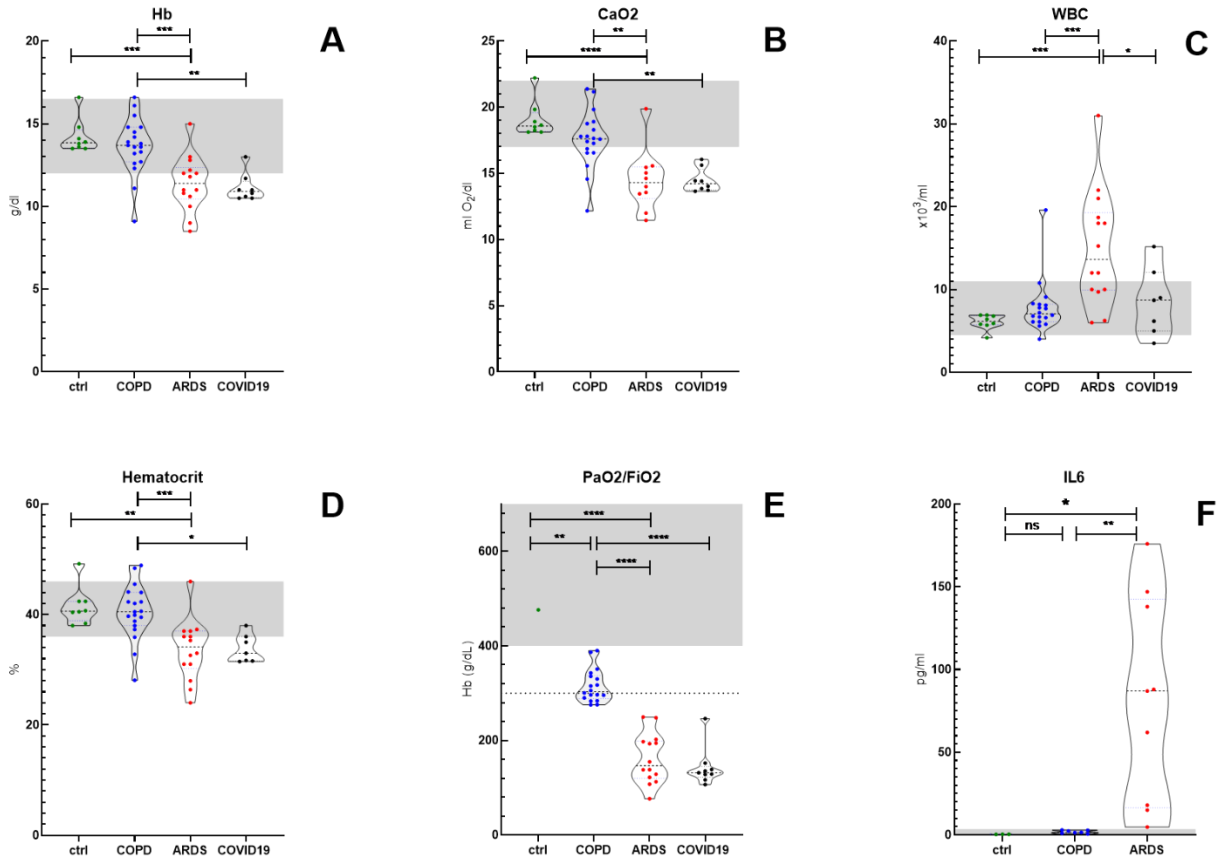

Figure 9: Hematopoiesis, hypoxia and inflammation in ARDS, COPD and control subjects (violin plots). *As arterial blood gas analysis was not performed in healthy controls (ctrl), CaO_2 was estimated assuming a paO2 of 100 mmHg. *p<0.05; **p<0.01; ***p<0.005, Tukey's range test. Grey areas delimit the reference healthy values per each parameter. The dotted line in panel A delimits the higher paO2/FiO2 threshold to define ARDS (300).*

4.1.3 Iron metabolism

4.1.3.1 Circulating levels of iron were decreased and Hep increased in ARDS patients

Iron metabolism was dysregulated in ARDS patients at all time points (figure 10): the total iron values were below the reference lower threshold of 49 µg/dL and Hep values were higher than COPD patients, healthy volunteers and reference values (figure 11). Soluble transferrin receptor was higher than reference values at each ARDS time point, while transferrin, always lower than reference values, increased 2 days after anemia onset. Just like ARDS, COVID19 patients had higher Hep than reference values and control subjects, and NTBI was higher than both controls and ARDS.

