University of Verona

PHD in Cardiovascular Science

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PhD Thesis

NITRIC OXIDE(NO) ADMINISTRATION DURING EXTRACORPOREAL LIFE SUPPORT (ECLS) IN DIFFERENT EXPERIMENTAL MODELS

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INTRODUCTION

ECLS/ECMO (extracorporeal life support / extracorporeal membrane oxygenator) is the application of mechanical devices to temporarily (days to months) support heart or lung function (partially or totally) during cardiopulmonary failure, leading to organ recovery or replacement. Different type of mechanical support is available for different clinical situation to deal for physicians in case of cardiac or pulmonary or cardio/pulmonary disfunction (figure 1):

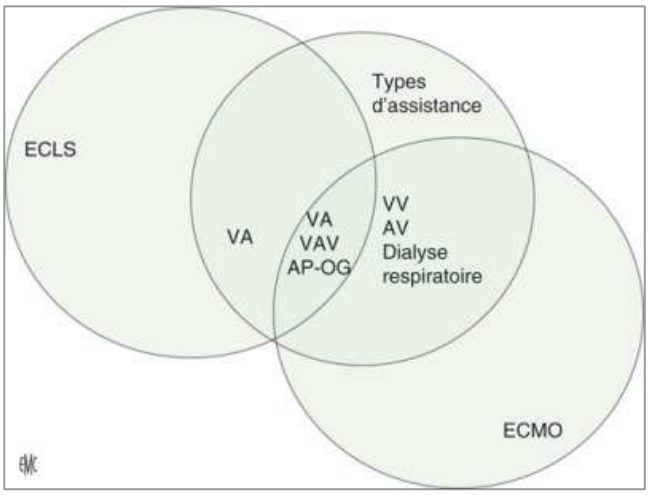


Figure 1 ECLS/ECMO support

Although to indicate this technique, in clinical practice it is used generically the ECMO denomination; it would be more appropriate to talk about Extracorporeal Life Support – ECLS for cardio-circulatory assistance, and Extracorporeal Membrane Oxygenation – ECMO for respiratory assistance.

ECLS:

Extracorporeal cardiopulmonary resuscitation (ECPR) is the application of extracorporeal membrane oxygenation (ECMO) in patients where conventional

cardiopulmonary resuscitation (CCPR) measures are unsuccessful in achieving a sustained return of spontaneous circulation (ROSC).

The primary purpose of ECPR is to restore the circulation and gas exchange. By providing organ perfusion, it provides time for the delivery of interventions necessary to regain an adequate native circulation. These may include percutaneous coronary intervention (PCI) and recovery from myocardial stunning, pulmonary thrombectomy, rewarming, or toxin clearance.

ECPR should be considered after 10–15 minutes of unsuccessful conventional resuscitation efforts, because organization and preparation for ECPR will take some time and time to ECMO start correlates with neurologic outcome.

Studies shows a survival range from 15%to 50%. Among adult ECPR patients recorded in the international ELSO dataset, survival to hospital discharge is 29%.

At the time being, we do not know whether the number of neurologic injured patients will increase with growing use of ECPR. A major task for the future will be to develop better neuroprognostication tools. In the current observational studies in selected populations >85% of survivors of cardiac arrest treated with ECPR had neurologic outcomes fall into favorable neurologic performance categories. Future trials involving ECPR should endeavor to report neurologic outcomes as well as mortality (1,2,3,4).

EXTRACORPOREAL MEMBRANE OXYGENATION

EXTRACORPOREAL MEMBRANE OXYGENATION (ECMO) IS A COMPLEX LIFE SUPPORT MACHINE, WHOSE PRIMARY GOAL IS TO TEMPORARY REPLACE THE NATIVE PULMONARY AND/OR CARDIAC FUNCTION IN PATIENTS AFFECTED BY LIFE-THREATENING CONDITION, SUCH AS SEVERE LUNG DAMAGE OR CARDIOGENIC SHOCK (5).

The first successful use of ECMO in the Intensive Care Unit (ICU) was reported in a 24-year-old trauma patient who was cannulated due to post-traumatic Acute Respiratory Distress (ARDS) (6).

After that, the use of ECMO became more ubiquitous in the late 2000s during the H1N1 epidemic, in fact it was successfully used in many patients for treatment of ARDS (7).

CURRENT APPLICATION

Veno-venous ECMO

Venovenous extracorporeal membrane oxygenation (VV ECMO) is a highly invasive method for organ support that is gaining in popularity due to recent technical advances and its successful application in recent epidemics. The use of venovenous extracorporeal membrane oxygenation (VV ECMO) among adults is rapidly increasing worldwide.

Veno-venous extracorporeal membrane oxygenation (VV-ECMO) is indicated in primary respiratory failure that is refractory to conventional medical therapy and mechanical ventilation (8) .

Utilizing ECMO in these patients allows "lung rest" through more protective ventilator settings. The primary goal of supporting a patient with VV-ECMO is to promote lung rest via lung-protective ventilation (9).
In addition to supporting oxygenation, ECMO may be a beneficial option in patients with hypercapnic respiratory failure that are unable to be managed with mechanical ventilation (10, 11)

Indications for VV ECMO
Acute Respiratory Distress Syndrome
Bronchopleural fistula
Status asthmaticus
Bridge to lung transplant
Refractory viral or bacterial pneumonia
Acute lung injury
Refractory hypoxia
Refractory hypercapnia

A more detailed table (nr 1) resume the indications and contraindication for VV ECMO.

Common indications for venovenous extracorporeal membrane oxygenation
One or more of the following:
1) Hypoxemic respiratory failure (PaO_2/FiO_2 < 80 mm Hg)*, after optimal medical management, including, in the absence of contraindications, a trial of prone positioning.
2) Hypercapnic respiratory failure (pH < 7.25), despite optimal conventional mechanical ventilation (respiratory rate 35 bpm and plateau pressure [P_{plat}] ≤ 30 cm H_2O).
3) Ventilatory support as a bridge to lung transplantation or primary graft dysfunction following lung transplant.

Specific clinical conditions:
- Acute respiratory distress syndrome (*e.g.*, viral/bacterial pneumonia and aspiration)
- Acute eosinophilic pneumonia
- Diffuse alveolar hemorrhage or pulmonary hemorrhage
- Severe asthma
- Thoracic trauma (*e.g.*, traumatic lung injury and severe pulmonary contusion)
- Severe inhalational injury
- Large bronchopleural fistula
- Peri-lung transplant (*e.g.*, primary lung graft dysfunction and bridge to transplant)

Relative contraindications for venovenous extracorporeal membrane oxygenation
- Central nervous system hemorrhage
- Significant central nervous system injury
- Irreversible and incapacitating central nervous system pathology
- Systemic bleeding
- Contraindications to anticoagulation
- Immunosuppression
- Older age (increasing risk of death with increasing age, but no threshold is established)
- Mechanical ventilation for more than 7 days with P_{plat} > 30 cm H_2O and F_iO_2 > 90%

*Clinical trials have utilized several cutoff points for the indication of the start of VV ECMO: PaO_2/FiO_2 < 80 mm Hg [EOLIA Trial[1]], Murray Score >3 [CESAR Trial[2]], without strong data indicating the superiority of any one.

Table nr 1: indication and contraindication for VV ECMO

(ASAIO Journal 2021)

Veno-arterial ECMO

Venoarterial extracorporeal membrane oxygenation (VA-ECMO), also referred to as extracorporeal life support (ECLS), is a form of temporary mechanical circulatory support and simultaneous extracorporeal gas exchange.
Analogous to veno-venous ECMO for acute respiratory failure, VA-ECMO provides circulatory support and allows time for other treatments to promote recovery or may be a bridge to a more durable mechanical solution in the setting of acute or acute on chronic cardiopulmonary failure.

Technological improvements and miniaturization have made this technique more accessible, in fact its application is increased over the past years.
In patients who suffers a cardiac arrest, VA ECMO enables treatment of the underlying cause while maintaining adequate perfusion (e.g. coronary catheterization etc).

Prolonged cerebral hypoperfusion leads to significantly worse neurologic sequelae and early initiation of ECPR with veno-arterial (VA) ECMO may be a useful adjunct to reducing the interval time from arrest to cerebral perfusion restore.

Table nr 2 represent the primary indication for VA ECMO (12).

Indications for VA ECMO
Cardiogenic shock
Acute myocardial infarction
Acute or chronic cardiomyopathy
Myocarditis
Pulmonary embolism
Refractory ventricular arrhythmia

Cardiac arrest
Postcardiotomy syndrome

Table nr 2: indication for VA ECMO

COMPONENTS OF THE EXTRACORPOREAL MEMBRANE OXYGENATION CIRCUIT

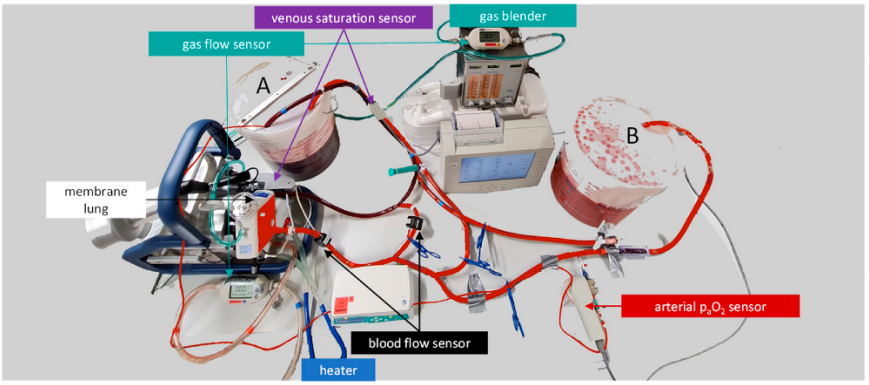


Figure nr 2: ECLS/ECMO circuit

Veno-venous (VV) or veno-arterial (VA) ECMO requires the drainage of deoxygenated blood from the venous system, moving it across a membrane oxygenator that removes carbon dioxide (CO_2), replenishes oxygen, and returns oxygenated blood back to the patient's venous or arterial system, depending on the use of VV or VA ECMO, respectively. Figure nr 2 illustrates the circuit components.
This process is facilitated by a simple system of cannulas, a blood pump, a non-microporous polymethylpentene oxygenator and a heat exchanger.
All are centrifugal pumps and provide an efficient flow for ECMO support. To provide oxygenation and ventilation for the patient, all ECMO circuits have an oxygen supply and "sweep gas" flowmeters in line with the circuit.
This oxygen flow will be the source of oxygen used for gas exchange in the membrane lung. The sweep gas allows for the removal of CO_2 in an efficient manner and allows low tidal volume ventilation and lung rest to allow for patient recovery.
Lung protective ventilation strategies are paramount to lung recovery and full utilization of ECMO can help facilitate recovery (7).

CONTROINDICATIONS, COMPLICATIONS AND ETHICAL CHALLENGES

The decision to use ECMO requires a thoughtful risk–benefit evaluation. Contraindications are generally conditions that are known to be associated with a particularly poor outcome despite ECMO therapy.
Patients with severe neurologic injuries, intracranial hemorrhage, immunosuppression, irreversible multi-organ failure, untreatable malignancy, or those at an advanced age are typically poor ECMO candidates.

Patients with aortic dissections or severe aortic regurgitation are not ECMO candidates due to the risks of propagating the dissection and over-distending the left ventricle, respectively.
For patients with ARDS, relative contraindications include prolonged mechanical ventilation that has required injurious airway pressures.
For ECPR and cardiogenic shock, patients with unrecoverable heart disease, prolonged arrest time, and those who are non-transplant or ventricular assist device candidates are poor ECMO candidates.

If cannulated, these patients are relegated to a "bridge-to-nowhere", which presents a complicated ethical dilemma in that they cannot survive without ECMO, but recovery is improbable, and they are not candidates for definitive therapy. Patients generally require anticoagulation while on ECMO and, while it is not an absolute contraindication, an inability to anticoagulate complicates both cannulation and long-term management strategies. Complication rates with ECMO are high. This is true during both cannulation and ongoing management (13).
Complications include hemorrhage, stroke, limb ischemia, thrombosis, and infection from the indwelling lines/tubes.
Data show at least one significant complication occurs in over half of patients on ECMO, with bleeding (30–40 %) and infection (31 %) being the most common (14, 15).
Hemorrhagic complications most commonly occur at the cannulation or surgical sites themselves and are generally related to anticoagulation, of which VA ECMO requires more aggressive anticoagulation due to the risk of arterial thrombosis. VA cannulation carries a high risk of arterial injury—recently reported in 18 % of patients requiring VA ECMO, with most of the injuries requiring surgical repair (16).
Neurologic complications such as intracranial hemorrhage or stroke are also well-recognized and can be devastating. Other complications include hemolysis, pulmonary edema, and lower extremity ischemia from occlusion of the arterial flow with placement of the arterial cannula, which can be prevented with the routine placement of an antegrade arterial cannula to that limb.
Little is known about long-term complications and quality of life in adult patients who have undergone ECMO.
While there are many anecdotal cases of complete recovery from critical illness in ECMO survivors, some studies report neurologic injury and long-term neurocognitive abnormalities in over 50 % of cases (17).
In addition, ECMO survivors may experience a poor quality of life, anxiety, depression, and post-traumatic stress disorder (18).
ICU lengths of stay tend to be long in ECMO patients and their costs are generally much higher than patients receiving conventional therapy (19) .
The challenges of complications, cost, and resource utilization, when taken together with the potential of creating a bridge-to-nowhere situation for some patients and lack of high-quality evidence, create an ethical obligation to consider the risks and benefits very carefully when considering ECMO in a patient's treatment plan.

NITRIC OXIDE (NO)

Nitric oxide (NO) is a molecule that has gained increasing attention in the vascular community since being named Science magazine's "Molecule of the Year" in 1992, followed by the awarding of the Nobel Prize in Physiology and Medicine in 1998 to Drs Robert F. Furchgott, Louis J. Ignarro, and Ferid Murad "for their discoveries concerning nitric oxide as a signaling molecule in the cardiovascular system."
Over the past two decades the vascular actions of NO have been investigated intensively.

Biologic characteristics of NO

NO is a gas with a half-life of several seconds. It is synthesized by a family of NO synthase (NOS) enzymes that produce NO and citrulline through a five-electron oxidation of the guanidine-nitrogen terminal of L-arginine. See figure nr.3.
Three distinct isoforms of NOS have been identified in human beings and other organisms. Two of these are constitutively expressed: neuronal NOS (nNOS; also known as NOS-1, because it was the first isoform discovered) and endothelial NOS (eNOS; NOS-3). Both are regulated by calcium and calmodulin and by posttranslational modifications of the enzymes.
The third isoform is inducible NOS (iNOS; NOS-2). It is regulated by cytokine stimulation and produces quantities of NO far exceeding those produced by the other two isoforms.
These enzymes all require several cofactors for proper function, including tetrahydrobiopterin (BH4), nicotinamideadenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide, and flavin mononucleotide.
NOS enzymes are expressed by a variety of cell types. eNOS has been identified in endothelial and smooth muscle cells (SMCs), cardiac myocytes, bone cells, and neurons. nNOS has been found in neurons, skeletal muscle, the pancreas, and the kidneys.
iNOS can be expressed in almost any cell type under cytokine stimulation but is also constitutively expressed in some tissues such as the bowel wall.
The list of tissues and cells that can express any or all of these NOS isoforms continues to grow, which supports the importance of NO in a variety of physiologic and pathophysiologic processes.
The first realization that NO was pivotal in biologic processes occurred when it was identified as endothelium derived relaxing factor (EDRF).
In 1980 EDRF was described as a factor that mediates vasodilation and is released by the vascular endothelium in response to acetylcholine. Independent work conclusively revealed that NO had identical properties and characteristics as those of EDRF. Since these early works, NO has been further characterized and found to possess several physiologic functions essential to vascular homeostasis.
The molecular signaling pathways through which NO transmits its message are many and are still being elucidated. However, one prevalent pathway in the vasculature involves the activation of soluble guanylyl cyclase, which then produces cyclic GMP (cGMP).
cGMP appears to be the second messenger responsible for mediating vasorelaxation and antiplatelet functions.
NO inhibits SMC proliferation and migration through cGMP, although cGMP independent pathways for these actions also have been found.

NO can also act directly on calcium-dependent potassium channels, leading to the relaxation of smooth muscle. The vasoprotective effects of NO extend beyond the SMCs, and include promotion of endothelial cell proliferation, protection of endothelial cells from apoptosis, and inhibition of adherence of inflammatory cells (20, 21) .

Medical applications of nitric oxide

The most recognized role of NO is mediated vascular relaxation by the action of guanylate cyclase and cGMP. Nitric oxide also has other biochemical effects such as the modulation oxidative stress, inflammation, permeability, and coagulation (22). However, while the effect of vascular relaxation is a certain hemodynamic event, these are influenced by tissue specificity; these are therefore less predictable effects.
In the medical field, NO is introduced into the body only by inhalation after dilution in a humidified air/oxygen mixture (23, 24). Direct intra-tracheal administration is prohibited as this may cause injury to the respiratory mucosa. It has been established that doses should be between 10 and 40ppm, higher doses, as well as offering no additional benefit, could be harmful (25, 26) .

Nowadays the most used indication for the use of inhaled NO is the treatment of patients with severe pulmonary arterial hypertension and/or severe refractory arterial hypoxemia.

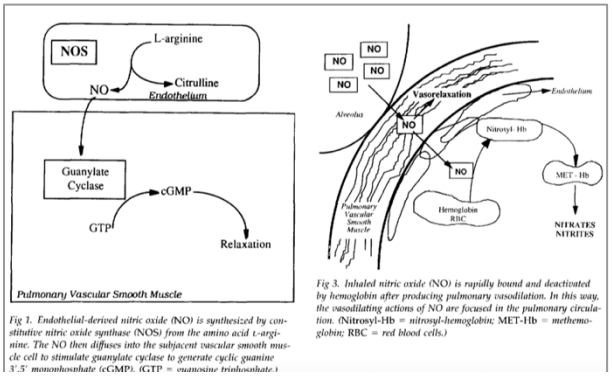


Fig 1. Endothelial-derived nitric oxide (NO) is synthesized by constitutive nitric oxide synthase (NOS) from the amino acid L-arginine. The NO then diffuses into the subjacent vascular smooth muscle cell to stimulate guanylate cyclase to generate cyclic guanine 3',5' monophosphate (cGMP). (GTP = guanosine triphosphate.)

Fig 3. Inhaled nitric oxide (NO) is rapidly bound and deactivated by hemoglobin after producing pulmonary vasodilation. In this way, the vasodilating actions of NO are focused in the pulmonary circulation. (Nitrosyl-Hb = nitrosyl-hemoglobin; MET-Hb = methemoglobin; RBC = red blood cells.)

Figure nr 3: nitric oxide mechanism of vasorelaxation

In particular, NO is indicated in the treatment of acute respiratory distress syndrome (ARDS), pulmonary hypertension in patients undergoing heart transplants or right ventricular assist device (RVAD) implants, and in neonates affected by idiopathic or post-surgery hypertension and congenital heart diseases.
Treatment with iNO demonstrates several advantages over the use of intravenous vasodilators (27).
Although the main indication of NO is the treatment of arterial hypertension and arterial hypoxemia, there are some evidences that patients with myocardial infarction refractory to conventional therapy positively responded to prolonged iNO therapy, showing improvements in clinical hemodynamic parameters and decrease in pulmonary systemic resistance (28) .
The biochemical properties of NO turned attention to the ability that NO could have in modulating the inflammatory and systemic response associated with cardiac surgery and the extracorporeal circulation system, highlighting the possibility of influencing intrinsic pulmonary and myocardial ischemia-reperfusion during surgery (29) .

PULMONARY ARTERIA HYPERTENSION (PAH)

Pulmonary arterial hypertension (PH) is defined as a mean pulmonary arterial pressure (mPAP) of 20 mm Hg or greater at rest, confirmed by right-sided heart catheterization. While there are many causes of PAH, it is almost always associated with deteriorating symptoms and increased mortality, regardless of the underlying disease.

Pulmonary hypertension affects approximately 1% of the global population, up to 10% of individuals older than 65 years, and at least 50% of patients with heart failure (HF) (30).

Classification and Hemodynamic definitions of PH

The World Health Organization has classified PH into 5 clinical subgroups.

1. Pulmonary arterial hypertension (PAH) is characterized by loss and obstructive remodeling of the pulmonary vascular bed. Pulmonary arterial hypertension features precapillary PH, defined as an mPAP of 20 mm Hg or greater, pulmonary artery wedge pressure (PAWP) of 15 mm Hg or less, and pulmonary vascular resistance (PVR) of 3 Wood units (WU) or greater. The chronic elevation of PVR can result in progressive right ventricular (RV) dysfunction and RV failure (RVF). In the presence of RVF, right atrial pressure may increase, and cardiac index may decrease. Pulmonary arterial hypertension is divided into 7 subgroups: idiopathic PAH (group 1.1), heritable PAH (group 1.2), drug- and toxin-induced PAH (group 1.3), PAH associated with various conditions including connective tissue diseases, HIV infection, portal hypertension, and congenital heart disease (group 1.4), PAH in long-term responders to calcium channel blockers (group 1.5), PAH with venous/capillary involvement (group 1.6), and persistent PH of the newborn (group 1.7). Drugs definitely associated with PAH include aminorex, fenfluramine, dexfenfluramine, benfluorex, methamphetamines, dasatanib, and toxic rapeseed oil. Drugs possibly associated include cocaine, phenylpropanolamine, L-tryptophan, St John's wort, amphetamines, interferon-α and interferon-β, alkylating agents, bosutinib, direct-acting antiviral agents against hepatitis C, leflunomide, and indirubin. There is an increased risk of persistent PH in newborns of mothers receiving selective serotonin reuptake inhibitors.