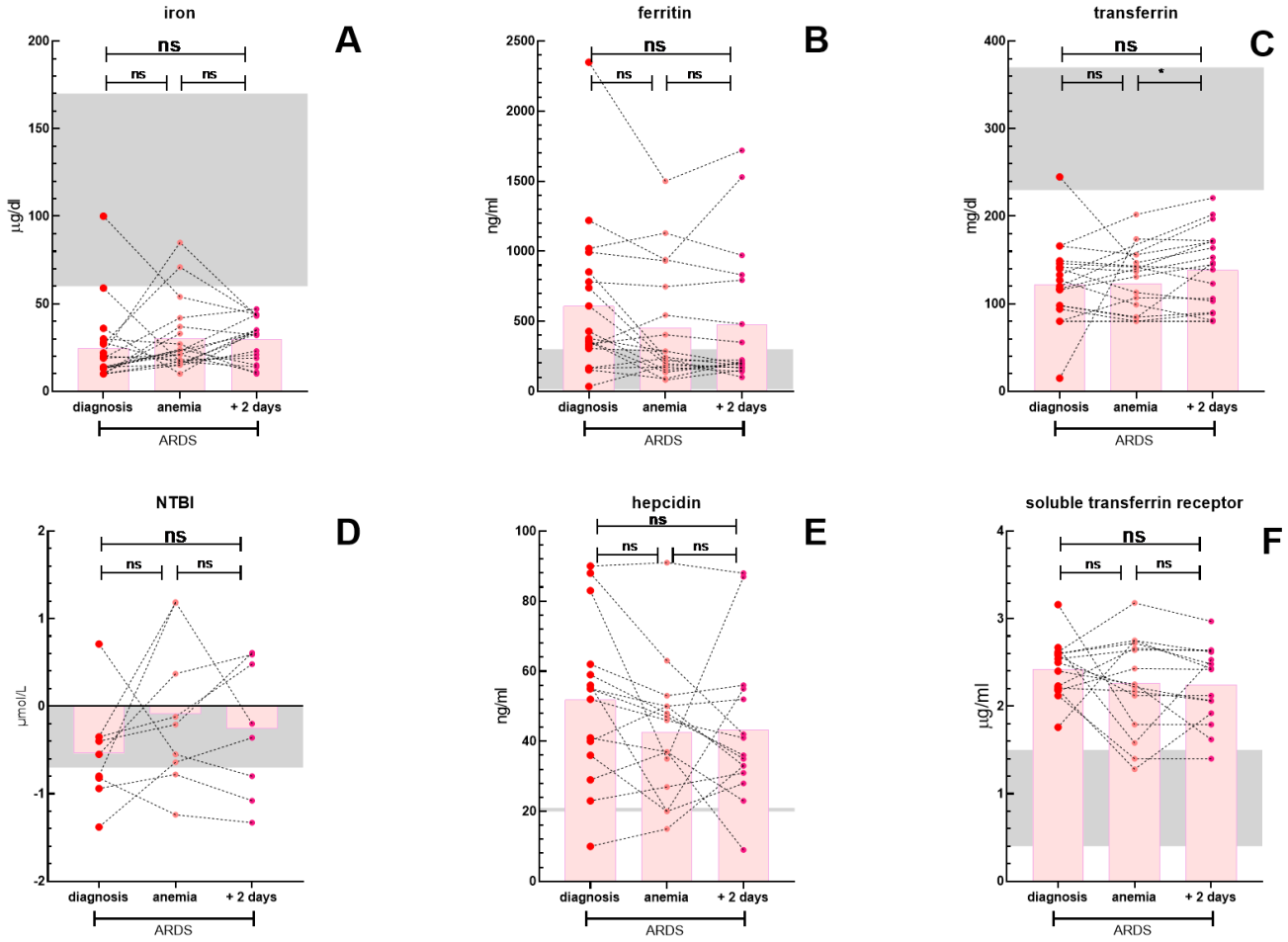

Figure 10: Iron metabolism parameters in ARDS patients. **p<0.05; **p<0.01; ***p<0.005, Dunnett's range test. Grey areas delimit the reference healthy values per each parameter. For Hep (panel E) such reference is defined through literature values and not through daily clinical practice [44].*

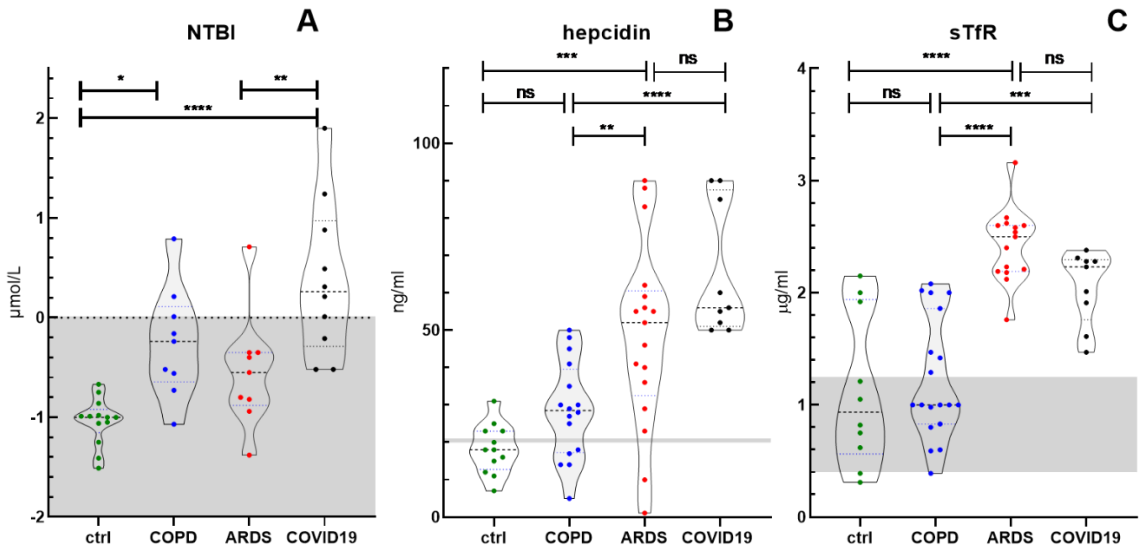

Figure 11: Iron metabolism parameters in ARDS, COPD and control subjects (violin plots). **p<0.05; **p<0.01; ***p<0.005, Tukey's range test. Grey areas delimit the reference healthy values per each parameter. For Hep (panel E) such reference is defined through literature values and not through daily clinical practice [44].*

4.1.4 Oxidative stress

4.1.4.1 dROMs values at the diagnosis predict mortality in ARDS patients

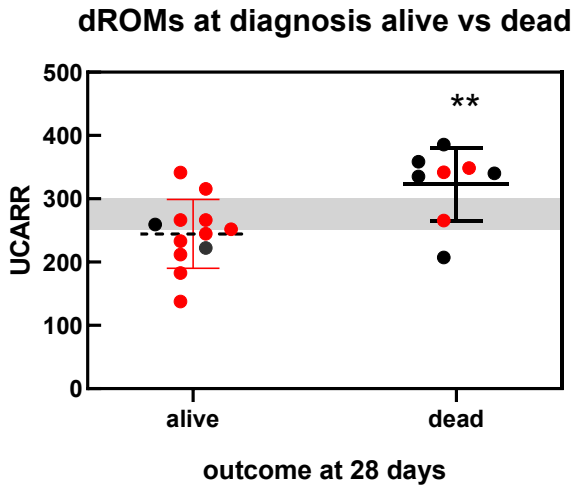

Figure 12: DROMs values at diagnosis predict mortality in ARDS and COVID19 ARDS patients. *In grey: Reference values provided by the manufacturer. In red: ARDS patients. In black: COVID19 patients. **p<0.01, Tukey's range test*

DROMs, one of the gold standards to assess oxidative stress in human serum, were higher in those patients who had a worse outcome at 28 days (figure 12). Data from non COVID19 ARDS and COVID ARDS patients were combined for this result, as both COVID19 and non COVID19 characteristics satisfied the Berlin criteria to define ARDS. Mortality among the COVID19 patients was higher than among the non-COVID19 patients.

4.1.4.2 Antioxidant barrier is consumed and decreases at anemia onset in ARDS patients

MDA values, a marker of lipid peroxidation, were higher in ARDS patients, when compared to COPD patients (figure 13b). FRAP values, an indirect measure of the antioxidant barrier strength, were

lower than normal in all hypoxic patients and lower in ARDS patients at the anemia onset, when compared to the moment of ARDS diagnosis (figure 13a).

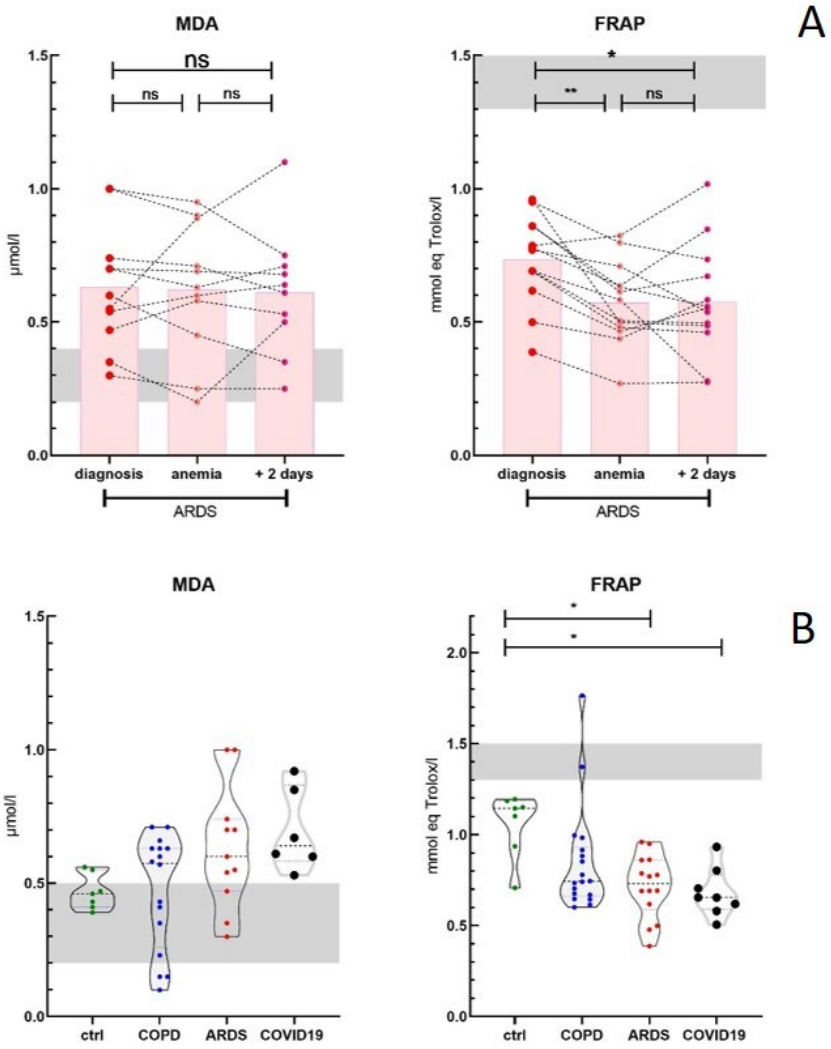

Figure 13: Oxidative stress parameters (MDA and FRAP) *in ARDS patients (A) and in ARDS, COPD and control subjects (B). *p<0.05; **p<0.01; ***p<0.005, Dunnett's (A) or Tukey's (B) test. Grey areas delimit the reference healthy values per each parameter.*

4.1.4.3 Coenzyme Q10 is reduced in ARDS patients

Coenzyme Q10, an important antioxidant and cofactor of the mitochondrial respiration, was depleted in both COPD and ARDS patients (figure 14).

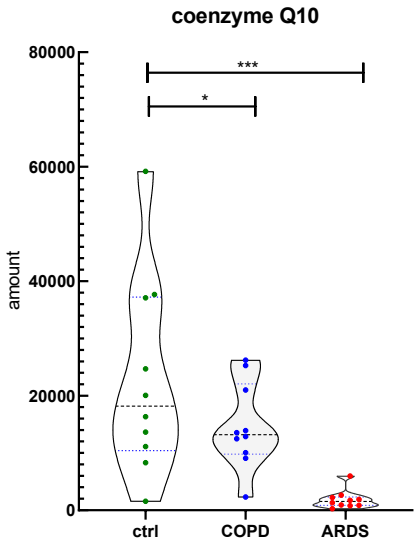

Figure 14: Coenzyme Q10 amounts estimated through untargeted metabolomics (violin plots)*. *p<0.05; **p<0.01; ***p<0.005, Tukey's range test.*

4.1.4.4 Glutathionyl-Hb is higher in COPD than ARDS

Glutathionyl Hb, a minor form of Hb that indicates oxidative stress, was measured with a mass spectrometry-based technique. As an example, figure 15 shows two extreme conditions observed in RBC analysed for this study: one of the highest (27%, panel A) and one of the lowest (<0.5%, panel B) levels of glutathionyl Hb. This interval spans the values observed in most studies [57].

The obtained values of glutathionyl Hb, shown in figure 16, highlight the higher fractions in the chronically hypoxic COPD patients. As glutathionyl Hb is one of the fractions in which the glutathione pool is reversibly partitioned, total soluble glutathione was also measured in the RBC (figure 16 panel B).