2. Pulmonary hypertension due to left-sided heart disease (LHD) (PH-LHD) occurs in response to an increase in left atrial (LA) pressure and is usually a consequence of an underlying cardiac disorder such as HF (with preserved or reduced ejection fraction) or valvular heart disease. Patients with PH-LHD usually have isolated postcapillary PH (PAWP >15 mm Hg and PVR <3 WU [≈240 dyn·s·cm^{-5}]), although some have combined postcapillary and precapillary PH (PAWP >15 mm Hg and PVR ≥3 WU).

3. Pulmonary hypertension due to chronic lung disease (CLD) (PH-CLD) and/or hypoxia can occur in many lung diseases including chronic obstructive pulmonary disease (COPD), interstitial lung disease, and sleep-disordered breathing. Elevation of mPAP in COPD may result from loss of lung vasculature, vascular distensibility, and reduced vessel recruitment. These patients have precapillary PH.

4. Chronic thromboembolic PH (CTEPH) is characterized by obstruction of the pulmonary vasculature by organized thromboembolic material and vascular remodeling, resulting from prior pulmonary embolism. Chronic thromboembolic PH is likely underdiagnosed, and its incidence and prevalence have not recently been established. Estimates based on older data are probably too low, as awareness has increased in recent years.

5. Patients with unclear and/or multifactorial mechanisms are listed as group 5. This group is divided into PH associated with hematologic disorders (group 5.1: chronic hemolytic anemia, myeloproliferative disorders), systemic and metabolic disorders (group 5.2: glycogen storage disease, Gaucher disease, sarcoidosis, pulmonary Langerhans cell histiocytosis, lymphangioleiomyomatosis, and neurofibromatosis). Pulmonary hypertension associated with fibrosing mediastinitis or chronic renal failure forms group 5.3, and PH associated with complex congenital heart disease is group 5.4. (30).

Prognosis of Patients With PH

Pulmonary hypertension is a life-threatening condition associated with increased mortality regardless of the classification and underlying etiology.
Pulmonary arterial hypertension registries report survival rates of between 68% and 93% at 1 year and 39% and 77% at 3 years.

Based on registry data, parameters that predict survival have been identified and they include exercise capacity, functional class, hemodynamic values, findings on imaging of the right side of the heart, and laboratory values. Because no single variable provides sufficient prognostic information, various clinical, functional, exercise, noninvasive, and invasive parameters have been combined to produce risk scores that predict survival and classify patients into low-, intermediate-, and high-risk groups.
These parameters include the REVEAL (Registry to Evaluate Early and Long-term PAH Disease Management) 2.0 risk score, based on data from REVEAL, the largest US PH registry.
The survival rates of patients across the risk groups differ substantially between scores. The 12-month mortality for patients with PH-LHD may be as high as 32%.

Predictors of worse prognosis include renal dysfunction, anemia, older age, RV dysfunction, and the presence of combined postcapillary and precapillary PH rather than isolated postcapillary PH. A cohort study in patients with PH-CLD reported 1-, 3-, and 5-year survival rates of 79%, 48%, and 31%, respectively. Patients with more severe lung disease and severe PH have a poor prognosis.
In an international CTEPH registry, 3-year survival was 90% in patients who underwent pulmonary thromboendarterectomy (PTE) and 70% in those who did not.
In patients with LHD (group 2) or CLD (group 3), the presence of PH greatly reduced survival.

Clinical presentation

Common symptoms of PH include exertional dyspnea, fatigue, weakness, angina, presyncope, and syncope. Fluid retention leading to abdominal distention and ankle edema can develop with progressive RVF (31).
Physical findings may include left parasternal lift or retraction, augmented second heart sound, an RV third heart sound, elevated jugular venous pressure with abnormal waveform, low volume arterial pulses, hepatomegaly, ascites, peripheral edema and a tricuspid regurgitant murmur.
History, physical examination, resting electrocardiography, and resting echocardiography in primary care can estimate the probability of PH.
The H2FPEF score, based on clinical and echocardiographic parameters, may help to identify HF with preserved ejection fraction.
Right-sided heart catheterization may be conducted in patients with PHLHD or PH-CLD if referred to an expert PH center but may not be necessary if it will have no bearing on management (32).
If no LHD or lung disease is present, a ventilation-perfusion scan is mandatory to exclude CTEPH (discussed subsequently). In patients with PAH, further tests to establish the underlying cause may include chest radiography, exercise echocardiography, pulmonary function tests, high-resolution computed tomography, contrast-enhanced tomography, pulmonary angiography, cardiac magnetic resonance imaging, laboratory tests (eg, thyrotropin, HIV, antinuclear antibodies, liver function tests, serum and urine protein electrophoresis), and sleep study or overnight oximetry (33).

Treatment of PAH

Patients with known or suspected PAH or suspected CTEPH should be referred to an expert PH center. Referral to a PH center of excellence is especially important in PAH because high-volume centers achieve better results in this rare disease. These centers can also expedite a diagnostic work-up for CTEPH, assess for operability, and perform PTE or balloon pulmonary angioplasty (BPA). Management of comorbidities (eg, sleep apnea and COPD) is essential in all patients with PH, and patients in any group may require supportive therapies such as diuretics, oxygen, and management of HF, including treatment of aggravating factors, optimization of fluid status, reduction of RV afterload, and cardiac inotropes if indicated.

Oral anticoagulation is indicated in all patients with CTEPH even if they do not have a known history of pulmonary embolism and may be appropriate in other types of PH.
Iron deficiency is common in PAH, and monitoring of iron levels, with iron substitution, when necessary, is indicated. Specific treatment of PH depends on the disease group. Insights into the pathophysiology of PAH28 led to the development of targeted treatments, which improve exercise capacity, hemodynamics, and outcomes compared with untreated patients.
The first agents introduced were the prostacyclin analogues, potent vasodilators that act on the prostacyclin receptor.
Available prostacyclin analogues include oral beraprost, intravenous epoprostenol, intravenous and subcutaneous treprostinil, and iloprost, available in intravenous, oral, and nasal aerosol formulations. Selexipag is a selective oral nonprostanoid prostacyclin receptor agonist.
Endothelin receptor antagonists, including bosentan, macitentan, and ambrisentan, prevent the vasoconstrictive and mitogenic effects of endothelin 1.29 The nitric oxide (NO)esoluble guanylate cyclase (sGC)ecyclic guanosine monophosphate (cGMP) pathway is also involved in the pathogenesis of PAH. Production of cGMP triggers vasodilation and inhibits cell proliferation, but in PAH, the NO-sGC-cGMP pathway is suppressed and phosphodiesterase type 5, which hydrolyzes cGMP, is induced, leading to vasoconstriction and cell proliferation.
Phosphodiesterase type 5 inhibitors prevent breakdown of cGMP, while the sGC stimulator riociguat stabilizes NO-sGC binding, sensitizing sGC to NO, and stimulates sGC directly, resulting in increased cGMP synthesis. The European Society of Cardiology/European Respiratory Society3 and the American College of Chest Physicians have published recommendations for the use of targeted therapies in PAH.
Patients should receive initial combination therapy, while high-risk patients should be considered for early escalation to triple therapy.
In high-risk patients (eg, RV dysfunction and/ or severe hemodynamic impairment), initial combination therapy should include parenteral prostanoids, with epoprostenol having the highest recommendation because of mortality reduction in a high-risk cohort of patients.
The combination of riociguat with phosphodiesterase type 5 inhibitor is contraindicated.
Lung transplant is an option for selected patients with severe PAH that does not respond to maximal medical therapy.
However, with the increase in PAH treatment options and the progressive approach with combination therapy, lung transplant should be used only as a last resort.
The cornerstone of PH-LHD therapy is management of the underlying heart disease.
Examples include repair of valvular heart disease and aggressive treatment of HF with reduced systolic function.
Nonspecific vasodilators may be beneficial, although the evidence is limited.
In severe HF, it is essential to optimize volume status, potentially through invasive monitoring.
Patients with PH-LHD have a poor prognosis and should be referred to expert PH centers for individual assessment and management, including consideration for clinical trials.
There is no evidence for benefit from PH-approved therapies in PH-LHD, and some have shown potential safety signals.
Guidelines recommend against PH-approved therapies in this group.

Treatment of PH-CLD primarily involves management of the underlying lung disease, and the only potentially curative treatment is lung transplant.
Pulmonary hypertension CLD is associated with increased mortality, and patients should be referred to expert PH centers and considered for clinical trials.
There is no established medical therapy for PHCLD and no evidence that PH-approved therapies are beneficial.
Ambrisentan is contraindicated in idiopathic pulmonary fibrosis and riociguat is contraindicated in PH associated with idiopathic interstitial pneumonias.
Pulmonary thromboendarterectomy is the treatment of choice for CTEPH as it is potentially curative, achieving substantial symptom relief and improvement of hemodynamics and RVF in most patients.
Surgery should therefore not be delayed in favor of medical therapy in patients who are candidates for PTE. However, up to 40% of patients are ineligible for PTE, and in up to 51%, persistent/ recurrent PH develops after PTE.
These patients are candidates for targeted medical therapy and should be considered for BPA at an expert CTEPH center.
Riociguat, the only approved medical therapy for CTEPH, is indicated for adults with inoperable or persistent/ recurrent CTEPH based on efficacy and safety data from the CHEST-1 (Chronic Thromboembolic Pulmonary Hypertension Soluble Guanylate CyclaseeStimulator Trial 1) study.
Several other PAH-approved agents have been evaluated in patients with CTEPH, most notably macitentan, which showed benefits on PVR in patients with inoperable CTEPH in the MERIT-1 (Macitentan for the Treatment of Inoperable Chronic Thromboembolic Pulmonary Hypertension) study and in preliminary results from its long-term extension, MERIT-2.
In selected patients with CTEPH, BPA can improve hemodynamics, symptoms, exercise capacity, and RV function (34).

ACUTE RESPIRATORY DISTRESS SYDROME

Acute respiratory distress syndrome (ARDS) is the acute onset of hypoxemia and bilateral pulmonary oedema due to excessive alveolocapillary permeability.
Although ARDS has a codified clinical definition, known as the Berlin definition with stages that estimate mortality risk, there is no single test to identify or exclude the diagnosis.
The heterogeneity of ARDS, evident in its causes, manifestations, and response to therapy, challenges clinicians and scientists to provide impeccable supportive care and discover new therapies (35). Characteristics are illustrated in table nr 3.

	Acute Respiratory Distress Syndrome
Timing	Within 1 week of a known clinical insult or new or worsening respiratory symptoms
Chest imaging[a]	Bilateral opacities—not fully explained by effusions, lobar/lung collapse, or nodules
Origin of edema	Respiratory failure not fully explained by cardiac failure or fluid overload Need objective assessment (eg, echocardiography) to exclude hydrostatic edema if no risk factor present
Oxygenation[b]	
Mild	200 mm Hg < Pao_2/Fio_2 ≤ 300 mm Hg with PEEP or CPAP ≥5 cm H_2O[c]
Moderate	100 mm Hg < Pao_2/Fio_2 ≤ 200 mm Hg with PEEP ≥5 cm H_2O
Severe	Pao_2/Fio_2 ≤ 100 mm Hg with PEEP ≥5 cm H_2O

Abbreviations: CPAP, continuous positive airway pressure; Fio_2, fraction of inspired oxygen; Pao_2, partial pressure of arterial oxygen; PEEP, positive end-expiratory pressure.
[a] Chest radiograph or computed tomography scan.
[b] If altitude is higher than 1000 m, the correction factor should be calculated as follows: [Pao_2/Fio_2 × (barometric pressure/760)].
[c] This may be delivered noninvasively in the mild acute respiratory distress syndrome group.

Table nr 3: characteristics of ARDS

Epidemiology and outcomes of ARDS

ARDS is more common than initially believed.
In 2016, a study of patients in 459 intensive care units from 50 countries reported that 10% of ICU patients and 23% of mechanically ventilated patients fulfilled criteria for ARDS (36).
Although the survey was done during the winter viral season and included ARDS that resolved rapidly, the hospital mortality of 35–45% closely resembled that described by the large datasets used to validate the Berlin definition.
Even patients whose ARDS resolved rapidly had a mortality rate of 31%.
Given that many patients with diffuse lung injury supported with high-flow nasal cannula (HFNC) do not meet the ARDS Berlin definition, which requires positive pressure ventilation, the incidence of ARDS is probably even higher.
The COVID-19 pandemic has highlighted this limitation, as many patients are treated without mechanical ventilation.
Men might be slightly more likely to develop ARDS, although outcome is largely similar between sexes (37).
Women—and patients of shorter stature—are less likely to receive lung protective ventilator tidal volumes.
For patients with severe persistent ARDS, women had higher mortality than men (38, 39).

Black patients might have a reduced risk of developing ARDS, and Black and Hispanic patients with ARDS had a higher mortality in at least one study, which seemed to be mediated by increased severity of illness.
Tobacco use, alcohol use, hypoalbuminemia, chemotherapy within the previous 6 months, and ambient air pollutant exposure can increase ARDS risk, whereas, in some studies, patients with diabetes were less likely to develop ARDS (40).
Mortality for ARDS remains sobering; observational studies consistently report greater than 30% hospital mortality, with one large trial of moderate to severe ARDS reporting 43% in-hospital mortality at 90 days.
The proportion of ARDS mortality that is attributable to the syndrome itself (as opposed to risk factors and comorbidities) has been challenging to determine but was estimated for sepsis-associated ARDS at 27–37%.
The cause of death is more commonly sepsis and multiple organ failure than respiratory failure (41, 42).
Although most ARDS survivors recover normal or near-normal pulmonary function, many remain burdened by functional limitations related to muscle weakness, deconditioning, or psychological sequelae of severe illness (43) .
Cognitive impairment is also distressingly common, affecting almost half of survivors at 2 years (44, 45).

Causes and risk factors

Since its initial description, ARDS has been recognized as a clinical condition that develops in the setting of various causes or risk factors.
The most common risk factors are pneumonia and non-pulmonary sepsis, followed by aspiration of gastric contents (46).
Trauma and blood product transfusion are less common ARDS risk factors in the modern era as ventilator, fluid, and transfusion management has evolved, whereas new causes such as e-cigarette or vaping product use associated lung injury (EVALI) have emerged (47, 48).
Bacterial and viral pneumonias frequently cause ARDS, with sporadic spikes in global ARDS incidence due to pandemic influenza31 and emerging viruses including SARS-CoV-2, and the coronaviruses responsible for SARS and MERS.
Identification of a specific cause for ARDS remains a crucial therapeutic goal to improve outcomes associated with ARDS.
Although genetic susceptibility to ARDS is suggested by the variability with which clinical risk factors predict ARDS development and by the replicated association of numerous genetic variants with ARDS risk, the attributable risk of any singular genetic polymorphism to ARDS risk or outcome seems small (49, 50)

Diagnostic considerations

No single diagnostic test confirms or refutes a diagnosis of ARDS. Furthermore, it must be emphasized that ARDS is a syndrome rather than a specific pathologic entity and is currently identified by purely clinical criteria. As elaborated by the Berlin definition, ARDS diagnosis requires that new or worsening respiratory distress and bilateral chest radiographical abnormalities be present for 7 days or fewer, that heart failure cannot

fully explain the hypoxemia and radiographical infiltrates, and that the impaired oxygenation be clinically significant. By comparison with previous definitions, the Berlin definition provided more specific guidance on chest radiograph patterns consistent with ARDS— bilateral opacities consistent with pulmonary oedema that can be patchy or asymmetric —and those that are inconsistent with ARDS, including isolated pleural effusions, atelectasis, or tumors (51).

Management of ARDS

There are no specific drugs or therapies available to directly treat or prevent ARDS. Mechanical ventilation with an aim to minimize Ventilator Induced Lung Injury (VILI) and management of refractory hypoxemia are the keystones in supportive management of ARDS.

Mechanical ventilation

ARDS is a heterogeneous process within the lungs in which some alveoli will never inflate, some will open and close cyclically while others will be continuously distended and damaged. Therefore, the effective lung being ventilated is much smaller than usual and is termed 'baby lung'. The primary mechanism of VILI is tidal hyperinflation of the 'baby lung' and cyclic atelectasis of already injured lung units.
Low tidal volume ventilation to prevent tidal hyperinflation and application of positive end expiratory pressure (PEEP) to improve hypoxemia and limit cyclic atelectasis are the key aspects of lung protective ventilation in ARDS.
Multiple other aspects of mechanical ventilation such as modes of ventilation, recruitment maneuvers, higher versus lower PEEP have all been studied. The current recommendations for mechanical ventilation in ARDS are represented in the table nr 4.

Mechanical Ventilation Intervention	Outcome	Guidelines
Lung protective ventilation (tidal volume of 4–8 mL/Kg predicted body weight and plateau pleasure of <30 cm H_2O)	Mortality benefit and all other measures	Strong recommendation in all ARDS patients
Higher PEEP	Mortality benefit in severe ARDS	Conditional recommendation
Recruitment maneuvers	Mortality benefit in some meta-analyses	Conditional recommendation

Volume control versus Pressure control	No difference in mortality or lung compliance or gas exchange	No recommendation
Driving pressure (Plateau pressure – PEEP)	Increased mortality with increasing driving pressures	No recommendation
APRV/BiLevel mode of ventilation	No benefit	No recommendation
High frequency oscillatory ventilation (HFOV)	Harm	Strong recommendation against the use

Table nr 4: mechanical ventilation in ARDS

Lung protective ventilation is the cornerstone of ARDS management (52).

Extracorporeal Membrane Oxygenation in patients affected by ARDS

ECMO is an extracorporeal life support modality used to temporarily support patients with respiratory and/or cardiac failure that are refractory to conventional treatment.
The venovenous ECMO (VV-ECMO) configuration is the choice in patients with respiratory failure with preserved cardiac function and the venoarterial ECMO (VA-ECMO) configuration is the choice in patients with cardiac failure with or without respiratory failure (53).
Even though ECMO was first used in adults in the 1970s, it started gaining popularity during the 2009 H1N1 pandemic when significant improvement in survival was noted in patients with ARDS and after two large RCTs reported some benefits when using ECMO in ARDS (54, 55, 56, 57).
The first landmark trial published in 2009 was a United Kingdom based multicenter RCT (CESAR trial) where 180 patients were randomized to receive conventional management or were referred to a single center for consideration for VV-ECMO.
Adult patients with severe (Murray score for acute lung injury >3 or pH <7.2) but reversible respiratory failure was included and patients with high FiO_2 (>0.8) or high peak airway pressure (>30 cmH_2O) or mechanical ventilation more than 7 days or intracranial bleeding or contraindications to heparinization or any contraindication to continued active treatment were excluded.
The study concluded that transferring patients with severe but reversible respiratory failure to a center with an ECMO based protocol improved survival (63 % in ECMO group vs 47% in control group) and was cost effective.
However, this trial had many limitations as 24% of the patients in the ECMO group never received ECMO after being transferred to an ECMO center. Only 70% of patients in the

control group received lung protective ventilation versus 93% in the ECMO group. Despite the limitations, the CESAR trials showed that VV-ECMO had a role in managing patients with severe ARDS and importance of transferring patients to specialized ECMO centers.

More recently, the EOLIA trial was published in 2018 which was a multicenter international RCT where 249 adult patients with severe ARDS (P/F <50 mmHg for >3 h or P/F <80 mmHg for > 6 h or pH < 7.25 with pCO_2 > 60 mmHg for > 6 h) were randomized to early VV-ECMO or standard lung protective ventilation.

This trial did address the limitations of the CESAR trial by implementing a strict lung protective ventilation protocol in both groups, ECMO initiation before transfer and crossover to ECMO was allowed for control group patients with refractory hypoxemia (defined as SpO_2 < 80% for > 6 h) and no irreversible multiorgan failure.

At 60 days, the difference in mortality rate was not statistically significant between both groups (35% in ECMO group versus 46% in control group, P = 0.09) and 28% of control group patients crossed over to ECMO group had a 57% mortality rate. It was hypothesized that one of the main reasons the trial was not able to demonstrate mortality difference between the groups was because the study was underpowered.

A meta-analysis of 3 trials with 504 patients using VV-ECMO versus standard care showed decrease in morality with VV-ECMO (RR, 0.64; 95% CI, 0.51-0.79).

Of note, in both the CESAR and EOLIA trials less than 25% of the screened patients were eligible for the study since it is considered unethical to withhold crossover to ECMO group from the control group, it might be difficult to perform a large study within a reasonable time frame that can show a significant survival benefit with using VV-ECMO in ARDS. Currently the most widely accepted indications ECMO consideration in respiratory failure are Murray score > 3, refractory hypoxemia (P/F < 100) despite lung protective ventilation, neuromuscular blockade, and prone positioning when indicated or persistent respiratory acidosis with pH < 7.2 (58, 59).

Absolute contraindication for ECMO include:

- terminal illness with life expectancy < 6 months,
- uncontrolled metastatic cancer,
- acute intracranial hemorrhage or infarction
- any contradiction to systemic anticoagulation.

The most common complications of VV-ECMO were bleeding (29.3%), neurological complications (7.1%) including intracranial hemorrhage, ischemic stroke, brain death and seizures.

Given the above evidence, the guidelines endorsed by British Thoracic Society suggest using ECMO in the selected patient group mentioned above and ATS/European Respiratory Society (ERS)/SCCM have no definitive recommendations for or against ECMO in severe ARDS (60).

In conclusion, the use of ECMO should be considered in a select number of patients with severe ARDS on lung protective ventilation with Murray Score >3 or pH < 7.2 due to uncompensated hypercapnia. Additional factors such as age, comorbidities, etiology of ARDS and availability of ECMO also need to be taken into consideration (61, 62).

CARDIAC ARREST

Cardiac arrest (CA) is a dramatic and potentially lethal event, which can occur suddenly and unexpectedly, its treatment requires a multidisciplinary approach.
Over time, different strategies have been proposed to obtain a return to spontaneous circulation (ROSC) and to optimize post-resuscitation care, with survival as the goal.
Among the latter, there is also the ECLS assistance, which today represents a useful resource to be used in highly selected patients presenting CA refractory to conventional treatment.
Despite recent and continuous progress in the medical field, cardiac arrest is the third leading cause of death, affecting approximately 350,000-700,000 people every year with an incidence of 67-170 cases per 100,000 inhabitants and representing more than 50% of fatal cardiovascular events.
The impact of this on society and health systems is evident.
Over the years there has been an increase in data collection, followed by the development of etiology-based therapies, continuous changes in medical practice and risk management.

Definition of cardiac arrest

Cardiac arrest, as defined by the American Heart Association and the American College of Cardiology, is the cessation of cardiac activity resulting in unresponsiveness, normal breathing, and signs of circulation (63) . Most cardiac arrests occur suddenly and are due to cardiac causes, including ischemic heart disease.
We speak of sudden cardiac death (Sudden Cardiac Death- SCD) in the case of sudden death due to a cardiac condition, almost always confirmed by an autopsy; SCD must occur within one hour of the onset of symptoms and it must have a cardiac cause to be defined. This condition has an enormous impact on the health system, in fact it is the leading cause of death in Western countries (15-20%) and is responsible for the majority of deaths from cardiovascular disease.
Despite this, its incidence is decreasing thanks to continuous developments in the prevention, treatment, and management of this. Condition (64).
In other circumstances, CA may be due to non-cardiac causes, including respiratory failure.
Various classifications are reported in this area, but the most frequent provides for a distinction between intra-hospital cardiac arrests, those affecting patients hospitalized and therefore occupying a hospital bed, from those outside the hospital, therefore in the case of subjects in the area who have reached first aid with cardiopulmonary resuscitation (CPR) in progress.
Based on this, CPR guidelines were outlined, which differ in some points (65).
CA can present with different cardiac rhythms, divided into two frequent categories: we speak of shockable rhythms (fibrillation and ventricular tachycardia) and non-shockable rhythms (asystole and pulseless electrical activity-PEA) depending on whether these may or may not be interrupted using electrical defibrillation. This distinction is fundamental for the therapeutic approach. The prognosis of cardiac arrest caused by shockable rhythms is significantly better.

Etiology of cardiac arrest

Once it has been established that the patient presents cardiac arrest, it is necessary to trace the cause.
The causes can be classified into primary cardiac causes and non-cardiac conditions, which include:

- Electrolyte imbalances
- Acute respiratory failure
- Acute bleeding
- Acute neurological disorders
- Drug abuse

Alternatively, CA may result from mechanisms of unavoidable death in terminally ill patients (end-stage cancer) (66). The cardiac event that most frequently causes cardiac arrest is ventricular fibrillation induced by acute ischemic events due to the presence of coronary artery disease.
During an ischemic episode, the onset of a ventricular hyperkinetic arrhythmia is sudden, unpredictable, frequently irreversible, and lethal (67).
There are further abnormal conditions of cardiac origin capable of determining CA including dilated and hypertrophic cardiomyopathy, myocarditis, and primary electrical disturbances.

Postcardiac arrest syndrome

Survival of patients affected by cardiac arrest remains significant, despite recent advances in resuscitation techniques; a recent report from North America indicated that only 7.9% of patients with CA survive to hospital discharge.
The first problem following the cardiac event is the negative neurological outcome, although it is evident that post CA treatments can beneficially influence the overall result.
The complex physiological processes that occur post CA and reperfusion after successful CPR have been defined as post cardiac arrest syndrome.
This can be divided into four different phases:

1. The immediate post-arrest: Occurs in the first 20 minutes following restoration of spontaneous circulation (ROSC)
2. The early post-arrest phase: 20 to 6/12 hours after ROSC. In this time frame, early interventions are effective.
3. The intermediate post-arrest phase: between 6/12 and 72 hours after ROSC. Here the pathways leading to the appearance of lesions are still activated and aggressive treatment can be initiated.
4. The recovery phase: extending from 3 days onwards, it is at this point that forecasts become more reliable.

Brain damage following cardiac arrest

Brain injuries account for 2/3 of deaths following out-of-hospital cardiac arrest.

This manifests as a persistent coma and, in some cases, as a myoclonic state or as brainstem death.
CA is responsible for brain injury through several complex mechanisms, including free radical formation, impaired calcium homeostasis, activation of cell death signaling pathways, pathological protease cascades, and excitotoxicity.
These processes may continue in the hours and days following ROSC, but this can be modified by proper treatment.
Impaired cerebrovascular autoregulation with elevated cerebral blood flow following ROSC can cause cerebral edema, often without significant increases in intracranial pressure. Despite this, cerebral blood flow may still be compromised.
Intravascular thrombosis is also thought to impair cerebral microcirculatory perfusion, despite the provision of adequate cerebral perfusion pressure.
Other factors affecting brain function after cardiac arrest are pyrexia, seizures, and hyperglycemia (68).

Post-cardiac arrest myocardial dysfunction

Myocardial dysfunction is the main cause of the low survival rate after CA.
In fact, CPR helps to cancel the ischemic process only partially, since cardiac output and systemic oxygen release, achieved during resuscitation procedures, is lower than that which occurs under normal physiological conditions.
Inadequate oxygen delivery to the tissue may persist even in the early phase of recovery of spontaneous circulation (ROSC), as the systolic and diastolic function of the myocardium has not fully recovered, resulting in continuous hemodynamic instability.
The cause of the persistence of these changes is not fully known, but the presence of a heart disease that led to CA and therapeutic interventions during CPR may contribute to this dysfunction (69).

Ischemia-reperfusion injury

Ischemia-reperfusion injury associated with an accumulation of oxygen debt (the difference between expected oxygen consumption and actual oxygen consumption multiplied by duration) causes activation of systemic inflammation and coagulation, leading up to the establishment of no-reflow phenomena and increasing the risk of multi-organ failure and infections.
Immediately after ROSC, blood pressure and heart rate may have an extremely variable range; these can in fact be increased, normal or decreased also due to the temporary increase, both local and systemic, of catecholamines (70). However, the post-CA dysfunction state is a transient condition that can be fully recovered (71).

Ischemia-reperfusion injury:
During cardiac arrest, blood flow is cut off, cutting off the oxygen supply to the tissues and the removal of metabolic waste products.
Cardiopulmonary resuscitation will only partially restore the microcirculatory flow (72).
Restoration of blood flow, if not spontaneous, can be achieved by therapeutic maneuvers, such as coronary thrombolysis, percutaneous coronary revascularization, or extracorporeal circulation (ECLS).

Reperfusion is essential to reduce the damage that occurs and above all the possibility of death after cardiac arrest much of the harmful impact of ischemia occurs precisely during this phase: it is the ischemia-reperfusion injury, which due to the release of circulating inflammatory molecules including cytokines, polymorphonuclear leukocytes, causes a change in coagulation, complement activation and cellular damage. These alterations are associated with endothelial DNA dysfunction, thrombosis, impaired fibrinolysis, and reactive oxygen species (ROS) release. During reperfusion, tissue reoxygenation induces excessive formation of ROS, such as superoxide anion radical (O_2^-), hydroxyl radical (OH-), and hydrogen peroxide (H_2O_2), which contribute to increase neuronal death by oxidizing the proteins, damaging DNA, and inducing lipid peroxidation. Furthermore, oxidative stress during reperfusion reduces the bioavailability of nitric oxide (NO), an intracellular signaling molecule.
During the ischemic period, the reduction in the availability of O_2 reduces the activity of NO synthase, producing O_2 instead of NO. Subsequently, during reperfusion, the arrival of O_2 increases the activity of NO synthase, which may exert a deleterious effect by promoting nitrative stress and decreasing NO availability to preserve endothelial integrity (73).
At cardiac tissue, ischemia-reperfusion injury can induce myocardial cell death by increasing the size of the infarcted area.
Myocardial injury during reperfusion causes several types of cardiac dysfunction.
Among these myocardial stunning, represented by a reduced mechanical function, despite the restoration of coronary flow and the absence of irreversible necrotic damage, can persist for a few days or weeks.
It is also possible to experience reperfusion arrhythmias, which are potentially harmful.

Pre-existing pathology

The pathophysiology of the post CA syndrome is often complicated by the persistence of the acute pathology that caused or contributed to the occurrence of the CA.
The diagnosis and management of this (including acute coronary syndromes, pulmonary pathologies, hemorrhage, sepsis…) can be complicated or in turn complicate the post arrest syndrome.
It is believed that these conditions may require treatment during the post CA period.

Cardiac arrest therapy and chain of survival

It is possible to survive a sudden cardiac event thanks to the use of a precise chain of actions (74):

1. Recognizing warning signs
2. Activation of the emergency system
3. Basic cardiopulmonary resuscitation
4. Rapid defibrillation
5. Basic and advanced Territorial Emergency System
6. Advanced life support and post arrest assistance

The sequence of these events constitutes the so-called "chain of survival", which to be defined requires a separate specialized program for each point, but simultaneously integrated with all the others, as demonstrated in figure nr 4.

Figure nr 4: chain of survival in cardiac arrest

A weakness in the connections decreases the possibility of survival of the patient, which after a sudden cardiac arrest is closely related to the underlying rhythm and the environment in which this occurs. Events caused by shockable rhythms have a significantly better prognosis. Cardiopulmonary resuscitation (CPR) maneuvers consisting of chest compression and ventilation, associated with early defibrillation when appropriate, ensure a favorable neurological outcome. The purpose of CPR is to ensure minimal blood flow to the heart and brain to reduce tissue ischemia time and delay cell necrosis. It is very important that the initiation of CPR is immediate because the probability of effective resuscitation decreases rapidly over time: for every minute that elapses between collapse and the first defibrillation, in the absence of CPR, the witnessed survival rate decreases by 7- 10%. Early initiation of effective CPR is defined as the application of chest compressions within 5/10 minutes of the patient losing consciousness. Current guidelines recommend that chest compressions be performed at a depth of excursion of approximately 5cm and at a frequency of 100/120 strokes per minute in adult patients. The basic cardiopulmonary resuscitation protocol also calls for chest compressions is accompanied by ventilations with a ratio of 30:2, starting from compressions. The protocol suggests promoting a better quality of CPR: at regular intervals of two minutes the operator who is performing the CPR is replaced by the one who takes care of the patient's manual ventilation. Ventilation is generally performed using an Ambu bag equipped with a mask for the delivery of O2; this system is characterized by various limitations, such as the need to interrupt the massage during insufflation and the possibility of gastric distension and therefore of regurgitation.
These problems are reduced in the case of positioning a supraglottic device or oro-tracheal intubation, which can only be performed by expert personnel.

During CPR supported by an advanced airway management, a ventilation rate of 10 breaths/minute with a volume of approximately 500-600 mL and an FiO2 of 100% is recommended. Cardiac defibrillation is the cornerstone for terminating ventricular fibrillation and pulseless ventricular tachycardia, so it is necessary to administer the first shock as quickly as possible. The aim is to interrupt the electrical activity of the heart for a short period of time to allow the sinus node to regain organizational control of the cardiac electrical activity. In the case of non-shockable rhythms (asystole or PEA) the maneuvers are limited to CPR and to the administration of drugs. Antiarrhythmic drugs and vasopressors, such as adrenaline, amiodarone and epinephrine are commonly used during resuscitation attempts. The chain of survival applies to both in-hospital and out-of-hospital arrests, but, despite this, some points differ depending on whether it is the first or second case.

ECLS in the treatment of cardiac arrest

Mechanical circulatory support (MCS) become overtime a cornerstone of therapy for patients with compromised cardiac output, refractory cardiac arrest, and cardiogenic shock following restoration of spontaneous circulation (ROSC).
The ability to improve or completely replace native cardiac function with a percutaneous device has enabled hemodynamic stabilization of these critically ill patients. However, this significant benefit must be compared to the risk of complications that can lead to significant morbidity or mortality.
Patient selection, insertion techniques for risk mitigation, and post-stabilization care remain critical (75). As previously mentioned, several studies suggest the feasibility and potential benefits of cardiopulmonary resuscitation in patients with cardiac arrest refractory to conventional therapy. In this situation, ECLS is useful for immediately restore circulation and plays a fundamental role in post-resuscitation, allowing the heart to rest and ensuring myocardial perfusion such as to favor the restoration of spontaneous rhythm. Additional benefits that ECLS Support can offer are the ability to conduct advanced radiological inquiries, surgical or percutaneous revascularization interventions before complete cardiac recovery and the management of the patient's body temperature according to the different therapeutic strategies.
Furthermore, in the event that cardiac arrest leads to cerebral death, extracorporeal membrane circulation provides peripheral perfusion to make patients potential organ donors (76). Studies have not yet provided clear improvements regarding the increase in the survival rate of patients affected by CA refractory to medical treatment and subsequent ECLS assistance, but it has been possible to highlight an improvement in the neurological outcome of these subjects compared to those who received basic cardiopulmonary resuscitation (77).
The decision to switch from CPR to eRCP must look at several aspects:

- The time of no flow, in which there is a total absence of circulation, which elapses between the appearance of CA and the start of CPR, must not exceed the maximum duration of 5 minutes. Indeed, it has been shown that the best candidates for ECLS assistance are those who receive immediate CPR.
- The low flow time, therefore, the duration of CPR, also known as the low flow period, must not be extended beyond 15 minutes.

Figure nr 5 illustrates time for CPR in CA flowchart.

A 30-minute cutoff for initiating ECLS care is generally indicated, but, despite this, some studies have described prolonged low-flow periods in subsequently assisted and surviving patients (78, 79).

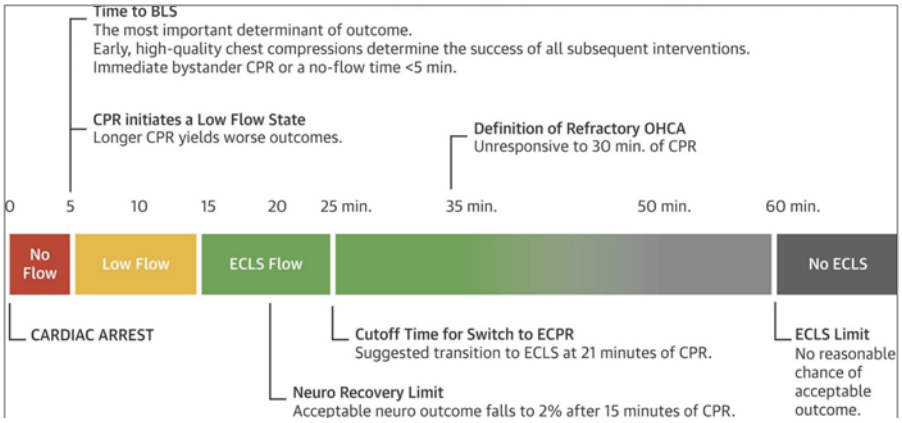


Figure nr 5:

AIM OF THE STUDY

The aim of the present preclinical study is to determine the effects of Nitric oxide when administered directly into the oxygenator of the ECMO machine, in different experimental model of ECLS/ECMO:

Experimental model nr 1. Nitric oxide administered through the oxygenator in a rat model of ARDS/ALI obtained through direct tracheal instillation of LPS (lipopolysaccharide). The aim is to value the pulmonary inflammation and oxidative damage and to obtain hemodynamic data in two different subjects:

a) Subjects affected by ARDS (tracheal LPS instillation of 5 mg/ kg) treated after 24-hours with 1-hour VV ECMO, completed with 6-hours of reperfusion
b) Subjects affected by ARDS (tracheal LPS instillation of 5 mg/ kg) treated after 24-hours with 1-hour VV ECMO associated with administration of Nitric Oxide directly through the oxygenator and completed with 6-hours of reperfusion

Hemodynamic data, histological and immunohistochemical pulmonary examination was obtained at 6-hours after reperfusion, assuming that the vasodilator and anti-inflammatory effects of nitric oxide has contributed to reduce the damage in the treated group.

Experimental model nr 2. Nitric oxide administrated through the oxygenator in an experimental model of pulmonary arterial hypertension (PAH).

The goal was to evaluate the effect of the administration of the nitric oxide directly into the oxygenator of ECMO by the analysis of several parameters such as hemodynamic parameters, recirculation fraction and oxygen delivery, inflammation, and oxidative stress in a preclinical rat model of PAH.

Experimental model nr 3. Nitric oxide administered into the oxygenator of ECMO in a rat model of cardiac arrest.

The aim is to verify the efficacy of nitric oxide in reducing the systemic inflammation response in a post cardiac arrest scenario, although the neuro and cardioprotective protective effect of nitric oxide in the ischemia/reperfusion was aim of study. We consider highly probable that the administration of nitric oxide through the oxygenator may reduce the inflammatory response and cerebral hypoxic c/ ischemic damage meanwhile ameliorate the post CA function. This study has involved about 50 subjects divided into two groups, one treated with inhaled NO and the other as a control group.

METHODS

Rats are excellent subjects for conducting research on inflammatory and oxidative mechanism regulated by different pathologies such as ARDS, also model of cardiac arrest and cardiopulmonary resuscitation. These, in fact, in addition of the low cost and easy maneuverability, are ideal for accurately reproducing clinical situations. Furthermore, the hemodynamic measurements in these species are comparable to those in humans. The benefit of such models is that the tissue damage induced is standardized and the resulting hemodynamic and respiratory measurements are relevant to the outcome (80). Male Sprague Dawley rats were used in these experimental studies, weighing about 500 grams and therefore approximately one year old to effectively access the cardiovascular system and ensure adequate assistance through extracorporeal circulation. All the rats used come from the Interdepartmental Service Center for Experimental Research using Laboratory Animals (C.I.R.S.A.L.) of the University of Verona.

EXPERIMENTAL MODEL NR 1 - VV ECMO IN ARDS

Experimental plan

The subject is initially sedated by inhalation of Sevoflurane 1.5% (Sevorane ABBVIE S.r.l.) until loss of consciousness is achieved, then orotracheal intubation is performed using a 14 Gauge venous cannula through which ventilation is maintained with FiO2 90% and a tidal volume of 6-8 ml/kg and a respiratory rate of 60 breaths per minute. The ventilator delivers a mixed miscella of air-Oxygen and sevoflurane 1.5% to guarantee continuous maintenance of sedation, all by means of a mechanical ventilator for rodents (INSPIRA ASV Harvard Apparatus Rodent Servo Ventilator). Analgesia is guaranteed by a subcutaneous administration of Ketoprofen 2mg/Kg, while muscle relaxation from rocuronium bromide 1 mcg/Kg. The cardiac electrical activity is monitored via a three-lead ECG; core temperature monitored via rectal probe. After accurate operation field, a lateral neck incision is performed, and carotid arterial and jugular vein are isolated. Blood pressure is monitored through Millar's catheter inserted through a 24-gauge cannula needle which is inserted in the carotid artery. The catheter is connected to a transducer Power Lab (AD Instruments, Colorado Springs CO USA) which in turn transmits the information to a computer through a USB input port and displayed in real time through the Lab Chart software with a signal sampling rate of 1000/sec. Then a left inguinal incision in made and the femoral vein is isolated taking good care of hemostasis to reduce to minimum the bleeding.

The cannulation is performed with a femoral (drainage cannula) – jugular (infusion cannula) vein approach, witch in our experience leads to better hemodynamics results, with reduction of the recirculation fraction and a better extracorporeal assistance. The jugular vein cannula used is a 4.5 Fr pediatric nasogastric tube, and the femoral vein cannula is a 5 Fr pediatric nasogastric tube made ad hoc (figure nr 6) for this experiment creating small incisions in the distal portion of the cannula to simulate a multistage cannula. The length of this cannula is estimated to access the intrahepatic vein to obtain a better drainage. Systemic heparinization is obtained with administration of 500UI/kg of heparin just after jugular vein cannula is inserted and advanced to the right atrium. The correct positioning of the cannula was confirmed by calculating the recirculation fraction, obtained by measuring the input and output saturation values of the

oxygenator and SvO2 of the patient; resulted < 30%, therefore related to the recommended values by the ELSO guidelines during assistance with ECMO VV (ELSO Guidelines for ECMO Centers, version 1.4 August 2017).

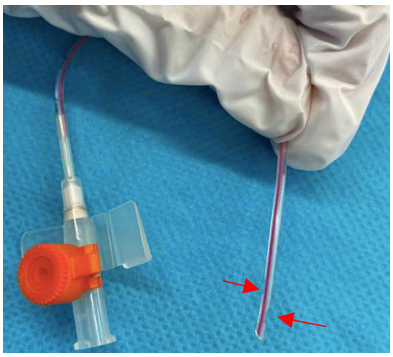


Figure nr 6: femoral venous cannula. Notice he small incision in the distal part of the cannula

The extracorporeal circulation circuit is prepared and connected with the femoral-jugular cannula as showed in figure 7.