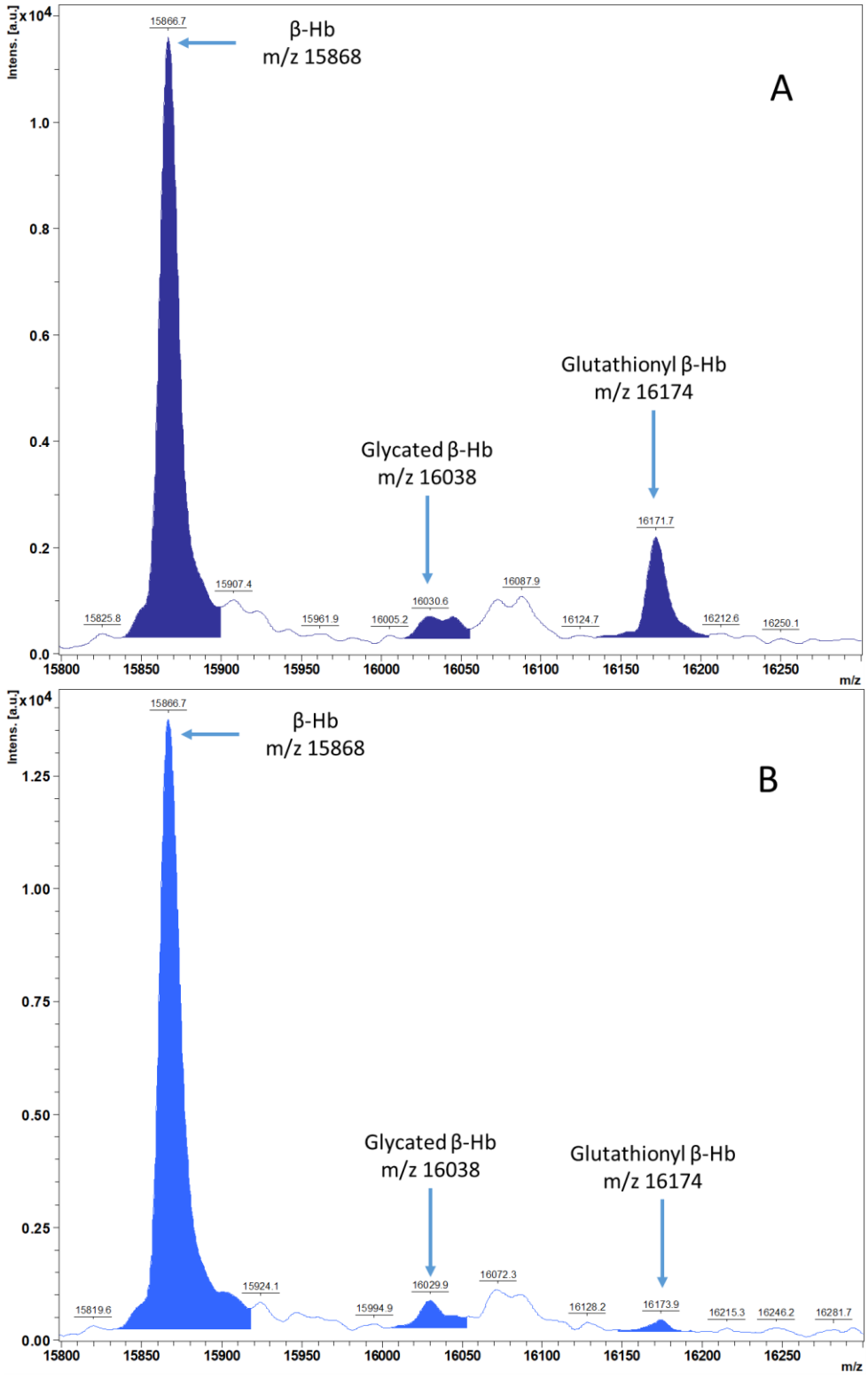

Figure 15: Partial mass spectrum (MALDI-TOF technique) of RBC hemolizates. *Shown are the signals of the β chain of Hb (m/z 15868) and of the glutathionylated form (m/z 16174) in a glutathionyl Hb rich (A) and a glutathionyl Hb depleted (B) sample.*

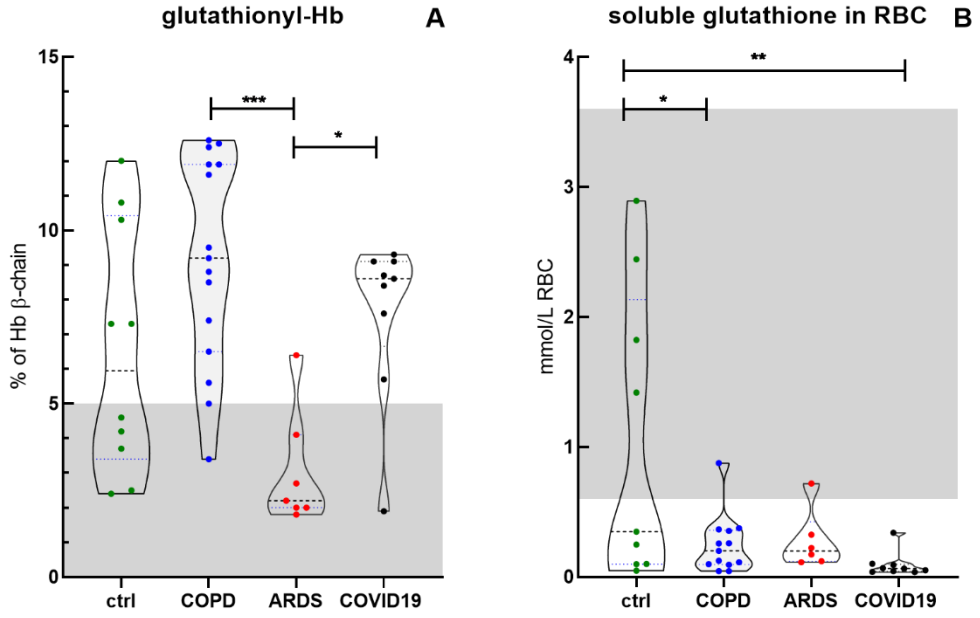

Figure 16: Glutathionyl-Hb and total glutathione in RBC. *p<0.05; **p<0.01; ***p<0.005, Tukey's range test. Grey area: literature reference values (in 18-48 y subjects) [53, 57].*

4.1.4.5 EPO values are mostly as high as reference values and do not vary despite hypoxia

Except for two ARDS patients, EPO concentrations were within the normal range, despite the hypoxic stimulus (figure 17).

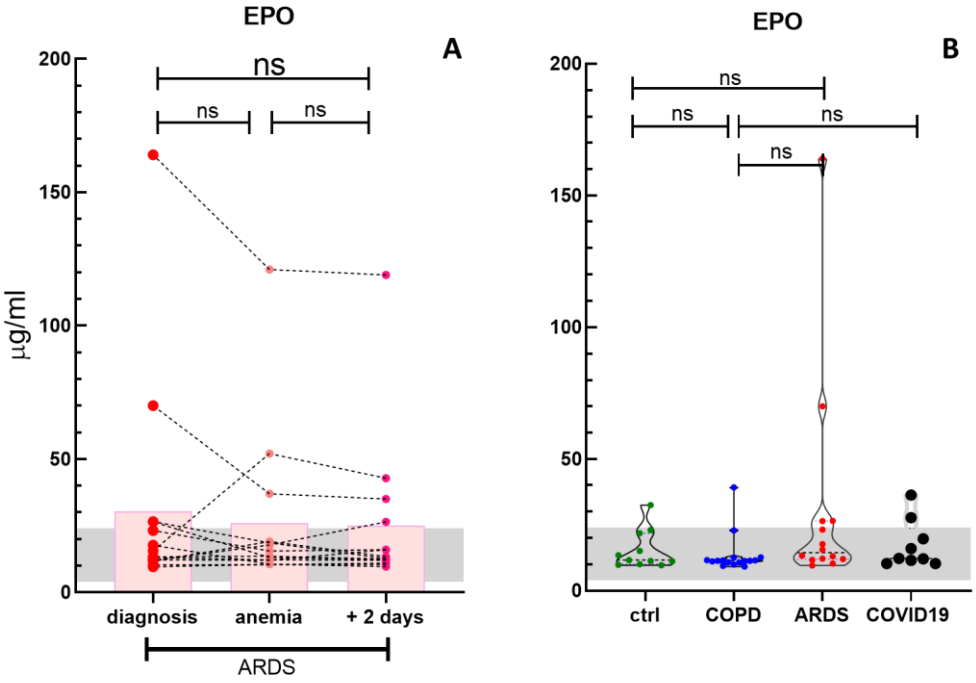

Figure 17: EPO concentration in patients' serum. *ns: non-significant difference, Tukey's range test. Grey area: reference values.*

4.1.5 Sphingolipid metabolism

4.1.5.1 Sphingosine 1 phosphate is decreased in ARDS and increases 2 days after anemia onset.

S1P, the main sphingolipid involved in the physiological response to high altitude hypoxia [69], was decreased in ARDS patients, compared to normoxic controls, and increased throughout the hospitalization (figure 18).