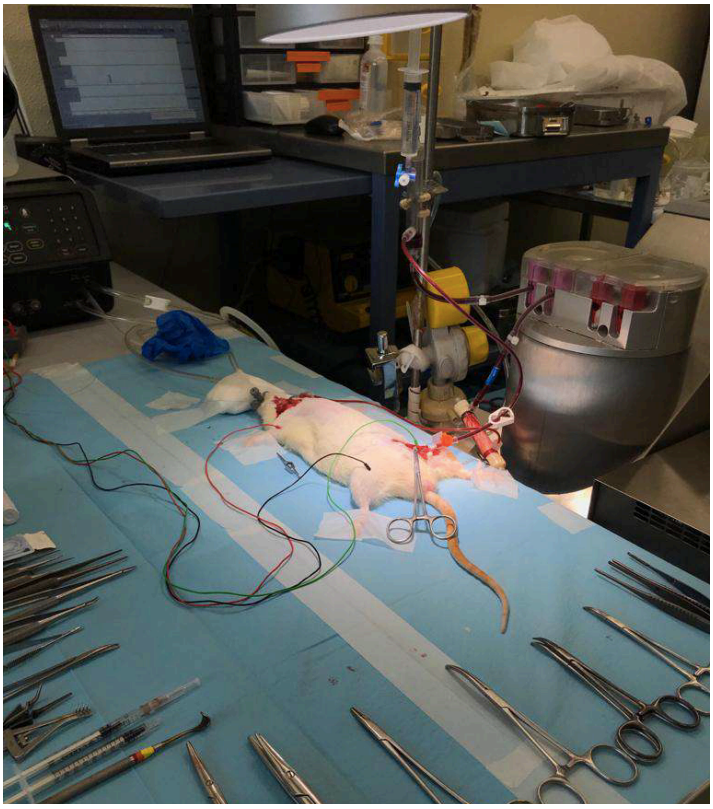


Figure nr 7: veno-venous ECMO model

ACUTE RESPIRATORY DISTRESS SINDROME (ARDS) INDUCTION

Acute lung injury/acute respiratory distress syndrome (ALI/ARDS) is a syndrome characterized by pulmonary edema and acute inflammation. Lipopolysaccharide (LPS), a major component in Gram-negative bacteria, has been used to induce ALI/ARDS. Ideal animal models should be able to duplicate the mechanisms and consequences of human diseases, including physiological and pathological features. Lipopolysaccharide (LPS) is a key constituent of Gram-negative bacterial cell walls. When delivered into animals and humans, LPS exposure displays major features of microvascular lung injury, including leukocyte accumulation in lung tissue, pulmonary edema, profound lung inflammation and mortality (81). It is a common cause in both direct (i.e., pneumonia) and indirect lung injury (i.e., sepsis), while it could also result in other chronic disorders (Figure 8).

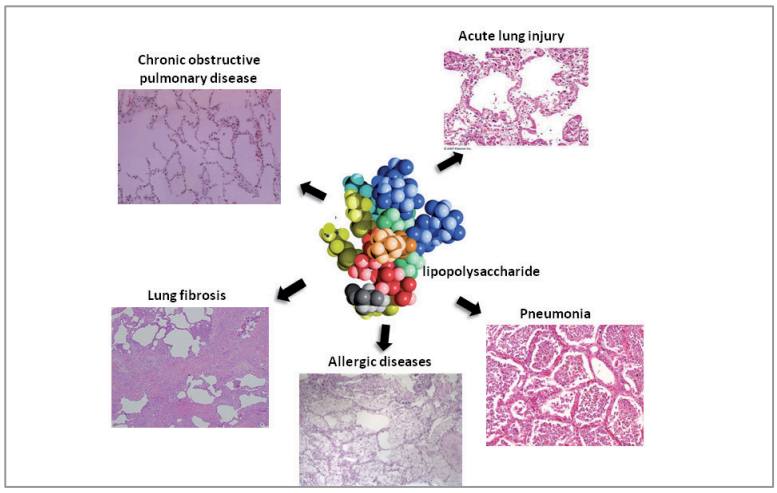


Figure nr 8: different pathological features of LPS

An LPS-induced animal model should reproduce the acute injury to the epithelial and endothelial barriers in the lungs and the acute inflammatory response in the air spaces (82) by either inhalation or systemic (intravenous and intraperitoneal) administration within a short period (usually no more than 48 h). LPS-induced injury is a very useful experimental in vivo model closely resembling ALI/ARDS in humans (83)

In our experiment, a lung damage was ascertained after 24 hours from the administration of LPS: the animals in the physical examination were in evident respiratory fatigue, tachypnoic, stunned and a blood hemogasanalysis evaluated

respiratory acidosis. The actual degree of hypoxia via the PaO2 / FiO2 ratio was possible to evaluate due to the absence of an air-oxygen mixer. Then the subjects were subdivided in two groups. 15 male rats of the Sprague-Dawley weighing 500 ± 50 gram were used. The first group 5 were induced with LPS and sacrificed to ensure the control-samples, while the remaining 10 Rats also subjected to ARDS, were treated according to two protocols:

1. Rats with ARDS, subjected to VV ECMO treatment (treated group)

2. Rats with ARDS subjected to ECMO VV and nitric oxide at 20 ppm treatment (group with experimental treatment). Figure 9 shows our

Figure nr 9 shows the dispenser iNO blender with the INOMAX revelator for the administration of nitric oxide used in our models.

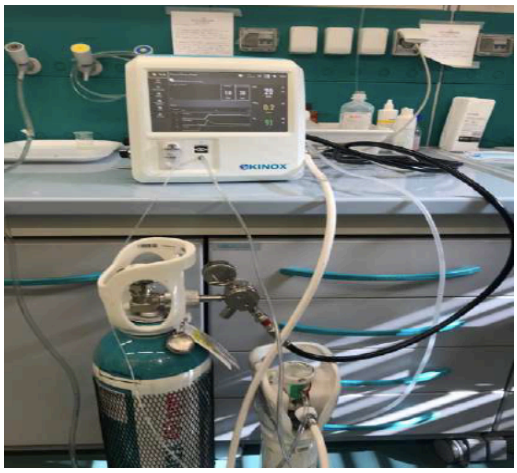


Figure nr 9: INOMAX system for Nitric oxide delivery circuit.

EXPERIMENTAL PROCEDURE

Extracorporeal circulation is started gradually until a speed of about 10 rpm in order to maintain a blood flow of 40-50 mL/kg/minute. Ventilation is set at a lower rate (30 breaths per minute) for protective ventilation. The extracorporeal circulation assistance is maintained for 60 minutes. 15 minutes after the start, a venous blood gas analysis of the subject is obtained, to evaluate the efficacy of assistance. At the same time, a pre and post oxygenator hemogasanalysis examination (through taps without interrupting

assistance) are performed to evaluate the recirculation fraction. After 60 minutes, weaning from extracorporeal circulation in started by progressively decreasing the assistance. Complete ventilatory assistance (60 breaths per minute) is resumed. In this way the subject will have a full recovery of respiratory function. A reperfusion period of 6 hours after ECMO in maintained. During and after the phase of ECMO the subject is monitored via ECG tracing and blood pressure values. An additional hemogasanalysis immediately after weaning is performed.

HEMODYNAMIC ANALYSIS

For hemodynamic parameter a full sternotomy and exposure of the mediastinal area is performed, this is needed to allow the placement of a clamp on the inferior vena cava to minimize the pre-load independent factors and have a monitoring of parameters concerning the effective cardiac contractility; this type of measurement requires the temporary interruption of the ventilation to eliminate the presence of artifacts given by the movement of the lungs. The Millar catheter is inserted into the right carotid artery and advanced through the aortic valve inside the left ventricle. The correct position of the catheter was checked by the shape of the pressure waveform (from a typical artery shape when placed in the carotid artery and then in the aorta to a ventricular shape when placed into the LV). The volume-pressure curve is monitored, together with the measurement of average blood pressure and heart rate, thus recording the left heart performance and the cardiac output after extracorporeal circulation assistance. The Millar catheter is then removed from the carotid artery and once the pulmonary artery was exposed, the Millar catheter was introduced directly into the right ventricle, perforating the wall near the apex of the right ventricle, therefore the same parameters are obtained as showed previously for the left ventricle. After that, the catheter was advanced to the pulmonary artery, to measure pulmonary artery pressure (PAP). The catheter was connected to the relative transducer and to the Power-Lab unit (AD Instruments, Colorado Springs, CO). The latter, through the USB port, was connected to a computer for real-time visualization of the pressure-volume curves and data logging using Chart software (AD Instruments). At the end of the evaluation phase, the lung was fixed in 10% formalin by injecting the solution into the pulmonary artery (after removal of blood components from the pulmonary circulation). Later, formalin is injected into

the trachea through the cannula used for the ventilation. Sampling of both lungs is completed and immersed in formalin, to obtaining total preservation of the organ.

HISTOLOGICAL AND IMMUNOLOGICAL EVALUATION

After 24 hours of storage of the samples in formalin, the process of dehydration in ethanol is executed, clarification with xylene and inclusion in paraffin, the samples were dissected in the microtome to obtain sections 7 μm thick, which were then fixed on a glass slide.

To evaluate the pulmonary histological alterations, rehydration was performed through decreasing concentrations of ethanol and then hematoxylin-eosin staining.

To evaluate the degree of oxidative stress and the inflammatory reaction; they have been furthermore, immunohistochemical investigations. In particular, the following have been used as markers of inflammatory damage antibodies:

- CCL5/RANTES Antibody
- IL-6 Antibody
- TNF-α (Tumor Necrosis Factors)
- Anti-Malondialdehyde Antibody

EXPERIMENTAL MODEL NR 2 VV ECMO IN PULMONARY ARTERIAL HYPERTENSION (PAH) MODEL

OBJECTIVE

Inhaled NO was demonstrated to selectively ameliorate PAH without impacting systemic blood pressure, since it has a very short eliminating half-life time, it can quickly diffuse into the blood flow and lose activity by binding hemoglobin. NO reduces pulmonary hypertension and improves RV systolic and diastolic function and RV-arterial coupling, with a positive effect on ventricular interdependence by increasing energetic reserve and reducing oxidative stress (84). Therefore, inhaled NO has been the milestone in the field of treatment for PAH in congenital heart diseases since 1992.

Also, VV ECMO has been used as a bridge to lung transplantation for end stage PAH. Giving these promises, our goal was to evaluate the effect of the administration of the NO directly through the oxygenator of ECMO by the analysis of several parameters such as hemodynamic parameters, recirculation fraction and oxygen delivery, inflammation, and oxidative stress in a preclinical rat model of PAH.

The present study was based on a rat model of right ventricle overloading induced by creation of systemic to pulmonary shunt. The Aorto-Caval shunt was standardized to produce a ratio of pulmonary to systemic blood flow superior to 2. The shunt is illustrated in figure 10. The success of the shunt was confirmed by observing mixing of arterial and venous blood in the IVC with distention and pulsations in the vein (85).

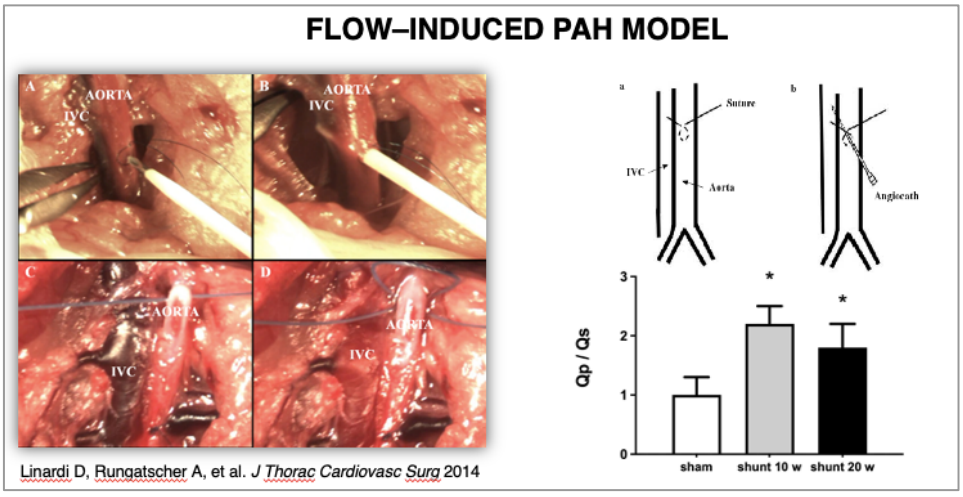


Figure nr 10: aorto-caval shunt

EXPERIMENTAL PROCEDURE

VV ECMO has been instituted in rat with standard clinical characteristics and with a hollow fiber oxygenator as in the previous study Blood gas analysis were obtained during VV ECMO support and Recirculation Fraction was kept within clinical standard (20-25%). The experimental plan used the previously PAH model as described which was validated by our group in order to demonstrate the subsequent pulmonary vascular remodeling and thus pulmonary hypertension due to the chronic exposure to the volume overload (85). After shunt creation and exposure to over circulation for 10 weeks, animals have been randomized and subdivided into three groups. The first

receive only VV ECMO treatment, the second received VV ECMO plus NO 20 ppm directly administered in the oxygenator, and the third refereed as sham underwent only mechanical ventilation. After 2 hours of treatment, all animals underwent hemodynamic analysis by miniaturized Pressure Volume conductance catheters and follow 6 hours of mechanical ventilation all animals were sacrificed, and tissue samples were collected for further analysis. A flowchart is described in the figure nr 11.

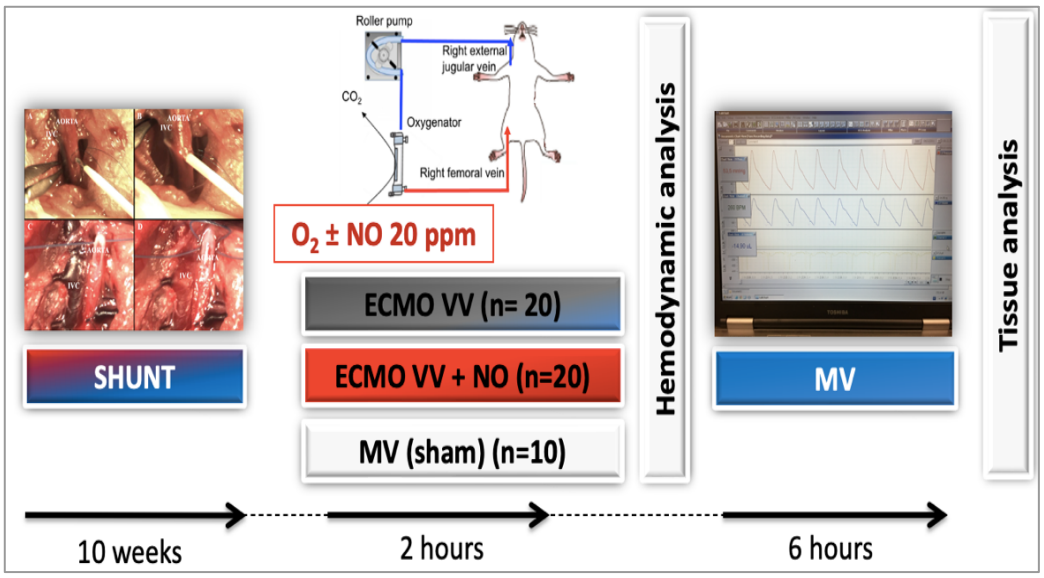


Figure nr. 11: methods of aorto-caval shunt for pulmonary hypertension

HEMODYNAMIC ANALYSIS

Biventricular contractility, RV-vascular coupling, and ventricular interdependence were assessed in vivo at different pre-loads by ventricular conductance catheters before and after. The laparotomy incision was reopened, and the previously applied 5-0 polypropylene suture that created a loop around the shunt was tightened to close the shunt. The right carotid artery was isolated and closed cranially. A 2F miniaturized, combined catheter-micromanometer (model SPR 838, Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the left ventricle. The correct position of the catheter was checked by the shape of the pressure waveform (from carotid artery and aorta to a waveform of left ventricle). The signals were

continuously recorded at a sampling rate of 1,000/sec using a P-V conductance system (Millar Instruments) connected to a PowerLab/4SP A/D converter (AD Instruments, Mountain View, CA) and a lap-top. Systolic pressure, end diastolic pressure, mean arterial pressure (MAP), stroke volume (SV), ejection fraction (EF), diastolic pressure decrement (dP/dt MIN) and maximal peak systolic pressure increment (dP/dt MAX), end-diastolic volume (EDV), time constant of ventricle pressure decay (Tau-Weiss), and stroke work were computed using the cardiac P-V analysis program (Millar Instruments). To obtain preload-independent parameters, such as pre-load recruitable stroke work (PRSW) and end-systolic pressure-volume relationship (ESPVR), and end-diastolic PVR (EDPVR), PVRs were measured by transitory inferior vena cava occlusion under the diaphragm with a small vascular clamp (86). Maximal slope of ESPVR defined ventricle systolic elastance (Ees). The vascular conductance catheter was then inserted into the RV through the jugular vein, and the same parameters previously described were recorded. In addition, effective pulmonary arterial elastance (Ea computed as RV end-systolic pressure/stroke volume) was calculated as the index of the pulmonary vascular load. The catheter was then slowly advanced into the RV outflow tract, and then in the pulmonary artery, pulmonary artery systolic and diastolic pressure was recorded.

Biochemical analysis

Freeze-clamped myocardial and lung biopsy specimens were snap-frozen. In particular, the tip of the freeze-clamp tong was pre-cooled in liquid nitrogen before the specimens were taken, and thereafter, the samples were stored at -80°C (protein isolation from tissue extracts) or in liquid nitrogen until analysis.

Measurement of high-energy phosphates

The sample preparation and high-performance liquid chromatography (HPLC) measurement of adenosine 50-triphosphate (ATP), adenosine 50-diphosphate (ADP), adenosine 50-monophosphate (AMP), and phosphocreatine (for heart samples) were performed.

Also, oxygen delivery by measuring ratio between P/F, immunohistochemical analysis, pulmonary arterial vasorelaxation by sampling a pulmonary arterial ring sample, lung oxidative stress and lung inflammation were studied.

EXPERIMENTAL MODEL NR 3 CARDIAC ARREST

Objective

The goal is to study the effects of the use of the Nitric Oxide (NO) in an experimental rat model of cardiac arrest subjected to ECLS (Extracorporeal Life Support) cardiocirculatory assistance. The aim is to verify the efficacy of NO in reducing the degree of post-cardiac arrest systemic inflammation; the neuroprotective and cardioprotective effect of nitric oxide (NO) in ischemia-reperfusion injury phenomena was also investigated. It is considered highly probable that the administration of NO into the oxygenator during ECLS could reduce the systemic inflammatory response and the hypoxic-ischemic brain damage and, at the same time, improve post-arrest cardiac function. To achieve these goals, it is necessary to study the inflammatory activation and the molecular mechanisms of cerebral ischemia during ECLS in the absence and in the presence of NO protection. Consequently, the results of the study could suggest the possibility of a new application of nitric oxide (NO), already available in intensive care and in the operating theatre, but in the medical field, administrated by inhalation in cases of acute pulmonary hypertension and post cardiotomy right ventricular dysfunction.

Experimental procedure

The subject is sedated by inhalation of Sevoflurane 1.5% (Sevorane ABBVIE S.r.l.) until loss of consciousness is achieved, then orotracheal intubation is performed using a 14 Gauge venous cannula through which ventilation is maintained with FiO2 90% and a tidal volume of 6-8 ml/kg and a respiratory rate of 60 breaths per minute. The ventilator delivers a mixed miscella of air-Oxygen and sevoflurane 1.5% to guarantee continuous maintenance of sedation, all by means of a mechanical ventilator for rodents (INSPIRA ASV Harvard Apparatus Rodent Servo Ventilator). Analgesia is guaranteed by a subcutaneous administration of Ketoprofen 2mg/Kg, while muscle relaxation from rocuronium bromide 1 mcg/Kg. The cardiac electrical activity is monitored via a three-lead ECG; core temperature monitored via rectal probe. After accurate operation field, a lateral neck incision is performed, and carotid arterial and jugular vein are isolated.

Blood pressure is monitored through Millar's catheter inserted through a 24-gauge cannula needle which is inserted in the carotid artery. The catheter is connected to a transducer Power Lab (AD Instruments, Colorado Springs CO USA) which in turn transmits the information to a computer through a USB input port and displayed in real time through the Lab Chart software with a signal sampling rate of 1000/sec. The cannulation is performed with a right carotid arterial – right jugular vein approach. The jugular vein cannula used is a 5 Fr pediatric nasogastric tube (multistage cannula) and advanced into the right atrium for better venous drainage. Systemic heparinization is obtained with administration of 500UI/kg of heparin just after jugular vein cannula is inserted and advanced to the right atrium. Then the carotid artery is cannulated as in the previous model with a 24 G cannula and advanced in aortic arch (figure nr 12). This peripherical setup reassembles the first treatment option in case of extracorporeal assistance for cardiac arrest, due to the easy access of the peripherical vessels. The ventilation is then suspended an hypoxic cardiac arrest is induced lasting for 10 minutes

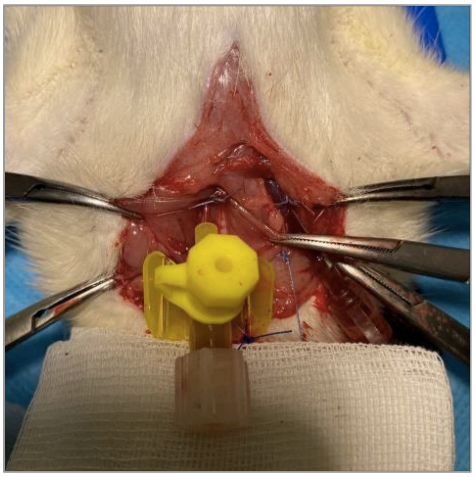


Figure nr 12: carotid and jugular cannulation model

The circuit of extracorporeal assistance is prepared and assembled and debottled with a prime of electrolyte solution of about 10 mL, to which about 400 IU of heparin is subsequently added to prevent clotting of the system. The circuit consists of a venous line, connected to the cannula of the right jugular vein, which drains the blood from the right atrium, a collection reservoir for the drained blood, an oxygenator (Euroset, Medolla, MO, Italy), a pump (Stocker SIII, Sorin, Germany) and an arterial line, through a cannula in the right carotid artery, which reinfuses the oxygenated blood into the arterial circulation. See figure nr 13.

Figure nr 13: model of extracorporeal circulation circuit

Before connecting the arterial and venous cannula to the extracorporeal circulation circuit, ventilation is suspended to induce a hypoxic cardiac arrest secondary to a respiratory arrest. The electrocardiographic tracing and blood pressure parameters are monitored. Progressively the cardiac rate decreases until the onset of cardiac arrest as shown in figure nr 14. From the absence of arterial pulse and cardiac electrical activity, 10 minutes of cardio-circulatory arrest are counted.

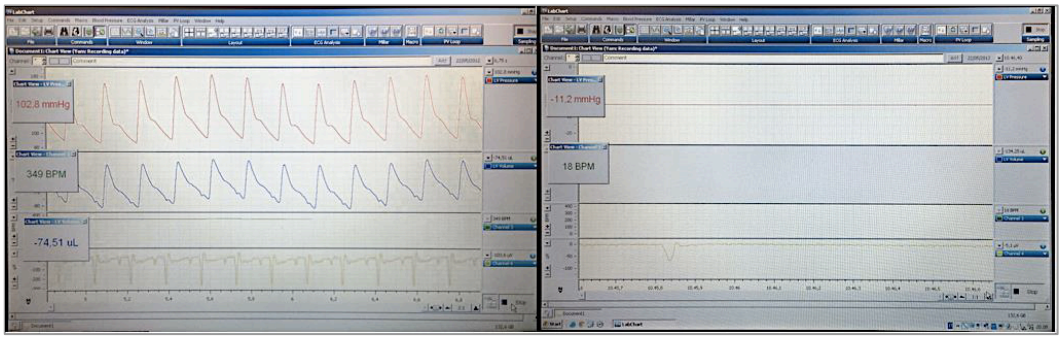


Figure nr 14: cardiac arrest induction

At this point the cannulas are connected to the circuit through the venous and arterial access, two blood samples are taken: the first for the basal analysis with flow cytometry

and the second for hemogasanalysis (EGA 1), to obtain the physiological values stablished during the ischemic period. In a small number of subjects of both groups, a further quantity of blood is taken for the subsequent dosage of the products deriving from the metabolism of Nitric Oxide. After 10 minutes of confirmed cardiac arrest, extracorporeal circulation is started gradually until reaching a speed between 15 and 20 RPM, to obtain a blood flow of 80-100 mL/Kg/min. The ventilation is restored with a protective rate of 30 breaths/min. The reperfusion is maintained for one hour, during which the spontaneous heartbeat resumes 10/15 minutes after the start of cardiocirculatory assistance. After 30 minutes, a second hemogasanalysis (EGA 2) is obtained. After one hour of ECMO, progressive weaning from extracorporeal is performed, restoring the complete ventilatory assistance at a rate of 60 breaths/minute. The hemodynamic parameters are monitored with an electrocardiographic tracing and arterial pressure values. Three further blood samples are then taken: the first is needed for a hemogasanalysis (EGA 3), the second for the Elisa test and the third for cytofluorimetric analysis. For hemodynamic monitoring, the Millar's catheter is removed from the femoral artery and inserted into the right carotid artery through the previous access used for the arterial cannula. The catheter is advanced to the left ventricle; the correct positioning is confirmed by the arterial shape as described. Hemodynamic parameter and data are measured and recorded as described in the previous model for later processing. At the end of the experiment, tissue sampling and conservation is performed (heart, lung and brain) in liquid nitrogen (-172°C) and are subsequently placed in a refrigerator at -80°C for analysis. For brain sampling, a posterior craniotomy is performed. Blood components from the circulation are removed by injecting 10 mL of phosphate buffered saline (PBS) and then 10 mL of 4% paraformaldehyde (PFA) and 4% sucrose to fix the brain tissue. This is stored in formalin to subsequently be subjected to Iba1 immunohistochemical analysis to examine the degree of tissue inflammation. A second methodology for brain sampling was applied to measure tissue oxygenation, in particular to analyze the oxidative state of protein thiols. In this case 10 mL of 1X PBS (phosphate buffered saline) enzymatic solution containing 100 mM N-ethylmaleimide (NEM) and 100 mM iodoacetamide (IAA) are infused into the cerebral circulation before sample extraction. Subsequently, 10mL of 4% PFA containing 100mM NEM and 100mM IAA are injected to fix the oxidized protein thiols in the tissue. The brain is then removed and stored in a solution of 4%

PFA and 4% sucrose for one night, and later placed in 1X PBS solution containing 30% sucrose, in which it is kept until the time of analysis. In subjects of both study groups, a circular portion of the pulmonary artery was sampled, from which traces of blood are previously eliminated thanks to the infusion of 10 mL of PBS (saline phosphate buffer) and conserved for analysis of vascular relaxation. Ach (acetylcholine inducted).

Immunofluorescence

Brain samples are object of immunohistochemical analysis. It is a specific and highly sensitive method, which allows the detection of certain antigens present in the tissue in relatively small sample sizes. The specific antibody for the antigen that need to be analyzed is placed on a prepared tissue section, in order to obtain an antigen-antibody immune reaction. Both antibodies directly conjugated to fluorescent molecules can be used (direct immunofluorescence), or the antigen-antibody reaction can be highlighted using a second antibody, conjugated to fluorochrome, specific for the first antibody (indirect immunofluorescence). The "colored" samples are then analyzed by fluorescence microscope.

For this procedure, the samples are initially stored in formalin at a temperature of -20ºC. To proceed with the analyses, the samples are removed from the solution and left to dry at room temperature for a few minutes. These are then covered with the OCT gel and placed in a cold environment to fix and freeze the tissues. OCT is a resin which at environment temperature has a gelatinous consistency, but at cold temperatures (from 4ºC) it solidifies. The OCT protects the sample from the trauma of freezing and constitutes a solid support to allow cutting. Once solidified, brain samples are cut through cryostat at a temperature of -22°C in 35 µm slices and then placed on microscope slides. See figure nr 15.

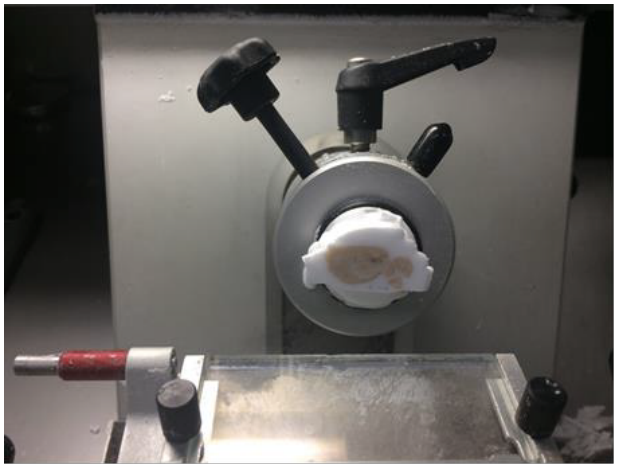


Figure nr 15: brain samples slice in cryostat

To analyze the degree of tissue inflammation, the ventricles are washed in 1X PBS solution for 10 minutes and incubated for 30 minutes in blocking solution (0.25% Triton X-100, 2% bovine serum albumin in 1X PBS). They are then incubated overnight at 4°C with a solution consisting of the primary anti-Iba1 antibodies (Ionized Calcium Binding Adapter Molecule 1) diluted in blocking solution (1:200). The Iba-1 antibody is specific for microglia cells, i.e., macrophages specialized in nervous-type tissue, determinants of the first and main line of immune defense of the central nervous system. The positivity to this type of marker indicates the presence of an inflammatory activation of the microglia cells and its quantity will be indicative of the degree of tissue inflammation. The following day, the slices present on the slides are washed 6 times for 5 minutes each with blocking solution and incubated in a secondary antibody solution, composed of Alexa Fluor 488 donkey anti-rabbit secondary antibodies dissolved in blocking solution (1:500) at room temperature for 4 hours. Then, the slides are washed three times for 5 minutes with blocking solution and washed again three times for 5 minutes each with 1X PBS. After this step, they are incubated for 10 minutes with DAPI (4,6-diamino-2-phenylindole dihydrochloride, Molecular Probes - Thermo Fisher Scientific, 1:3000) and TO-PRO (tmetc) (TO-PRO-3, Molecular Probes - Thermo Fisher Scientific, 1:3000) for nuclei staining. the slices placed on the slides are washed with 1X PBS and overlaid with additional slides using 1,4-Diazabicyclooctane (DABCO, Sigma-Aldrich).

To measure the tissue oxidative state, the sample is prepared to quantify the thiols. Quantification of thiols takes place in relation to reactive oxygen species (ROS). When

ROS production in cells exceeds neutralization by antioxidants, the cell is subjected to oxidative stress. Prolonged or excessive oxidative stress is harmful, causing significant DNA or RNA damage, enzyme deactivation, and damage to cellular structures that can lead to apoptosis. The natural production of an antioxidant, glutathione (GSH), in cells acts as a defense mechanism to counteract the adverse effects of cellular oxidative stress. Consequently, thiol quantification can be used to detect thiol groups in glutathione and monitor their rate of production in cells as a response to high levels of oxidative stress from cellular activities. For this analysis, 3 samples from the control group and 3 samples from the treated group are taken into consideration. The 35 µm brain slices present on the slides are washed in 1X PBS solution for 10 minutes and incubated for 1 hour with PBS solution containing 4mM Tris(2-carboxyethyl) phosphine hydrochloride (TCEP), a reagent used for the selective reduction of disulfides. Subsequently, they are incubated with 250 mL of dimethyl sulfoxide (DMSO) containing 7-diethylamino-3-(4- maleimidylphen)-4-methyl-coumarin (CPM) at a concentration of 4 mg/mL, for one and a half hours. The CPM is a fluorescent probe, which is used for monitoring thiol release by staining of nucleolar proteins. Finally, the slices placed on the slides are washed with 1X PBS and closed with a coverslip using 1,4-Diazabicyclooctane. This is followed by the phase of analysis under a fluorescence microscope. Iba1-immunolabelled cells were examined at the fluorescence microscopic level, in association with NeuN-labeled nuclei. Three samples were taken into consideration for the control group and three samples treated with nitric oxide (NO), analyzing two slides for each sample, and considering as a reference point 5 specific areas, identified and analyzed under the microscope for each slice. For the quantification of thiols, portions of the entire cortex were captured for each slice present on the slides. The images obtained for each area are then transferred to the computer for analysis. Content assessment was performed using ImageJ software (U.S. National Institutes of Health) under blinded conditions. For the different quantifications, a region of interest (ROI - Region of Interest) was delimited in the colored sections and a threshold was set to evaluate the percentage of the average gray level in each area (pixels positive for a specific color in the region considered in comparison to the pixels of the entire area of the region under consideration). The percentage of marked areas with the antibodies of interest was thus quantified.

Cytofluorimetry

To study the degree of leukocyte activation, the expression of the adhesion molecules CD-11b, CD54 and CD-62L was examined, by flow cytometry (BD FACSCANTO), in the monocytes of the samples of the two groups (group treated with nitric oxide (NO) and control group), after 10 minutes of cardiac arrest i.e., before starting the extracorporeal circulation and at the end. For the analysis, 2 ml of whole blood in tubes coated with K2EDTA are necessary. Leukocytes were detected using allophycocyan (APC) conjugated anti-lymphocyte common antigen (anti-CD45; Miltenyi Biotec). For cell detection in cytofluorimetric analysis (FACS), monoclonal antibodies anti-CD11b (Miltenyi Biotec), anti-CD54 (BioLegend) and anti-CD62L (BioLegend) conjugated with phycoerythrin (PE) were used. The blood sample (100 µl) is incubated with saturating concentrations of APC-conjugated anti-CD45 monoclonal antibodies and PE-conjugated monoclonal antibodies for 20 minutes at environment temperature. Subsequently, the erythrocytes are lysed, and the leukocytes are fixed with a solution (FACS Lysing Solution, BD Biosciences). Then, the samples are incubated for 10 min in the dark and then centrifuged at 200 x g for 10 min; the leukocytes obtained are washed with phosphate-buffered saline (BD Biosciences) and centrifuged again, to then be resuspended in phosphate-buffered saline and examined by flow cytometry. Results are expressed as mean fluorescence intensity (MFI) of CD11b, CD54, and CD62L on monocytes.

RESULT

EXPERIMENTAL MODEL NR 1

GAS BLOOD SAMPLE (EGA)

During the VV ECMO in the ARDS rat model, three blood gas sample for hemogasanalysis (EGA) were obtained:

1. EGA 1: at the beginning with the subject intubated and mechanically ventilated at FiO2 90%, before VV ECMO was started
2. EGA 2: after 15 minutes of VV ECMO assistance

3. EGA 3: after weaning from VV ECMO

The results of the blood gas analysis are illustrated in the table nr 5.

	ECMO LPS			ECMO LPS + NO		
	EGA 1	EGA 2	EGA 3	EGA 1	EGA 2	EGA 3
pH	7.39±0.04	7.37±0.04	7.35±0.04	7.39±0.04	7.40±0.04	7.35±0.04
pCO_2	37±2.5	30±3	29±3	37±2.5	27±3	29±3
pO_2	200±10	365±50	190±20	150±10	400±50	190±20
Hct (%)	45±2.5	29.6±2.3	41±2.3	45±2.5	30±2.3	38±2.3
Lat	0.7±0.2	2.8±0.7	10±2.7	0.7±0.2	2.8±0.7	9±3.5

Table nr 5: blood gas analisis.

No significant differences were found between the two subject groups.

For the validation of the model, further analyzes were useful through venous blood gas samples from the subject and from the oxygenator, to calculate the recirculation fraction (RF), the in our model was between 20-25%, therefore was kept into clinical standard to values recommended by ELSO guidelines (< 30%) as shown in the table nr 6.

	Arterioso	**Pre-ossigenatore**	**Post-ossigenatore**	**Venoso**
pH	7.40±0.04	7.36±0.03	7.46±0.03	7.26±0.03
pCO_2	37±2.5	52±5.3	30±3.7	48±5.2
pO_2	370±10	33±5	376±30	31±3
Sat (%)	99±1	52±4	100±1	38±4
Hct (%)	30±2.5	32±2.5	29±2.5	30±2.8
Lat (mmol/L)	0.8±0.2	1.1±1.2	1.3±1	1.5±1.4

Table nr 6: EGA analysis for recirculation fraction calculate

RF= [(SO2 pre-oxygenator - SvO2) / (SO2 post-oxygenator – SvO2)] x 100.

Histologic analysis

Hematoxylin-eosin stain evidence an exudative infiltrate around the alveolar-bronchial areas in the control group. These infiltrations are likely to remain if not even increase in the VV ECMO group (image G with objective 10X), probably due to the indirect damage on inflammatory activation that the extracorporeal circulation exercise on the endothelial damage due the release of proinflammatory cytokines. The oedema in the group treated with NO is significantly reduced as demonstrated in figure 16.

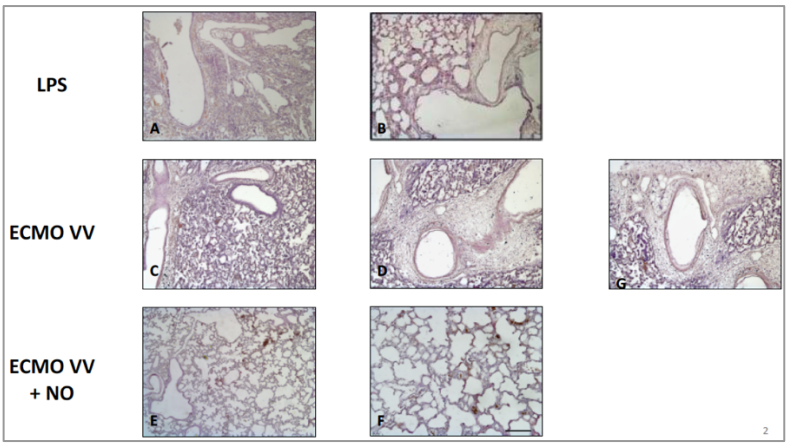


Figure nr 16: histologic sample in scale bar: 125 µm (A, B, C, D, E, F); 60 µm (G)

Immunohistochemical analysis

As described before, lung samples were obtained and analyzed for immunohistochemical examination, by detection of antibodies TNFα, CCL5 and IL-6. The results demonstrate a decrease of the pulmonary inflammatory state when nitric oxide (NO) is administrated in the VV ECMO group, showing a reduction in the lymphocytes infiltrate and inflammatory cytokines as showed in figure nr 17, 18, 19, 20.

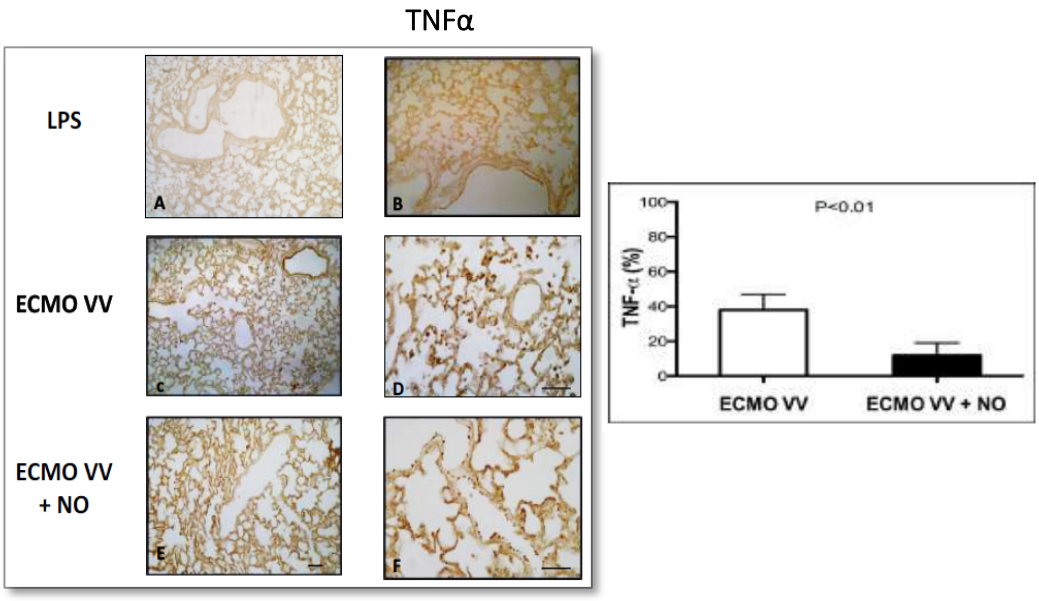


Figure nr 17: Left: samples marked with TNF Scale bar: 100 µm (A, B, E); 125 µm (B, D); 60 µm (F). Right: semi-quantitative comparison analysis between the two groups

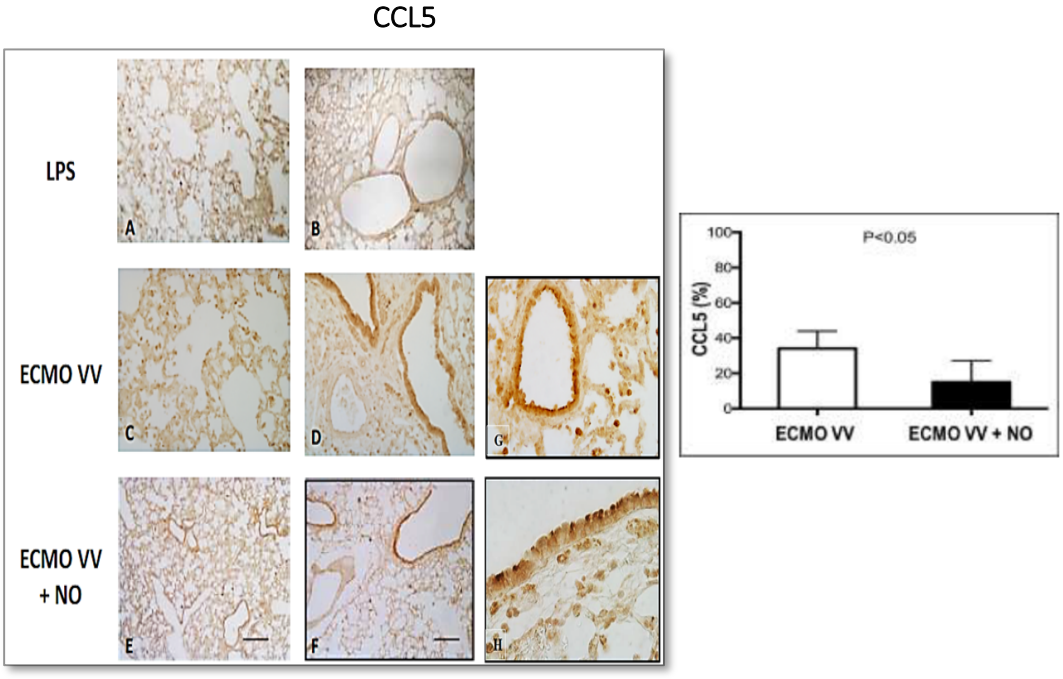


Figure 18: left: samples marked with CCL5. Scale bar: 125 µm (A, C, E); 60 µm (B, D, F). Right: semi-quantitative comparison analysis between the two groups

CCL5 / rantes is a chemokine responsible for lymphocyte T recruitment, also for basophiles and eosinophile in the inflammation site. During the immunohistochemical analysis, in the VV ECMO group, it was possible to observe a damage of the bronchial epithelial cell, therefore in the samples marked with CCL5 appears damaged and a functional loss of the component cell.