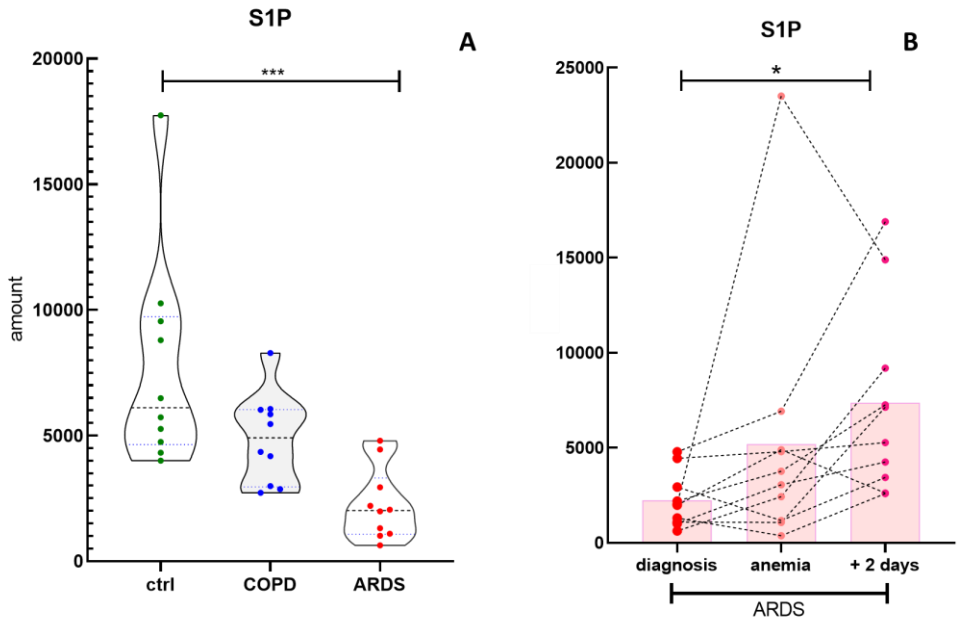

Figure 18: S1P amounts estimated through untargeted metabolomics. *p<0.05; **p<0.01; ***p<0.005, Tukey's range test.

4.2 In vitro: non hematopoietic antioxidant role of EPO in SHSY5Y cells

4.2.1 Erythropoietin partially rescues SHSY5Y mitochondria from damage

To clarify the mechanisms of the increase or decrease of the selected markers and their potentiality as therapeutic targets, this observational study in blood samples from human subjects was complemented with in vitro models. To characterize non hematopoietic and potentially protective roles of EPO, as one of the important hematopoietic factors induced by physiological response to hypoxia, we induced mitochondrial damage in SHSY5Y cells and treated with EPO.

Acute injection of MPP+ caused a rapid and significant decrease in the consumption of oxygen (OCR), a measure of mitochondrial activity. Treatment with EPO promoted a small but consistent rescue of OCR which was evident with 4 U/ml and became more pronounced with EPO at 10 and 40 U/ml (figure 20A). Addition of MPP+ also increased glycolysis (seen as rate of extracellular acidification, ECAR), which was further enhanced only by the combination with EPO at 4 U/ml but not with MPP+ in the presence of EPO at 10 and 40 U/ml (Fig. 20B). EPO alone did not change OCR or ECAR at any of the concentrations tested. These data confirm that acute treatment with MPP+ significantly diminishes respiration and increases glycolysis in neuroblastoma cells and indicate that EPO at low concentrations counteracts this effect to a small extent.

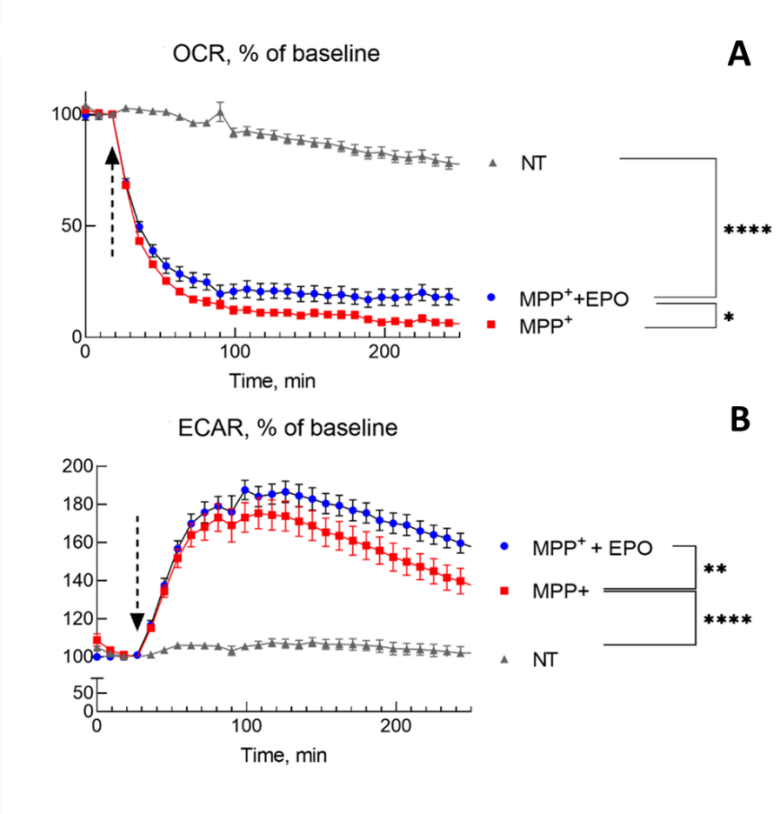

Figure 19: Acute effect of MPP+ and EPO in the cell metabolism of SH-SY5Y cells*. OCR and ECAR were measured in SH-SY5Y cells 200 mins after injection of 500 µM MPP⁺ in the presence or absence of 4 U/ml EPO. The dotted arrow shows the moment of the injection. Data are represented as average of three independent experiments. *p<0.05, ****p<0.0001 vs NT.*

We also examined the effect of long-term exposure of cells to MPP+ and EPO. As shown in Fig. 21A at time=0min, 24 h exposure of cells to MPP+ elicited a dramatic inhibition of respiration. Under these conditions, a Mitostress assay (which can be interpreted as pictured in figure 21B) revealed that MPP+ completely abolished ATP-dependent mitochondrial respiration, since no response was observed when oligomycin, an inhibitor of ATP synthase, was added. In addition, the mitochondrial uncoupling activity was markedly reduced (response to FCCP). In contrast to the results obtained after acute MPP+ and EPO treatment, basal respiration and other parameters of mitochondrial function were not ameliorated in the presence of MPP+ and EPO (4 U/ml). Concerning glycolysis, we observed that MPP+ caused a rise in ECAR that was significantly enhanced in cells treated with MPP^+ and EPO (figures. 21C and 21D). These results indicate that MPP+ induces a profound and log-lasting inhibition of mitochondrial function that is not rescued in the presence of EPO. The concomitant increase in glycolysis in cells incubated with MPP+ or MPP+ in the presence of EPO suggest a compensatory mechanism of survival and energy production as a result of mitochondrial dysfunction.

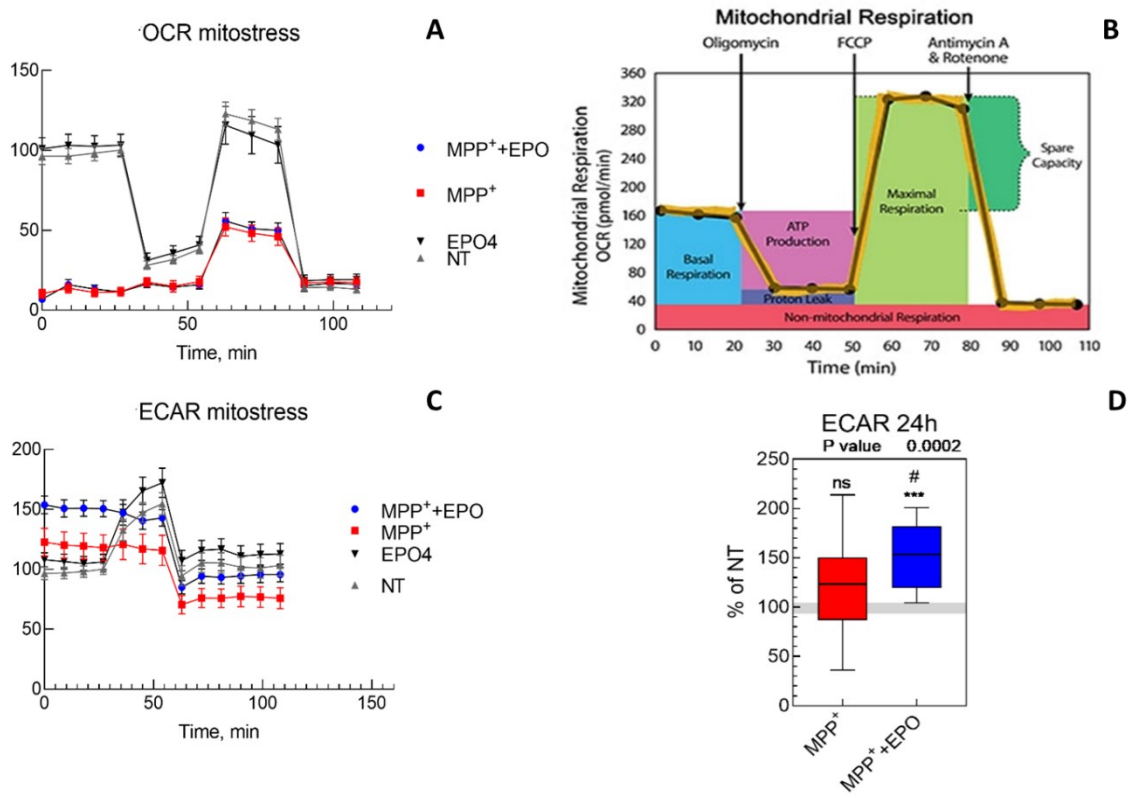

Figure 20: Mitostress assay and 24h effects of MPP+ and EPO on cell metabolism. *OCR (A) and ECAR (C) values of SH-SY5Y cell line, after 24 hours in MPP+ 500 µM treatment, and MPP+ plus EPO 4 U/ml co-treatment. A Mitostress was performed through the sequential injection of olygomycin, FCCP and Rotenone/Antimycin. The meaning of each treatment of the Mitostress assay is pictured in panel B (from Agilent Seahorse XF Cell Mito Stress Test Report Generator User Guide). Data from 3 independent experiments per condition, normalized to the control baseline (*p<0.05 vs NT, #p<0.05 vs MPP+). ECAR values of SH-SY5Y cells, after 24 h of treatment with MPP+ or MPP++EPO are shown in panel D. Data from 3 independent experiments per condition, normalized to the control baseline. *p<0.05 vs NT, # p<0.05 vs MPP+. The grey area delimits the value (mean±SD) obtained in NT.*

4.2.2 Loss of SHSY5Y cell ATP induced by MPP+ is partially prevented by erythropoietin

ATP levels were significantly decreased by MPP+ 4 and 24 h after treatment. EPO at 4 U/ml and 10 U/ml significantly counteracted this effect at 4 h but not at 24 h.

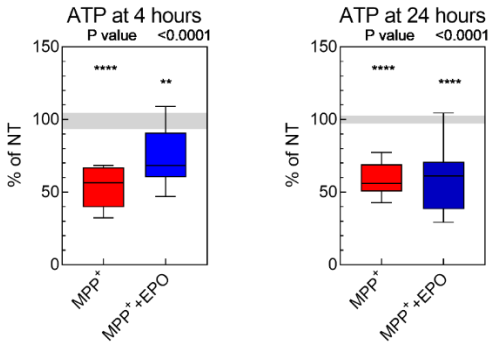

Figure 21: ATP concentration 4 h and 24 h after MPP+ and EPO treatments. *Data refer to 4 independent experiments and results are shown as percent of NT. **p<0.01, ****p<0.0001 vs. NT. The grey area delimits the value (mean±SD) obtained in NT.*

4.3 In vitro: model of hypoxia, inflammation and Hep overload

4.3.1 Inflammation and hepcidin affect glycolysis and mitochondrial respiration.

The effect of inflammation (LPS), Hep and hypoxia on A549 cell metabolism was assessed as changements in mitochondrial (OCR) and glycolityc (ECAR) activity.

Twenty-four hours of 5% O_2 hypoxia did not have any effect on the overall viability of A549 cells. LPS was administered at the concentrations of 1 and 5 mg/ml to induce inflammation, but decreased viability only when at 5 mg/ml, when cells were hypoxic. (figure 22A). In hypoxic conditions, the combination of LPS 5 µg/ml and Hep 50 ng/ml increased ECAR (figure 22B), a measure of glycolytic activity and decreased OCR (figure 22C), a measure of mitochondrial activity.