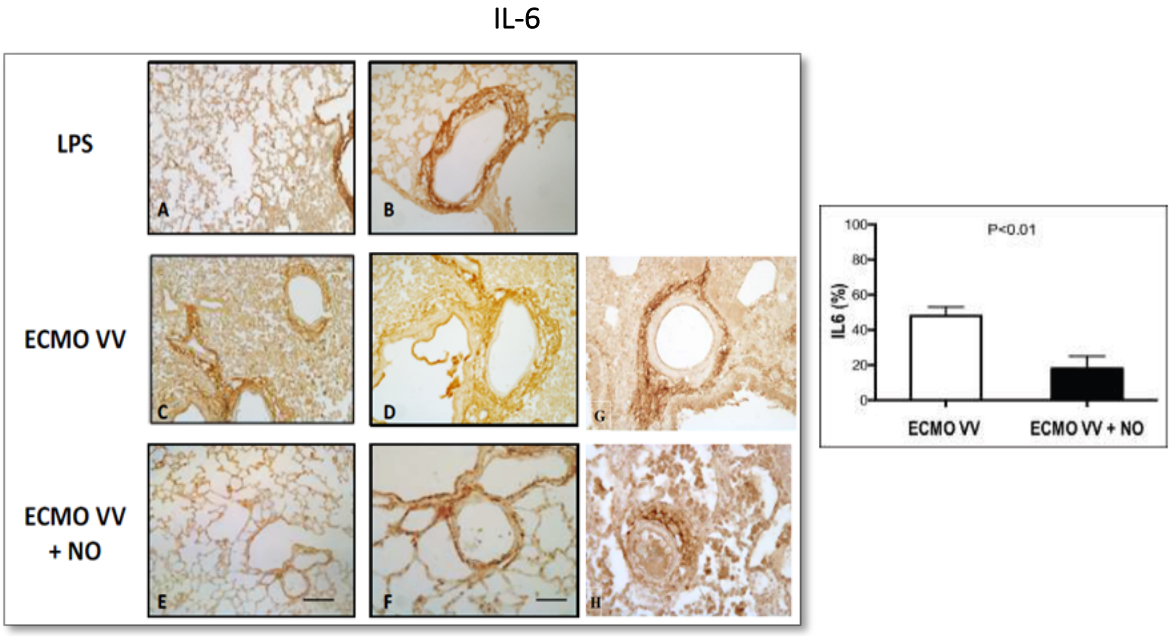


Figure 19: Left: samples marked with IL-6. Scale bars: 125 µm (A, B, C); 60 µm (B, D, F). Right: semi-quantitative comparison analysis between the two groups.

In the treated group with NO, the tunica adventitia expression of IL-6 was significantly reduced as showed in the figure below nr 20.

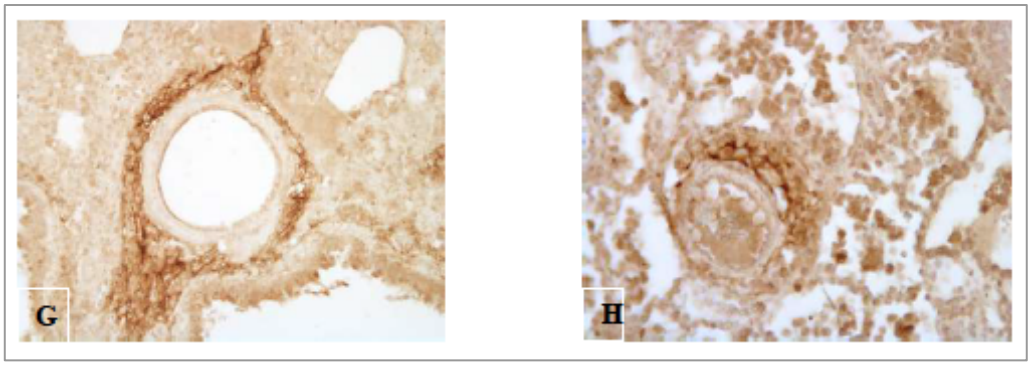


*Figure nr 20: **G**: pulmonary blood vessel of the VV ECMO group Scale bar 60 µm. **H**: pulmonary blood vessel of the treated NO VV ECMO group Scale bar 60 µm*

Further confirmation of the protective role of NO derives from samples that have been tested for Anti-Malondialdehyde Antibodies. Malondialdehyde is a marker of the peroxidized state of biological tissues. Lipid peroxidation caused by oxygen free radicals leads to formation of lipid hydroperoxides, which when broken give rise to aldehydes, including Malondialdehyde. The presence of a greater number of this antibody in the control group compared with the NO-treated group shows a significant reduction of oxidative stress in the treated group as shown in the figure 21.

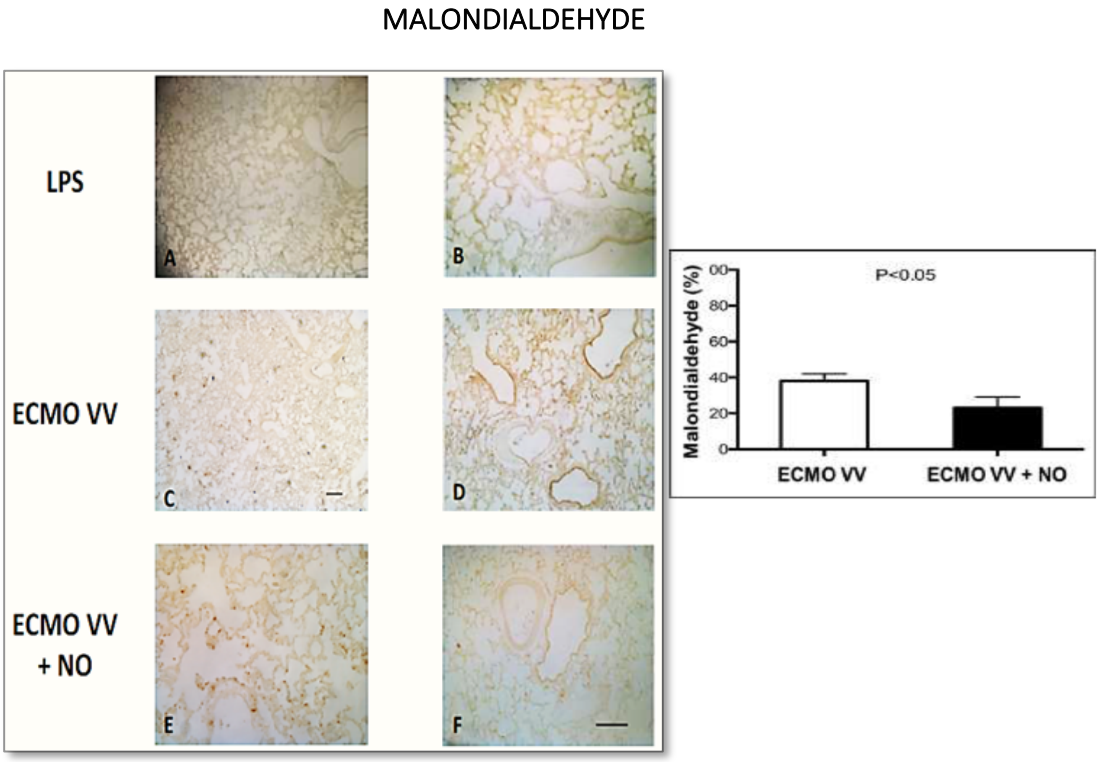
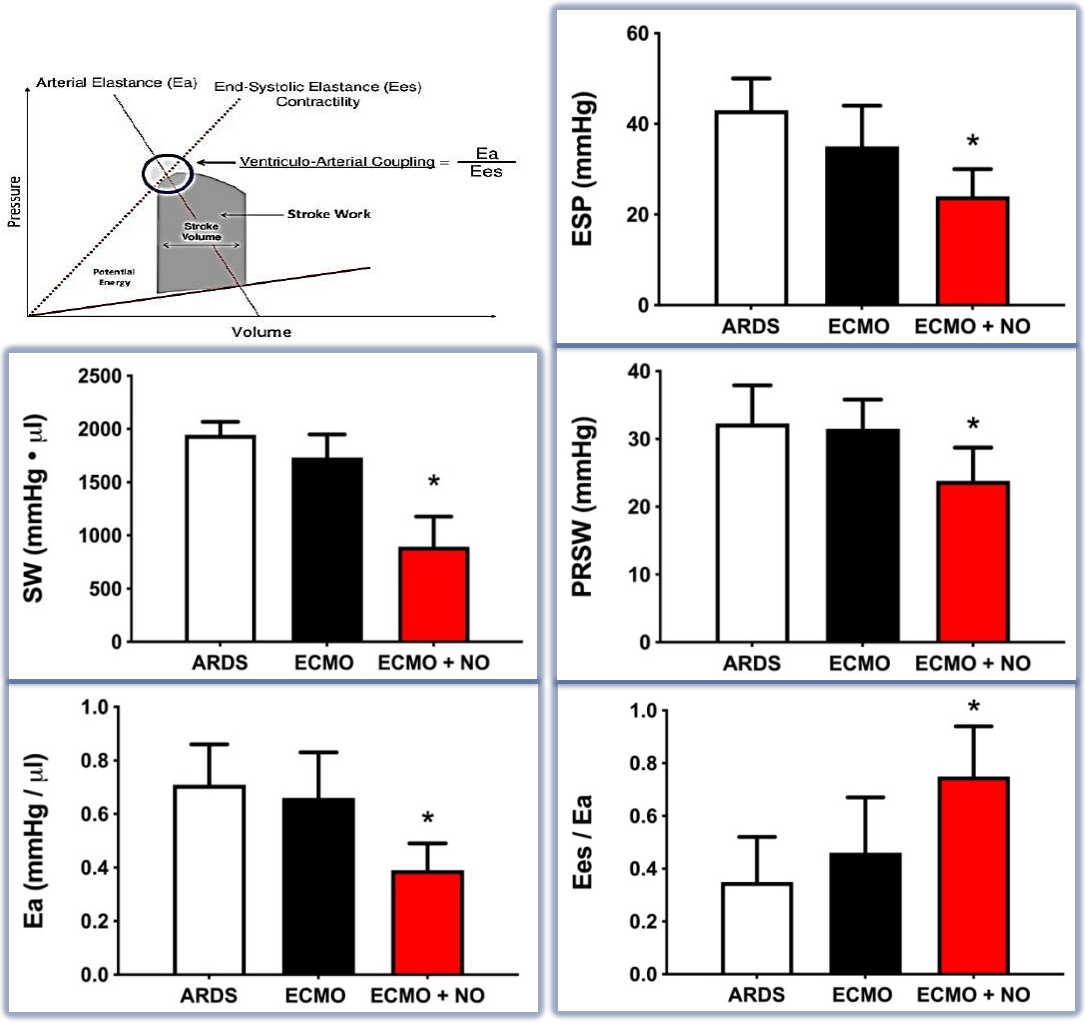


Figure nr 21: Left: samples marked with malondialdehyde. Scale bar: 100 µm (A, B); 125 µm (B, D, E, F). Right: semi-quantitative comparison analysis between the two groups.

Hemodinamic analysis

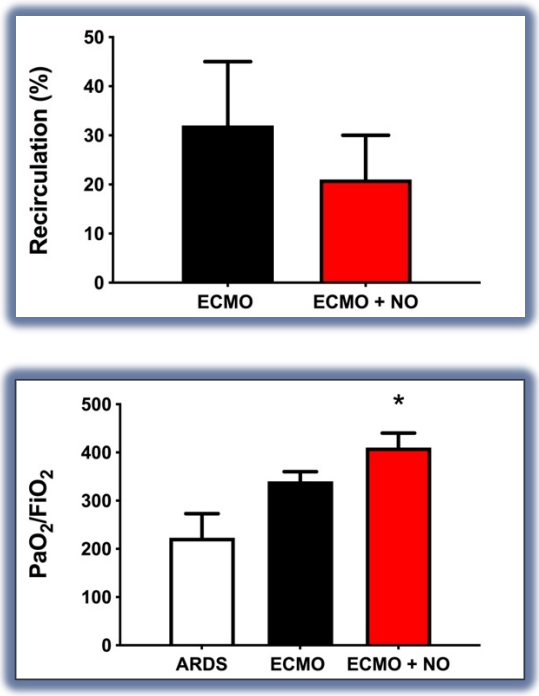
Hemodynamic analysis of the Pressure-Volume (P-V) loops revealed an improvement of independent parameter such as Stroke work and preload recruitable stroke work. Revealed an improvement of the right ventricle contractility in the treated group with

VV ECMO and nitric oxide (NO) group. Moreover, end systolic pressure (ESP) and Pulmonary artery elastance were reduced in the VV ECMO and nitric oxide (NO) group; revealed in diminished right ventricle afterload with a preserved right ventricle – arterial coupling (measured as the ratio between the arterial elastance and ventricular elastance) as illustrated in graphic nr 1. Consequently, the recirculation fraction is minimized in the VV ECMO and nitric oxide (NO) group (< 25%), as described before, and the oxygen delivery is improved. See graphic nr 2



Graphic nr 1: hemodynamic parameters analysis

Oxygen delivery



Graphic nr 2: analysis of recirculation fraction and oxygen delivery

EXPERIMENTAL MODEL NR 2

VV ECMO IN PULMONARY ARTERIAL HYPERTENSION (PAH) MODEL

Right ventricle function

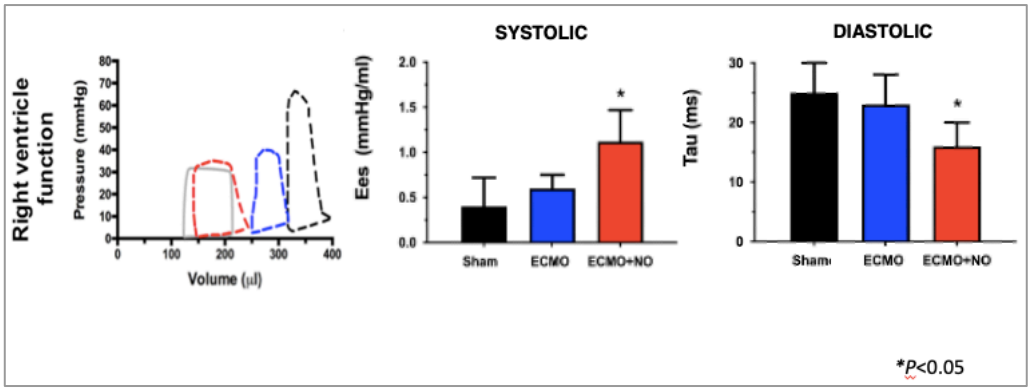
Hemodynamic analysis was obtained by pressure volume analysis. Preload-independent parameters were derived from PV. In the graphs ECMO+NO shown in red, ECMO in blue and sham operated animals in black. Right ventricle systolic function is expressed by maximal slope of End Systolic Pressure Volume Relationship (in the center) and diastolic function by Tau which is the time constant of pressure decay. In the right. Preserved systolic and diastolic function is significative only in the ECMO+NO group as shown in graphic nr 3.

RV afterload was assessed measuring mean Pulmonary Arterial Pressure and PA effective Elastance which was increased in the shunt animals. Only in the ECMO+NO group RV afterload was reduced. The relationship between pulmonary artery elastance and RV contractility gives the right ventricle arterial coupling which was preserved only in ECMO+NO group as shown in graphic nr 4.

Ventricular interdependence means the effects of RV on LV function. RV End diastolic diameter and LV Cardiac Output express this concept and were optimized in ECMO groups with higher significance in ECMO+NO group as shown in graphic nr 5.

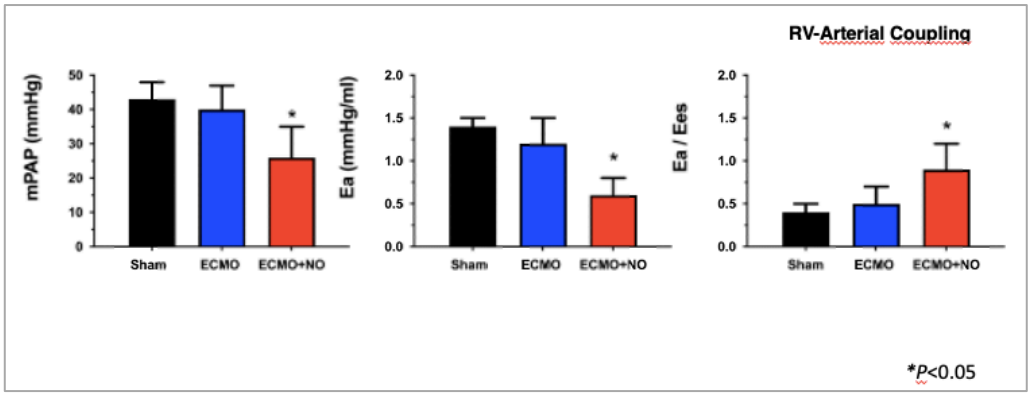
As shown in graphic nr 6, RV high energy phosphate content was preserved in ECMO+NO group.

RV FUNCTION

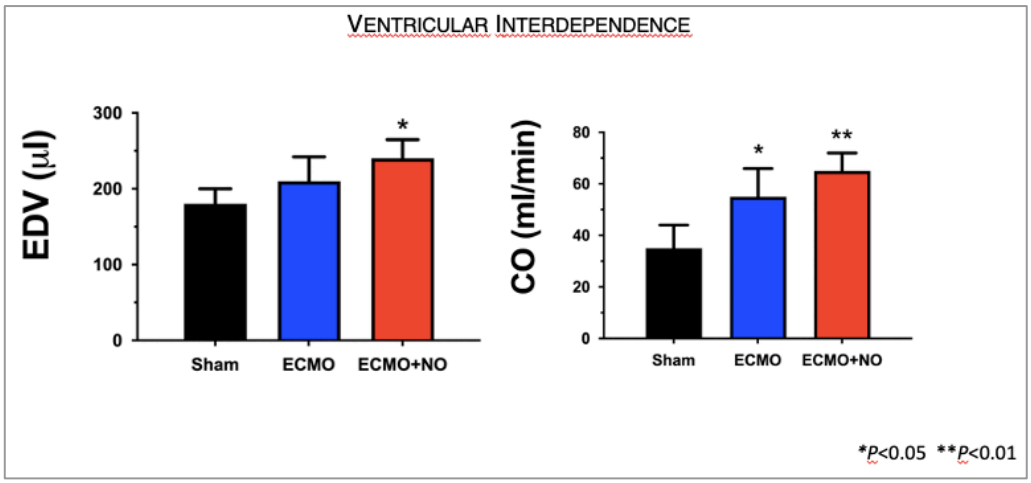


Graphic nr 3: sistolic and diastolic function is preserved in ECMO+NO group.