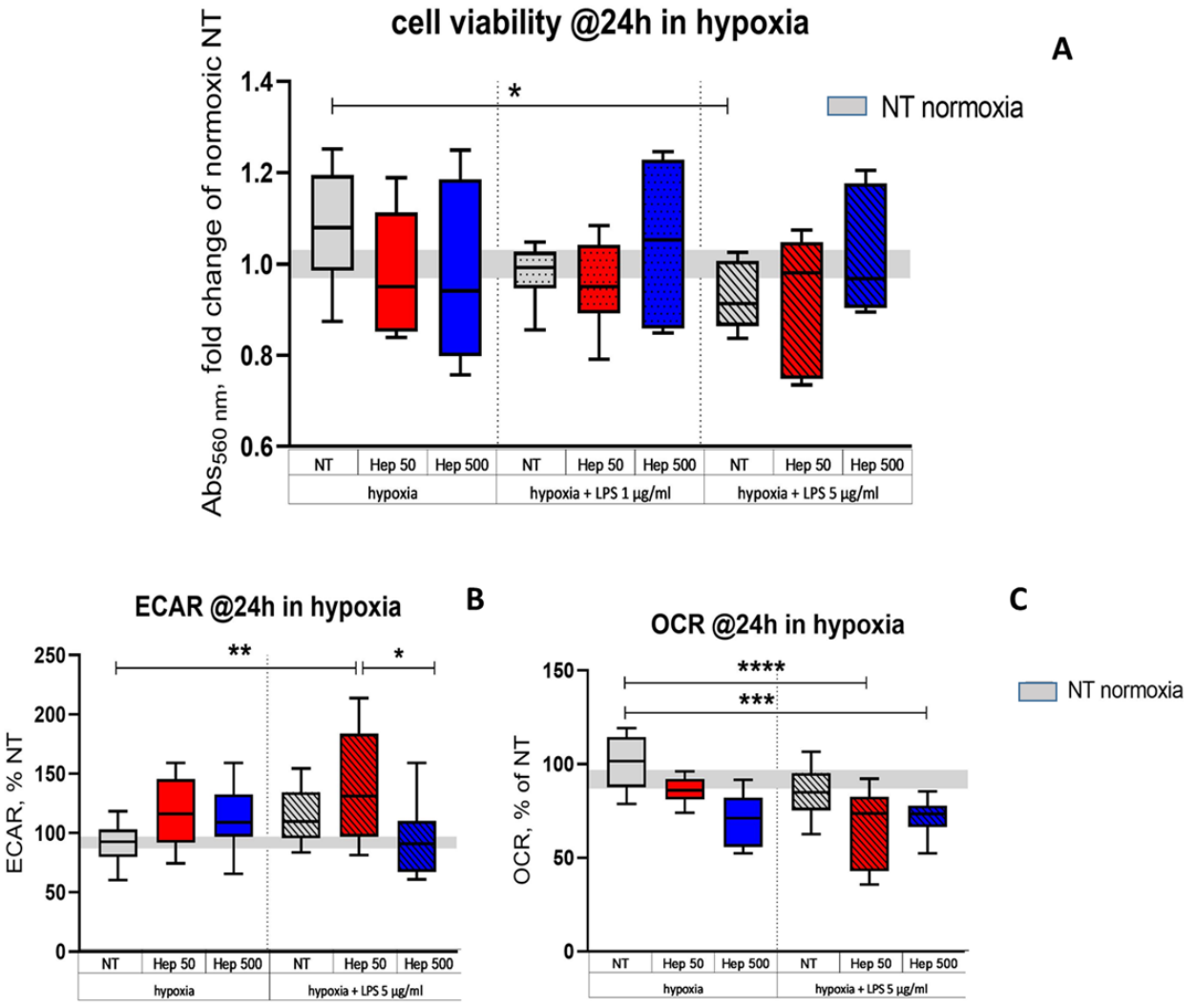

Figure 22: Cell viability (A), ECAR (B) and OCR (C) of hypoxic A549 cells treated with LPS (inflammation) and Hep. **p<0.05; **p<0.01; ***p<0.005, Tukey's range test. The grey area delimits the value (mean±SD) obtained in NT.*

5 Discussion

In the observational part of the study, we aimed at characterizing the responses to hypoxia and the anemia onset in ARDS patients, through both a follow up from ARDS diagnosis to anemia onset, and a comparison with stable hypoxic COPD patients. The systemic release of inflammatory mediators observed in ARDS interferes with the hematopoietic adaptation to hypoxia[83]. The acute exposure to inflammation and oxidative stress leads to Hep increase, resulting in less release of iron in the plasma and subsequent severe anemia. The increased Hep and soluble transferrin receptor confirm the hypothesis that these patients develop a condition similar to the anemia of chronic disease, as also suggested by increased CRP and IL-6 levels [78]. For this condition, one of the potential adjuvant treatments are Hep inhibitors [39]. These data suggest that those inhibitors might be useful in preventing anemia onset in the ARDS and possibly in COVID19, in which anemia is also an important comorbidity [25].

Despite the increased need for hematopoiesis to optimize O_2 transportation [7], EPO levels are not increased in proportion to hypoxic conditions in most ARDS and COPD patients. EPO is administered as a drug in anemic patients [35] and was recently proposed as an adjuvant drug in both ARDS and COVID19 [118].

Oxidative stress plays a key role in the pathogenesis and development of ARDS [50]. D-ROMs are also often considered as a gold standard for assessing oxidative stress [119]. These compounds, mainly hydroperoxides, are generated by the early interaction of ROS with organic molecules. In our study, assessed at the ARDS diagnosis, higher dROMs values were able to predict 28 days mortality. The increased MDA values in ARDS patients are signs of one of the main consequences of oxidative stress, the lipid peroxidation. The consumption of the antioxidant barrier (FRAP test), observed since the ARDS diagnosis and especially at the anemia onset, is a direct consequence of the increased oxidative stress [113, 120]. Such consumption is seen also as low concentration of total glutathione in RBC. This suggests that MDA and FRAP could be useful markers to investigate etiology and progression of anemia in patients exposed to acute (ARDS) or chronic (COPD) hypoxia and to suggest adequate therapeutic targets**.** The glutathionyl Hb values, higher in COPD patients, demonstrate the long-term effects of chronic hypoxia in unbalancing the RBC redox equilibrium.

The metabolomic and lipidomic untargeted approach in serum samples gives important new elements to identify other potential therapeutic targets and prognostic factors in hypoxic patients. The observed significantly lower amount of Q10 coenzyme is a sign of mitochondria dysfunction in all the hypoxic patients, especially ARDS. As Q10 coenzyme plays an important role in preventing lipid peroxidation[121], its administration can be proposed to reduce oxidative stress in those ARDS and COPD patients with increased oxidative stress. The role of sphingolipids is also assuming growing importance in the physiological adaptation to inflammation and hypoxia- related oxidative stress [83]. In this context, the low levels of S1P in ARDS patients are a sign of maladaptation to hypoxia. In fact, healthy individuals at high altitude show elevated levels of S1P [122], as the protective effect of S1P on hypoxia-induced oxidative stress and endothelial dysfunction results from proliferative and antiapoptotic effect [123]. In RBC, S1P acts by enhancing the glycolytic

metabolic fluxes leading to the generation of more 2,3-biphosphoglicerate, which promotes O_2 release [106]. This , and the slow increase in S1P throughout the hospitalization in parallel to the decrease of inflammatory markers, may also candidate S1P as a potential drug in hypoxic patients, as recently seen in animal models of hypoxia [124].

To identify the non-hematological potential positive effects of EPO and the intracellular effects of increased Hep, we used two cell lines (SY-SY5Y neuroblastoma cells and A549 lung cells) to reproduce some of the conditions observed in the patients.

As we observed that hypoxia is an important source of oxidative stress and mitochondrial damage, with one in vitro model we induced mitochondrial damage (through MPP^+) in a neuroblastoma cell line, the SH-SY5Y, and observed the potential beneficial effects of EPO. In fact, it was observed that ARDS may result in long term (at least 2 years) neurocognitive morbidity and decreased quality of life[21]. Since MPP^+ is known to damage mitochondrial respiration via inhibition of complex I of the electron transport chain [125], we questioned whether EPO plays a neurotrophic role at the mitochondrial level. As expected, MPP+ reduces mitochondrial respiration (observed as OCR) both acutely and after 24 hours, as already described by Giordano et al [103]. The acute protection of EPO may not be completely due to a rescue of mitochondrial activity. In fact, an EPO induced increase in the ATP production, significantly decreased after MPP+ treatment, is maximally observed after 4 hours. Such rescue may be due to an enhancement of glycolysis, as supported by the increase in ECAR observed 24 hours after treatment.