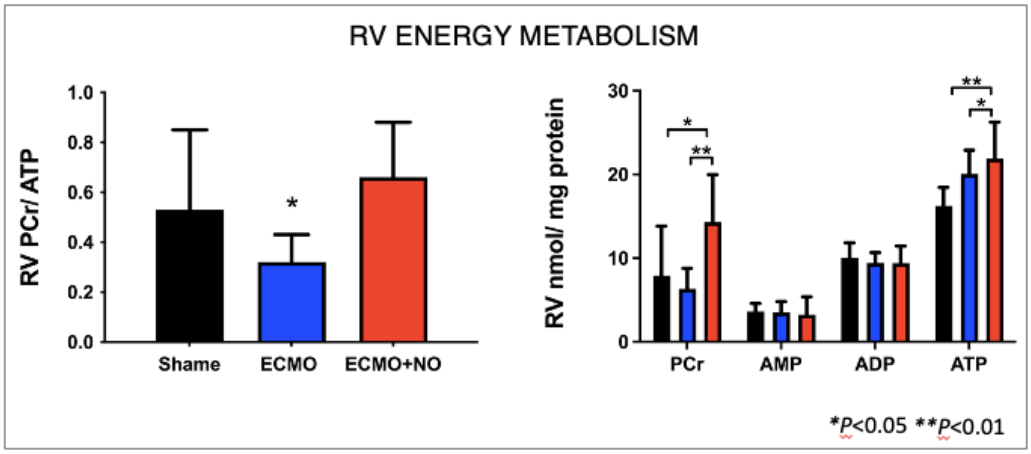
RV AFTERLOAD



Graphic nr 4: RV afterload is reduced in ECMO+NO group with a preserved ventricular-arterial coupling

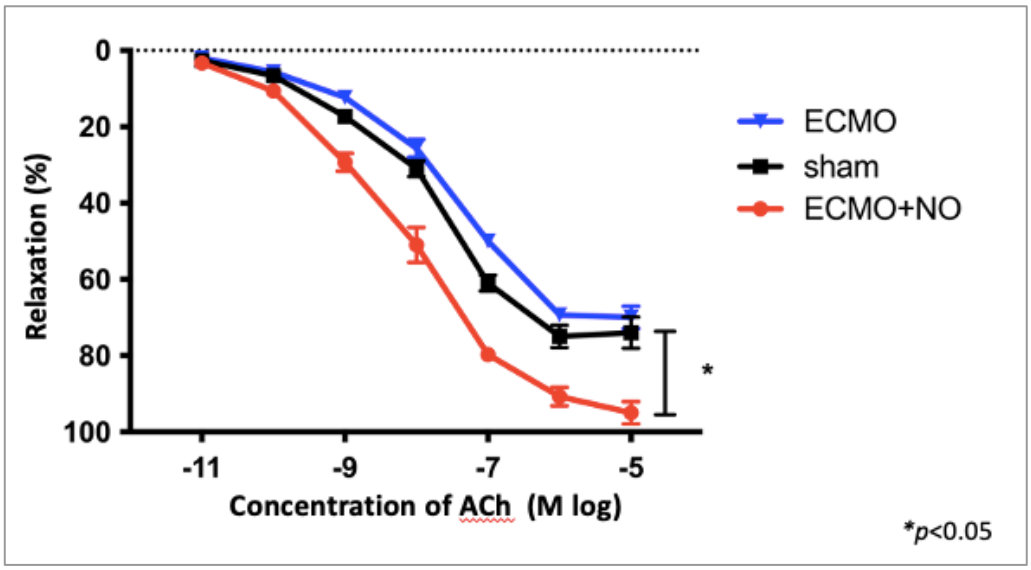


Graphic nr 5: RV EDV and LV COwere higher in ECMO+NO group with an optimized ventricular interdependence



Graphic nr 6: RV energy phosphate is preserved in ECMO+NO group.

To assess the endothelium dependent relaxation, Pulmonary artery vascular rings were obtained. The analysis revealed an almost complete vasorelaxation per concentration of Acetylcholine was observed in the group treated with NO when compared to conventional ECMO group, as illustrated in graphic nr 7.



Graphic nr 7: Pulmonary artery endothelium-dependent vasorelaxation acetylcholine induced was near 100% in the group treated with ECMO+NO.

LUNG INFLAMMATION

In a chronic over circulation, it was possible to demonstrate by immunohistochemistry a reduction of lung expression on inflammatory cytokine such as IL-6. To reinforce the possible effects of nitric oxide, other markers were studied such as ICAM1 and nitrotyrosine. ICAM1 (intercellular adhesion molecule 1) is an endothelial and leukocyte associated transmembrane protein known for its importance in stabilizing cell-cell interactions and facilitating leukocyte endothelial transmigration. Nitrotyrosine is a product of tyrosine nitration mediated by reactive nitrogen species such as peroxynitrite anion and nitrogen dioxide. Nitrotyrosine is identified as an indicator or marker of cell damage, inflammation as well as NO (nitric oxide) production. Nitrotyrosine is formed in the presence of the active metabolite NO. Generally, in many disease states, oxidative stress increases the production of superoxide (O_2^-) and NO forming peroxynitrite ($ONOO^-$) a destructive free radical oxidant. The production of peroxynitrite$^-$ can oxidize several lipoproteins and of nitrating tyrosine residues in many proteins. Usually, nitrotyrosine in proteins are the detectable marker for indirectly detecting peroxynitrite ($ONOO^-$). It is detected in large number of pathological conditions and is considered a marker of NO-dependent, reactive nitrogen species-

induced nitrative stress. In the present study both ICAM1 and nitrotyrosine analysis revealed a significant decreased inflammation in the ECMO+NO treated group compared to the other groups as shown in figure nr 22.

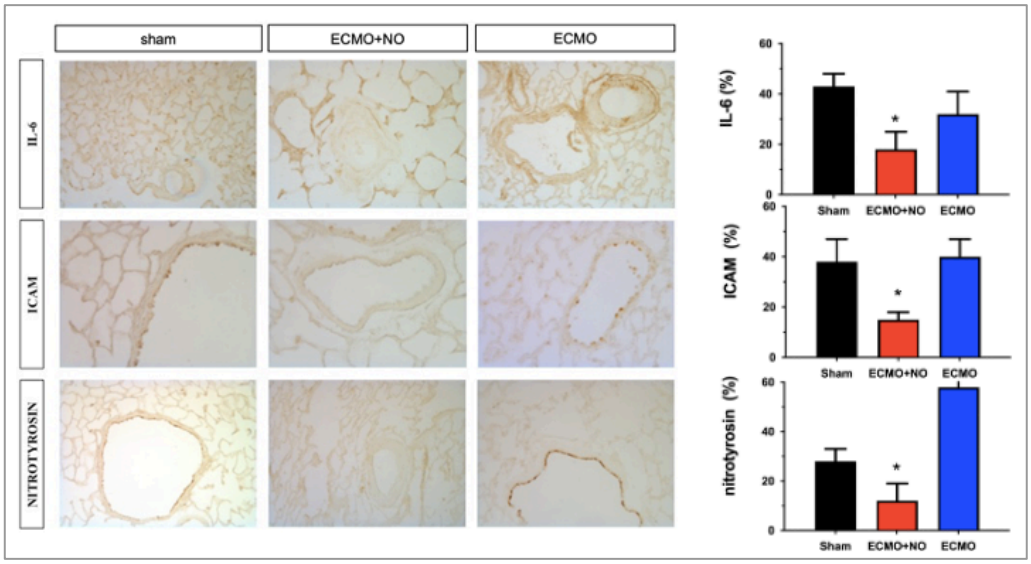
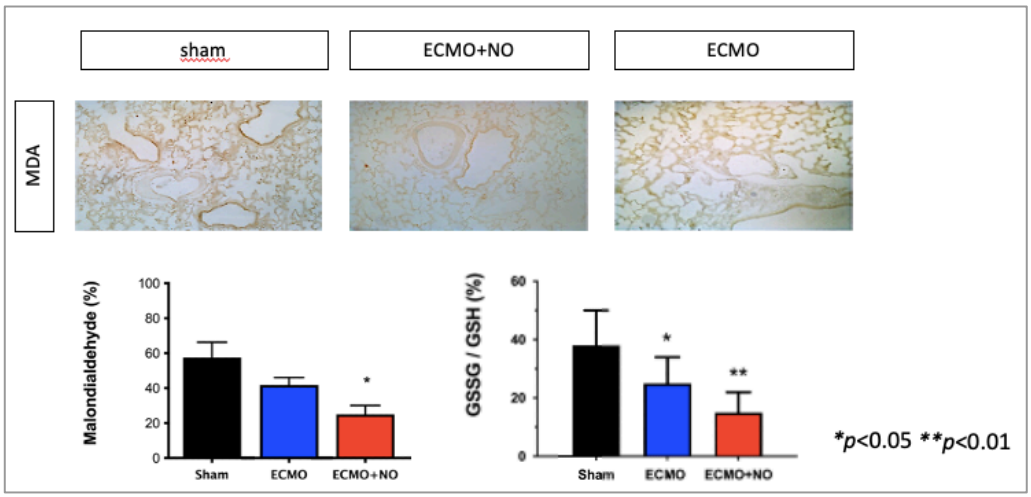


Figure nr 22: IL-6, ICAM1 and Nytrotirosin are less expressed in the ECMO+NO treated group

Moreover, the analysis of the oxidative stress markers in the lung tissue (malondialdehyde and of the ration between the oxidated and reducted glutation forms) showed a decreased oxidative stress in ECMO VV plus NO group compared to the other groups. See graphic nr 8.



Graphic nr 8 : oxidative stress markers are less expressed in the ECMO+NO treated group

It was possible to identify nitric oxide derivates and metabolites through blood sample. Analysis was conducted with specific nitrites kit, and we were able to identify and certify presence nitrites in the venous blood reflow.

EXPERIMENTAL MODEL NR 3 CARDIAC ARREST

BLOOD GAS ANALYSIS (EGA)

Hemogasanalysis were obtained in three phases of the experiment in the control group and in the treated group with ECMO + NO.

1. After cardiac arrest induction (EGA 1)
2. After 30 minutes of ECMO (EGA 2)
3. At the end of the ECMO assistance (EGA 3)

Results are shown in table nr 7

A hypoxic condition is observed in both groups with hypercapnia as attended with a reduction during the ECMO to normal values. The hyperglycemic state is attended for the condition of metabolic stress. The pO2 remains elevate due to the high concentration of oxygen administrated inside the oxygenator. The reduction of lactate value at the end of the experiment, is a sign of adequate perfusion.

	CONTROL GROUP			ECMO+NO GROUP		
	EGA 1	EGA 2	EGA 3	EGA 1	EGA 2	EGA 3
pH	7,04	7,28	7,3	7,26	7,38	7,33
pCO_2 (mmHg)	101,4	31,5	39,7	95	24,5	36
pO_2 (mmHg)	12,1	448,4	259,8	11,6	432	238,7
Glu (mg/dL)	244,7	454,9	442,7	325,17	453,17	431,7
Lat (mmol/L)	9,07	10,58	7,23	7,81	9,63	6,45
Hct (%)	46,1	27,6	29,5	44	29,7	30,7

Table nr 7: blood gas analysis

Hemodinamic data

Hemodynamic monitoring shows an improvement in systolic function in the group treated with NO. Stroke volume (SV), cardiac output (CO), cardiac index (CI) and dP/dtmax (systolic contractility index) measured in the NO group did not deviate much from the baseline conditions. On the contrary, in the control group there is a significant reduction of the same compared to the baseline conditions.

Administration of NO into the ECLS oxygenator also results in improvement in diastolic function as indicated by end-diastolic blood pressure (LVEDP) and diastolic release (dP/dt min). In particular, the LVEDP normalized to a greater extent after conventional extracorporeal circulation, while it appears to be similar to the baseline conditions after assistance with NO administration.

The reduction in diastolic blood pressure per unit of time (dP/dtmin) is lower after conventional ECLS, indicating impaired diastolic function. A reduction of arterial resistance (TPRI) was also demonstrated in the group treated with NO.

The reduction of the afterload together with the improvement of the systolic function explains the better hemodynamic profile, determined by a favorable ventricular-arterial coupling (ventricular-arterial coupling) in the treated subjects, which demonstrates a hemodynamic picture not dissimilar from the basal conditions. These findings agree with what was found in James's study which highlighted a reduction in low cardiac output syndrome in children treated with NO during cardiac surgery (87).

The recording of hemodynamic preload independent parameter are obtained during inferior cava occlusion. Results are shown in table nr 8.

	BASAL CONDITION	ECLS	ECLS + NO
HR (bpm)	334 ± 13	362 ± 18	351 ± 15
MAP (mmHg)	95 ± 11	92 ± 15	76 ± 13
LVESP (mmHg)	89 ± 9	75 ± 12	86 ± 10
LVEDP (mmHg)	8.1 ± 0.5	14.1 ± 0.7	9.5 ± 0.4*
SV (µl)	60 ± 5	26 ± 9	55 ± 8*
CO (ml/min)	42 ± 4	20 ± 8	38 ± 9*
CI (ml/min/100g bw)	9.01 ± 1.65	4.70 ± 0.81	9.05 ± 1.41*
dP/dt_{max} (mmHg/s)	7315 ± 120	5124 ± 205	6870 ± 290*
dP/dt_{min} (mmHg/s)	7520 ± 125	5284 ± 185	7420 ± 210*
TPRI (mmHg/ml/min/100g bw)	2.81 ± 0.23	2.95 ± 0.42	2.14 ± 0.35 *

Table nr 8. hemodynamic parameter measured with Millar's catheter: CI, cardiac index; CO, cardiac output; dP/dtmax and dP/dtmin maximal slope of the systolic pressure increment and the diastolic pressure decrement, respectively; HR, heart rate; LVESP, LV endsistolic pressure; LVEDP, LV end diastolic pressure.

Immunohistochemistry

To evaluate whether the administration of NO into the oxygenator during ECLS may reduce the inflammatory degree secondary to ischemia-reperfusion injury, an immunofluorescence and fluorescence microscope quantification analysis was performed. The activated microglia-specific marker Ionized Calcium Binding Adapter Molecule 1 (Iba1) was used for this analysis. Microglia are macrophages specialized in nervous-type tissue, determinants of the first and main line of immune defense of the central nervous system. The positivity to this marker therefore indicates the presence of an inflammatory activation of this cell, furthermore the dose of this marker indicates the degree of tissue inflammation.

For the quantification of Iba1, 3 samples were considered for the control group and 3 samples for the group treated with NO.

In particular, two slides per sample containing 4 slices each were analyzed, considering 4 different areas for each slice of the brain, figure nr 23.

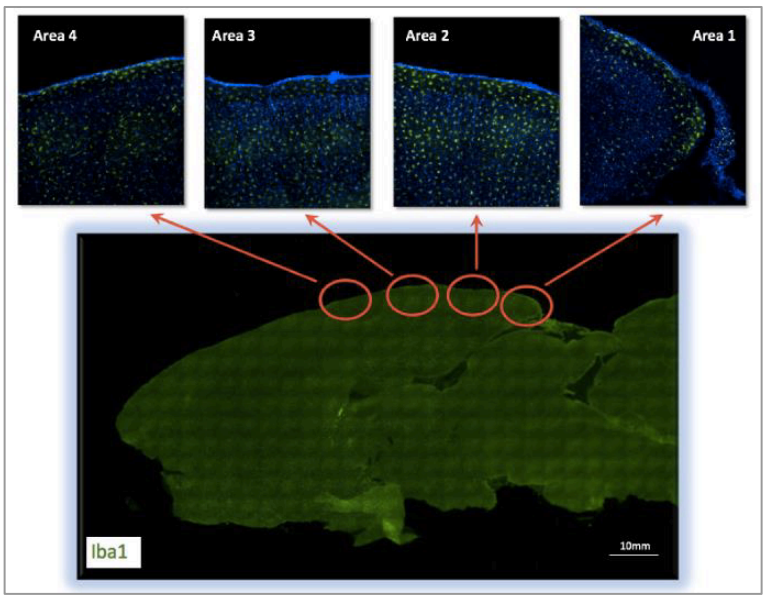


Figure nr 23: Representation of Area 1,2,3,4 in correspondence with the cerebral cortex, analyzed for the quantification of Iba1

For every image obtained, a region of interests (ROI) is delimitated, and the area is calculated. The number of cell nuclei labeled with TOPRO-3, which highlight a blue fluorescent color, is then quantified. Subsequently, the percentage of anti-Iba1 marker is determined, which highlights a green fluorescent color, present individually within the ROI, see figure nr 24. We then proceed to carry out a colocalization between nuclei and Iba1, i.e., the proportion of overlapping nuclei and Iba1 is determined, obtaining a percentage.

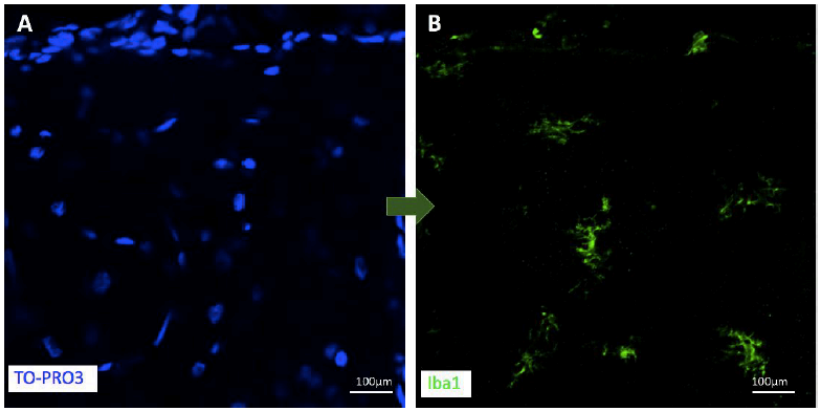


Figure nr 24 (A) quantification of Nuclei marked with nuclear marker TO-PRO3; (B) quantification of Iba1

The numerical values given by these procedures in the samples of the two groups are then processed obtaining a graph, which represents the numerical difference between the two groups with a mathematical representation. Subjects treated with ECLS extracorporeal circulation and Nitric Oxide had a lower concentration of the Iba1 marker than subjects treated with only conventional extracorporeal circulation.

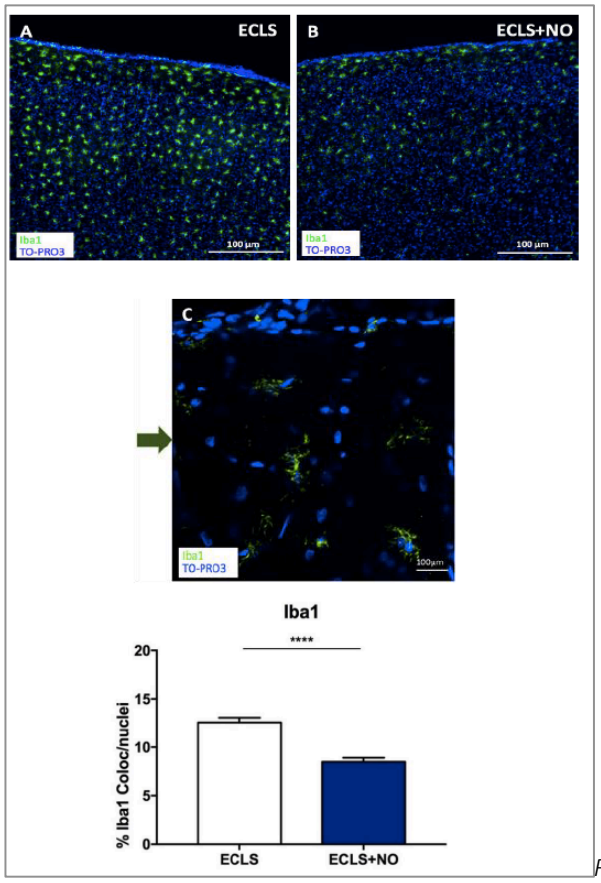
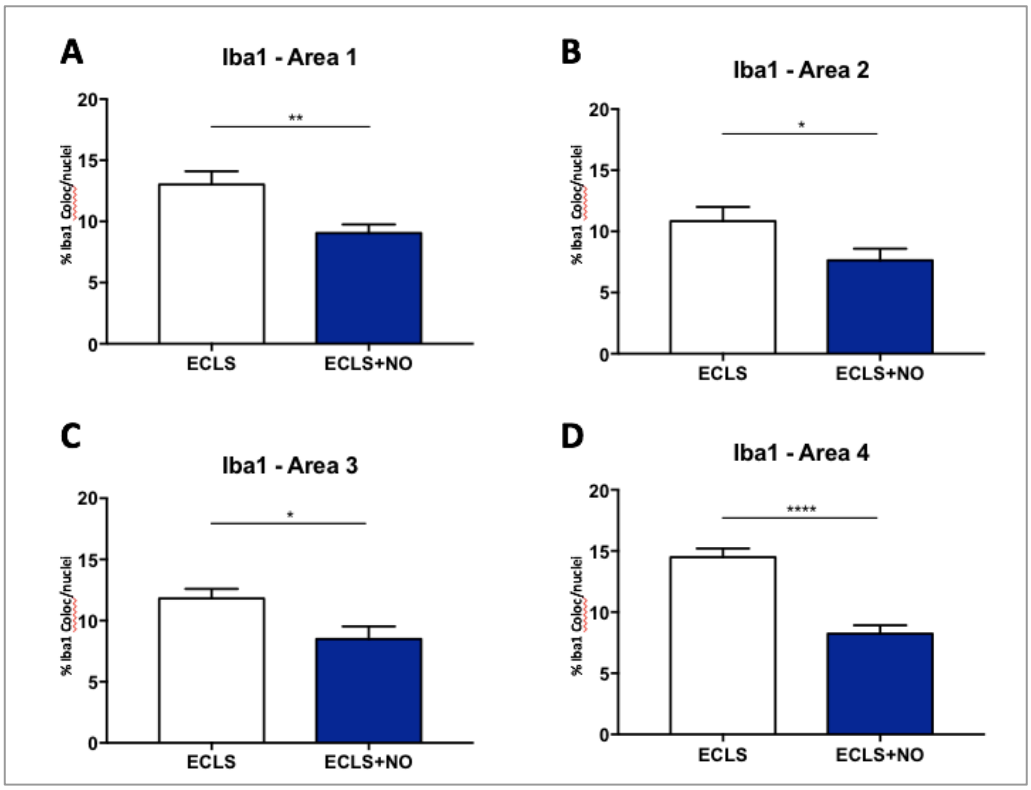


Figure nr 24

In figure nr 24 are illustrated (A-B) Areas of sagittal brain slices immunostained with the specific neuronal marker Iba1 (green) and TO-PRO3TM (blue) in control group (A) and ECLS +NO-treated group (B). (C) Representative graph of the percentage of Iba1 positive cells in the different brain areas of the control group - ECLS (white) and treated group - ECLS+NO (blue). As shown in the figure, the percentage of Iba1 positive colocalized to nuclei is higher in rats treated with conventional ECLS (12.58% ± 0.48%, n = 3) than in rats treated with ECLS with administration of NO (8.37% ± 0.41%, n = 3), and the difference was statistically significant. The differences between the experimental conditions were analyzed with the Unpaired t-test. Data are shown as mean ± SEM; 4 areas in 8 slices for 3 animals were analyzed for each group. **** $p < 0.0001$. Stairs: 100 μm.