With the second in vitro model, we reproduced three conditions present in ARDS patients (hypoxia, inflammation and increased Hep production), in order to investigate the metabolic changes induced by these conditions in A549 cells, as a model of type II alveolar cells. We observed that the combination of those conditions has a major effect on both increasing lung cell glycolytic activity (increase in ECAR) and in downregulating mitochondrial respiration (decreased basal OCR and basal respiration). Different fractions of O_2 (21% vs 5%) influenced the 24 hours response to LPS inflammatory stimulus in opposite ways. As suggested by Cabrera-Benitez and colleagues [96], reparative phenomena as proliferation of type-II alveolar cells prevail during the early phase of ARDS, as opposed to cell loss phenomena or extensive apoptosis, that prevail in the more advanced phases. The exposure to hypoxia (5% O_2) and Hep limits such proliferation and, conversely, increases LPS toxicity, as we also observed through MTT assay.

Further investigations should be performed to test Coenzyme Q10, S1P, EPO and Hep suppressors as potential adjuvants in the treatment and prevention of anemia onset in hypoxic patients. A comparison between a larger cohort of non- COVID19 and COVID19 ARDS patients may give new insights on the pathophysiology of this emerging pandemic disease.

6 Conclusions

With this PhD thesis, we identified potential markers and therapeutic targets to prevent or treat oxidative stress and iron metabolism alterations in hypoxic patients, which may eventually lead to anemia. Among these markers, the main one involved in the iron metabolism identified and investigated in vitro is Hep. ARDS is characterized by systemic release of inflammatory mediators, consumption of the antioxidant barrier and oxidative stress, which interfere with the hematopoietic adaptation to hypoxia. Oxidative stress and inflammation lead to Hep increase, which results in increased intracellular free iron, anemia, and mitochondrial damage. In this context, an increase in endogenous EPO would exert its antioxidant and hematopoietic functions, but such increase was not observed.

Our results in human subjects was complemented with two in vitro models, which confirmed the antioxidant and neuroprotective effect of EPO and the potential noxious effects of Hep in hypoxic lung cells. In summary, it appears that Hep increase induce mitochondrial damage in inflamed lungs and anemia onset especially in ARDS patients. These data suggest that the administration of Hep inhibitors and/or EPO may be beneficial to prevent or treat anemia in such patients.

7 Acknowledgements

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Study conception and sample and clinical data collection were done in collaboration with an expert team of clinicians from San Paolo Hospital: in particular Prof. Davide Chiumello , Dr. Silvia Coppola and Dr. Rocco Rinaldo, whom I would like to thank for their important contributions since the first steps of protocol drafting.

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A final thank goes to family and friends.

8 Review process

This thesis work was evaluted by two independent reviewers:

- Professor Daniele Mancardi, Department of Clinical and Biological Sciences, University of Torino, Italy.
- Professor Roberta Foresti University Paris Est Créteil, Mondor Institute of Biomedical Research 8, rue du Général Sarrail 94010 Créteil,

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Scientific activity

PUBLICATIONS IN PEER REVIEWED JOURNALS:

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Rey F, Ottolenghi S, Giallongo T, Balsari A, Martinelli C, Rey R, Allevi R, Giulio AMD, Zuccotti GV, Mazzucchelli S, Foresti R, Samaja M, Carelli S. Mitochondrial Metabolism as Target of the Neuroprotective Role of Erythropoietin in Parkinson's Disease. Antioxidants (Basel). 2021 Jan 15;10(1):121. doi: 10.3390/antiox10010121. PMID: 33467745; PMCID: PMC7830512

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Ottolenghi S, Rubino FM, Sabbatini G, Coppola S, Veronese A, Chiumello D, Paroni R. Oxidative Stress Markers to Investigate the Effects of Hyperoxia in Anesthesia. Int J Mol Sci. 2019 Nov 4;20(21). pii: E5492. doi: 10.3390/ijms20215492. PubMed PMID: 31690051.

Ottolenghi S, Sabbatini G, Brizzolari A, Samaja M, Chiumello D. Hyperoxia and oxidative stress in anesthesia and critical care medicine. Minerva Anestesiol. 2019 Oct 28. doi: 10.23736/S0375-9393.19.13906-5.PubMed PMID: 31680497.

Rey F, Balsari A, Giallongo T, Ottolenghi S, Di Giulio AM, Samaja M, Carelli S. Erythropoietin as a Neuroprotective Molecule: An Overview of Its Therapeutic Potential in Neurodegenerative Diseases. ASN Neuro. 2019 Jan-Dec;11:1759091419871420. doi: 10.1177/1759091419871420. PubMed PMID: 31450955; PubMed Central PMCID: PMC6712762.

POSTER PRESENTATIONS:

Ottolenghi S, Duca L, Coppola S,Nava I, Rinaldo R, Cappellini MD, Samaja M, Chiumello D, Motta I, "INFLAMMATION, OXIDATIVE STRESS AND IRON: ARE THEY RESPONSIBLE FOR ANEMIA IN ACUTE RESPIRATORY DISTRESS SYNDROME?" at the 25th Congress of the European Hematology Association (virtual edition), 2020, EHA Library. 06/12/20; 294070; EP1586

Ottolenghi S., Coppola S., Duca L, Cappellini M.D., Chiumello D., Samaja M, Anemia in hypoxic patients: the role of iron metabolism and oxidative stress. European Md/PhD Conference, Geneva, September 2019

ORAL COMMUNICATIONS:

Ottolenghi S, Duca L, Coppola S,Nava I, Rinaldo R, Cappellini MD, Samaja M, Chiumello D, Motta I, "Infiammazione, stress ossidativo e ferro: quali responsabili per l'anemia nella Sindrome da Distress Respiratorio Acuto?" at Congresso del Dipartimento di Scienze della Salute (DISS), Università degli Studi di Milano, virtual edition, 2020

Ottolenghi S., Paroni R., Veronese A., Sabbatini G., Chiumello D., Samaja M. "Role of perioperative hyperoxia on the oxidative stress markers in patients undergoing laparoscopic surgery" at European Md/PhD Conference, Paris, June 29th-July 1st 2018

Ottolenghi S., Paroni R., Veronese A., Sabbatini G., Chiumello D., Samaja "Role of perioperative hyperoxia on the oxidative stress markers in patients undergoing laparoscopic surgery" at Congresso del Dipartimento di Scienze della Salute (DISS), Università degli Studi di Milano, 2018

CHAIR AND ORGANIZER:

"La Giornata del Medico Ricercatore" (Day of the Physician Scientist) , November 18th 2019, Aula Magna, University of Milan. Speaker for the session: ""I Percorsi MD-PhD in Italia"