Now a graphic for each single area is derived, highlighting the differences between the two groups, as shown in figure. Representative graph of the percentage of Iba1 positive cells in the different brain areas of the control group - ECLS (white) and treated group - ECLS+NO (blue). We note a relevant difference between the two groups in each of the 4 areas. (A) The percentage Iba1 positive colocalized to nuclei, per total section area, is higher in rats treated with conventional ECLS (13.02% ± 1.09%, n = 3) than in rats treated with ECLS with NO (9.05% ± 0.71%, n = 3), ** $p<0.0034$. (B) The percentage Iba1 positive colocalized to nuclei, per total section area, is higher in rats treated with conventional ECLS (10.85% ± 1.17%, n = 3) than in rats treated with ECLS with NO (7.65% ± 0.95%, n = 3), *$p<0.041$. (C) The percentage Iba1 positive colocalized to the nuclei, for total section area, is higher in rats treated with conventional ECLS (11.8% ± 0.78%, n = 3) than in rats treated with ECLS with NO (8.50% ± 1.02%, n = 3), * $p<0.013$. (D) The percentage Iba1 positive colocalized to nuclei, per total section area, is higher in rats treated with conventional ECLS (14.49% ± 0.71%, n = 3) than in rats treated with ECLS with NO (8.25% ± 0.68%, n = 3), **** $p<0.0001$. See graphics nr 9.



Graphics nr 9: differences of Iba-1 percentage in different brain areas in the ECLS model and ECLS+NO model.

The results demonstrate a higher Iba1 positive response in subjects treated with conventional ECLS when compared with the group treated with ECLS and nitric oxide. The difference resulted statistically significative, highlighting the neuroprotective effect of the nitric oxide.

Furthermore, to better evaluate if the administration of nitric oxide during ECLS may reduce the hypoxic state after ischemia-reperfusion injury, other specific markers were object of studies. For this analysis we used the specific marker for the oxidation of Thiols, 7-diethylamino-3-(4-maleimidylfen)-4-methyl-coumarin (CPM). Quantification of thiols takes place in relation to reactive oxygen species (ROS). The natural production of an antioxidant, glutathione (GSH), in cells acts as a defense mechanism to contrast the adverse effects of cellular oxidative stress. Consequently, thiol quantification can be used to detect thiol groups in glutathione and monitor their rate of production in cells as a response to elevated levels of oxidative stress from cellular activities. For the quantification of oxidized thiols, 3 samples were considered for the control group and 3 samples for the group treated with ECLS and nitric oxide (NO).

One slide per sample containing 4 slices each was analyzed, considering the entire area of the cerebral cortex, identifying the presence of thiols by the blue fluorescent staining determined by the CPM fluorescent probe, as shown in figure nr 25.

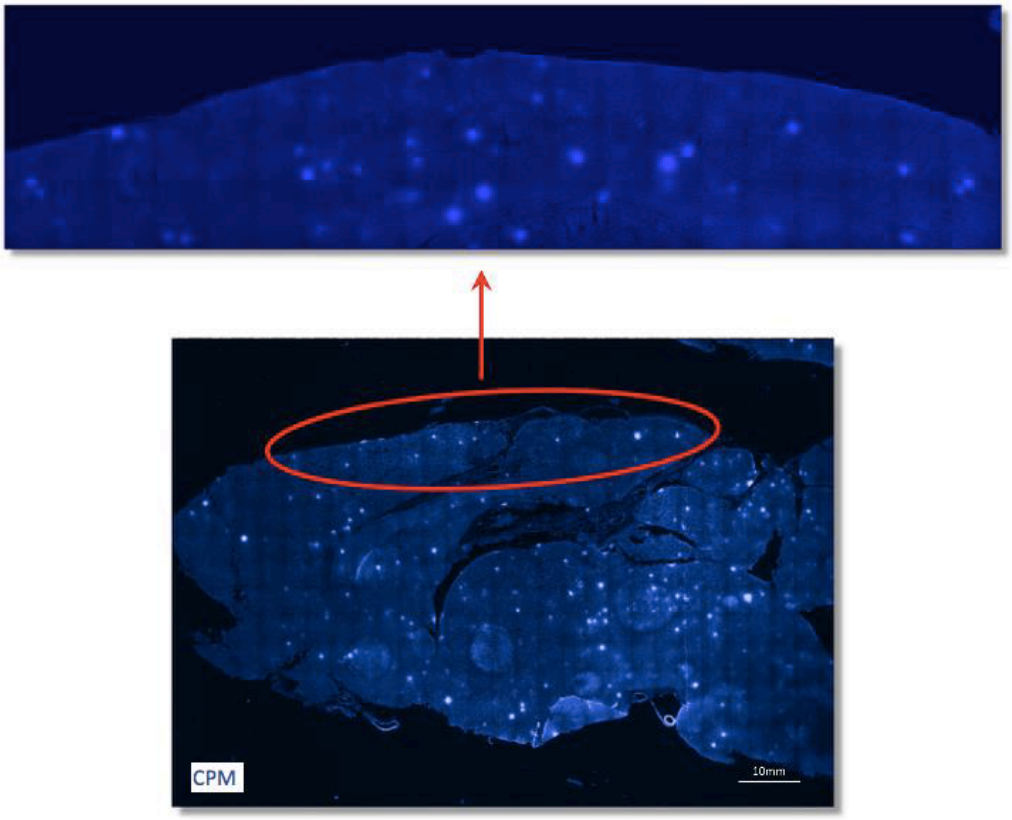


Figure nr 25: area of cerebral cortex analyzed for thiol quantification

For every image obtained, a region of interests (ROI) is delimitated, and the area is calculated. Inside the ROI, the percentage of the average gray level in each area is evaluated. The mean values for each sample are then compared, resulting in a graphical representation

The results, illustrated in figure nr 26, demonstrates that subjects treated with ECLS and Nitric Oxide have a lower concentration of thiol oxidation than subjects in the control group. (A-B) Areas of the cerebral cortex immunostained with the CPM fluorescent probe in control (A) and ECLS and NO-treated rats (B). (A1-B1) Quantification of hypoxia using a color scale, representative of the maximum intensity by red (= maximum degree of hypoxia), passing through green to the minimum intensity by blue (=absence of hypoxia). (C) Representative graph of the percentage of oxidized Thiols positive in the brain areas of the control group - ECLS (white) and treated group - ECLS+NO (blue), see

graphic nr 10. As shown in the figure, the percentage of oxidative state, per total section area, is higher in rats treated with conventional ECLS (11.72% ± 1.03%, n = 3) than in rats treated with ECLS and NO (4.07% ± 0.45%, n = 3), and the difference results statistically significant. The differences between the experimental conditions were analyzed with the Unpaired t-test. Data are shown as mean ± SEM; 4 slices for 3 animals were analyzed for each group. **** p<0.0001. Stairs: 100m. CPM= 7-diethylamino-3-(4-maleimidylphen)-4-methyl-coumarin. ECLS = Extracorporeal Life Support. NO = Nitric Oxide.

The results demonstrate a higher positive response to oxidative stress in subjects treated with conventional ECLS when compared with the group treated with ECLS and nitric oxide. The difference resulted statistically significative, highlighting again the neuroprotective effect after ischemia-reperfusion injury of the nitric oxide.

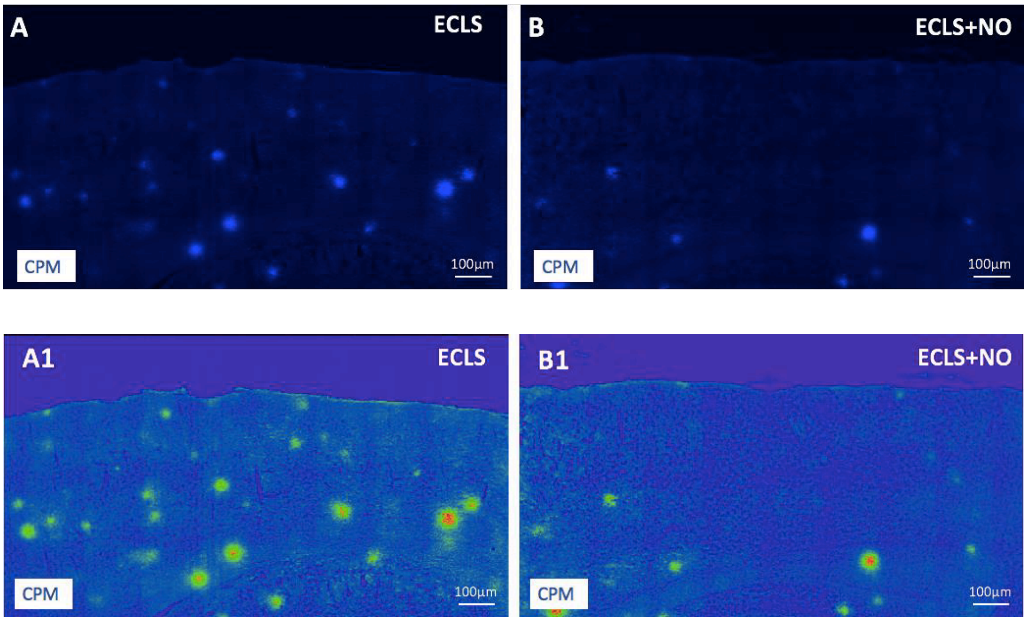
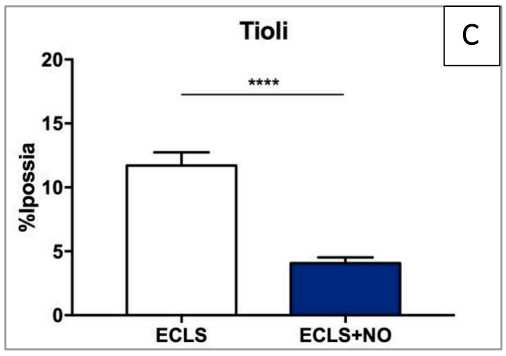


Figure nr 26. A-B areas shows concentration of thiol oxidation (A) ECLS group, (B) ECLS+NO group; A1-B1 : color scale quantification of hypoxia, RED maximum hypoxia, BLUE absence of hypoxia.



Graphic nr 10: percentage of oxidized thiol in ECLS group (white) and ECLS+NO (blue). The percentage results higher in the conventional ECLS group.

DISCUSSION

ECLS/ECMO is a mechanical assistance therapy supporting heart and/or pulmonary recovery in an acute organ disfunction, reversible and refractory to maximized medical therapy. In the last decades its applications are increased in different scenarios of life threatening such as cardiac arrest, end stage pulmonary hypertension or severe hypoxemia refractory to mechanical ventilation.

In severe respiratory insufficiency or PAH and end stage pulmonary function, VV ECMO has more often been used to consent a protective ventilation mode and acquire time for medical decision. A major cause of severe hypoxia is alveolar damage as consequence of the endothelial dysfunction in acute respiratory distress syndrome (ARDS). A strong inflammatory response is triggered, and endothelial damage increases proinflammatory cytokine such as IL-6 and TNF which leads to leukocytes recruitment and the releasing of lytic enzymes becomes responsible for cellular membrane damage. The migration of inflammatory cytokines, such as TNFα, IL-8, IL-6, IL-1β, in the systemic circulation may induce a systemic inflammatory response leading to a multi organ failure and increase mortality (88). The inflammatory insult is accompanied by an abnormal activation of the coagulation cascade, with hyper-activation of pro-coagulant

phenomena and depression of fibrinolysis in a sepsis-like scenario. The formation of vascular thrombi can induce alteration of the ventilation perfusion ratio (V/Q), as well as an increase in pulmonary pressure with overload of the ventricle right. In the acute phase of ARDS, the pulmonary oedema is secondary to endothelial disfunction with an alteration of the alveolar function, surfactant production, and so increasing the pulmonary shunt. As consequence, a pulmonary vasoconstriction, atelectasis, and a reduction of pulmonary distensibility are manifested. Therefore, nitric oxide (NO) has an important role, as demonstrated, to inhibit the expression of adhesion molecules and the infiltration of vascular immune cells, leading to a reduction in the expression of pro-inflammatory cytokines at the site of injury (26).

Still ARDS has high mortality and no specific treatment. As recent epidemic demonstrates, treatment for severe ARDS (as similar in COVID-19) is still an ongoing challenge. As maximum mechanical therapy appliable, ECMO in ARDS has still high mortality and its efficacy remains controversial (EOLIA trial).

Inhaled nitric oxide (NO) has demonstrated to selectively ameliorate PAH without impacting systemic blood pressure, since it has a very short eliminating half-life time, it can quickly diffuse into the blood flow and lose activity by binding hemoglobin. Therefore, inhaled nitric oxide (NO) has been the milestone in the field of treatment for PAH in congenital heart diseases since ‘92. Also, VV ECMO has been used as a bridge to lung transplantation for end stage PAH (89).

Cardiac arrest (CA) consists in the immediate and sudden cessation of the mechanisms of cardiac mechanical activity, which leads to the total cessation of blood flow. It remains one of the leading causes of death in industrialized countries, accounting for over 50% of all fatal cardiovascular events. Despite modern technologies and treatment such as ECMO, the mortality rate after hospital discharge remains high (40% of AC cases arrive alive in hospital after conventional resuscitation maneuvers, but only 12% of these survive one month after the ischemic event), with a major cause as post cardiac arrest syndrome. This process is a consequence of ischemical lesion during RCP and instauration of ischemia-reperfusion injury. There is an increased of cytokines such as interleukin and TNF. Their expression is a signal for leukocytes recruitment which liberates lytic enzymes causing a cell membrane damage. During reperfusion with ECMO or after ROSC, because of proinflammatory molecules release, a systemic

inflammatory response syndrome may establish. No reflow phenomena aggravates myocardial disfunction ad cerebral damage already present. Reperfusion exacerbates the oxidative stress through DNA damage and lipidic peroxidation as shown before. The reduction of oxidative stress, hypoxic injury and mediators of inflammatory response are potential therapeutic targets. Consequently, agents capable of modulating most of these abnormal processes may constitute a promising therapeutical solution.

Giving these promises, our goal was to evaluate the effect of administration of NO directly into the oxygenator of ECLS/ECMO by the analysis of several parameters such as hemodynamic parameters, recirculation fraction and oxygen delivery, inflammation, and oxidative stress in a preclinical rat model of ARDS, PAH and cardiac arrest. The subjects were grouped and subjected to different protocols, in order to evaluate the real effectiveness of nitric oxide as a protective mediator.

The ECLS/ECMO is already used as “bridge to therapy” in case of a cardiac arrest refractory to conventional therapy, also nitric oxide is used via inhalation in intubated patient as a treatment for pulmonary hypertension with right ventricular disfunction ad severe hypoxia.

For the VV ECMO in the ARDS model the histological analysis with hematoxylin-eosin stain demonstrated a reduction of the alveolar infiltration in the group treated with nitric oxide when compared with the conventional group. The immunohistochemical results revealed an increased alveolar and bronchiolar expression of antibodies anti IL-6, TNFα and CCL5 in the group that received only conventional VV ECMO, which resulted significantly reduced in the treated group with VV ECMO and nitric oxide. it was possible to visualize a better preservation of the epithelium in the group treated with nitric oxide. Indeed, in the conventional ECMO group, the CCL5 marker revealed a damaged bronchial epithelium and a functional constituent cellular lose, thus nitric oxide may modulate the epithelium preservation by reducing the inflammatory response. Further confirmation of the protective role of nitric oxide was the attenuation of the oxidative stress. The evidence of positivity of anti-Malondialdehyde antibody in the control group compared to the group treated with NO, in which the expression of this marker is negative, highlights a notable reduction of oxidative stress in the nitric oxide treated group. The hemodynamic aspect in ARDS is discussed in a study conducted in the 2017 (90) where the authors clarify the role of ARDS and an increased

right ventricle afterload and consequently the ventricular-vascular coupling is altered. Moreover, the right ventricle distension by overload, contributes to alterate the ventricular interdependence with hemodynamic instability.

In our model, hemodynamic analysis revealed an improvement of independent parameter such as Stroke work and preload recruitable stroke work. Also, right ventricle contractility was improved in the treated group with VV ECMO and nitric oxide (NO) group; end systolic pressure (ESP) and Pulmonary artery elastance were reduced in the VV ECMO and nitric oxide (NO) group; revealed in diminished right ventricle afterload with a preserved right ventricle – arterial coupling, the recirculation fraction was maintained in standard values in the VV ECMO and nitric oxide (NO), and the oxygen delivery (P/F ratio) was improved.

In the cardiac arrest model, brain samples were analyzed through immunofluorescence, quantifying inflammatory markers such as Iba-1 and markers for thiol reduction, useful to evaluate the ischemic condition. The immunohistochemical analysis reveals that the administration of nitric oxide into the oxygenator reduces the cerebral cortex inflammation and ischemia with a reduction of the microglia cell activation, when compared to the conventional ECMO group. Also, further confirmation is obtained with analysis of thiol for oxidative stress. The evidence of a greater number of Thiols in the control group compared to the group treated with nitric oxide shows a significant reduction in oxidative stress in the group subjected to nitric oxide. Hemodynamic analysis demonstrates an improvement of systolic and diastolic function in the group treated with nitric oxide when compared with the conventional group. The reduction of the right ventricle afterload associated with the improvement of the systolic function explains the better hemodynamic profile, determined by a preserved ventricular-arterial coupling in the nitric oxide treated subjects, which demonstrates a hemodynamic picture not dissimilar from the baseline conditions. These findings are in line with James's study (87) which highlighted a reduction in low cardiac output syndrome in children treated with NO during cardiac surgery.

It is important to note that the samples analyzed underwent a relatively short period of ischemia (10 minutes of induced cardiac arrest) and the time necessary for inflammation to generate (60 minutes of extracorporeal circulation) was minimal as the animal is immediately sacrificed for sample analysis. The inflammatory process requires

a period ranging from a few hours to days to develop completely. A 24-hour experimental model would allow greater sensitivity to immunohistochemical markers and therefore the possibility to obtain a more significant evaluation of systemic inflammation and neuroprotection.

The ECMO remains an essential treatment to support cardiac and respiratory function until recovery in critical patient (such as ARDS or PAH end stage), but it may aggravate systemic inflammatory response itself, because of the blood contact with external structure such as ECMO circuit. This inflammatory response may further aggravate the myocardial disfunction and manifest a low cardiac output syndrome, as demonstrated in different studies *(87, 91).*

In the pulmonary arterial hypertension (PAH) model, was also possible to demonstrate in the group treated with ECMO+NO a reduction of RV afterload through selective pulmonary vasodilatation; preserved PA endothelium-dependent relaxation; improvement of RV systolic and diastolic functions; preserved RV-arterial coupling; LV preload restored by the mechanism of ventricular interdependence; improvement of RV energetic metabolism and a reduction of lungs inflammation and oxidative stress.

It was possible through blood sample at the end of the experiment, and specific laboratory nitrites kit, to demonstrate the presence of nitric oxide metabolites in the venous blood. This confirms the correct administration and presence of nitric oxide during extracorporeal circulation.

Therefore, the protective role played by nitric oxide may reduce the systemic inflammation that generates after weaning from ECMO. If we consider that nitric oxide is a treatment disponible and used in ICU by inhaled through the ventilator, we propose a clinical commitment of nitric oxide through the oxygenator of the ECMO. The present preclinical study suggests clinical randomized studies to confirm the present findings and to correlate the benefits of nitric oxide during ECMO.

Further analyses are needed to investigate a possible antimicrobic/antiviral effect of nitric oxide during VV ECMO (especially ARDS).

A future objective is the survivor of the subjects after ECLS/ECMO assistance after 24 h, to proceed with additional analysis such as magnetic resonance and neurobehavior studies.

Based on our results, we can also hypothesize a future apply of nitric oxide for neuroprotection purpose during ECLS/ECMO after cardiac arrest or cardiac disfunction post cardiotomy.

CONCLUSION

The encouraging data obtained suggest an important role of Nitric Oxide (NO) when administrated into the oxygenator, regarding myocardial function, attenuation of inflammation and oxidative stress. Based on hemodynamic analysis we can deduce an improvement of systolic and diastolic function in the group treated with ECMO and nitric oxide. Immunohistochemical analysis shows a reduction of inflammatory degree and ischemic state in the group treated with nitric oxide in terms of microglia activation and thiol peroxidation. Oxidative stress is also reduced in all models in the nitric oxide treated group. Based on the results, a future application of nitric oxide for myocardial and neuroprotection purpose during ECLS/ECMO after cardiac arrest or cardiac disfunction post cardiotomy is to take in consideration. Further studies are needed to evaluate the survivor of the subjects for radiologic analysis (magnetic resonance) and neurobehavior after ECLS/ECMO.

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