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Preclinical Optimization of Treatment with Inhaled Argon to Improve Neurological Outcome and Survival After Cardiac Arrest

Thesis submitted by the student

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Discipline of Life and Biomolecular Sciences

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ABSTRACT

Introduction. Treatment of post cardiac arrest (CA) syndrome represents a clinical priority. Inhalation of the noble gas Argon may represent an attractive option. The purpose of the current thesis is to determine the efficacy, safety and mechanisms of action of Argon in different experimental models.

Methods. In order to assess the efficacy of argon treatment on post CA syndrome, a pig model of CA with underlying acute myocardial infarction and cardiopulmonary resuscitation (CPR) was used. Following resuscitation, animals were randomly assigned to receive ventilation with 70% Argon (Ar 70%) or 70% Nitrogen (N₂70%) in oxygen. Argon effects were studied in models of CA with different level of severity (i.e. short or long duration of untreated ventricular fibrillation). Furthermore, administration of low Argon concentration (i.e. 50%) was evaluated in a subset of animals. Hemodynamics, myocardial function, neurologic recovery, brain and cardiac histological injury and biomarkers together with survival were evaluated. The safety of Argon treatment was assessed in healthy pigs. In parallel, mechanisms of action were explored. Specifically, brain protection was investigated in a rat model of CA followed by CPR. After resuscitation, animals were randomized to receive Ar 70% or N₂70%. Brain glutamate, gamma-amino butyric acid, pyruvate and lactate were measured by *in vivo* micro-dialysis. In addition, myocardial protection by argon was explored in rats subjected to coronary artery occlusion followed by reperfusion and receiving Ar 70% or N₂ 70% during reperfusion. Six and 24 h after reperfusion, myocardial injury, plasma troponin T concentration and myocardial neutrophil infiltration were evaluated.

Results. Efficacy of argon on preventing post CA brain injury has been demonstrated in swine. Indeed, a faster and complete neurologic recovery following CA and CPR was achieved in Argon-treated animals compared to controls, without detrimental effects on hemodynamics and respiratory gas exchanges. Beneficial effects of Argon tended to be higher at a concentration of 70% than of 50%. Results obtained in small rodents suggest that ventilation with Argon reduces brain lactate/ pyruvate level and troponin T release. However, further studies are needed in order explain the mechanism underpinning Argon protective effects.

Conclusion. The consistency of the results is highly suggestive to consider ventilation with Argon as a promising therapeutic approach after CA and CPR.

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1. “Studi di Validazione degli Effetti Protettivi dell’Argon ed Ottimizzazione del Trattamento Inalatorio con Argon dopo Infarto Miocardico Acuto ed Arresto Cardiaco: Studio Preclinico sul Maiale”.
2. “Studi sui meccanismi di azione dell’Argon su modello arresto cardiaco e rianimazione sul ratto e microdialisi cerebrale”.

Conflict of Interest

The student and supervisors declare no conflicts of interest.

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Major abbreviations

ALT, serum alanine aminotransferase

ANOVA, analysis of variance

ATP, adenosine triphosphate

BBB, blood–brain barrier

CA, cardiac arrest

Ca^{2+} , calcium ion

CBF, cerebral blood fluid

CO, cardiac output

CPP, coronary perfusion pressure

CPR, cardiopulmonary resuscitation

DAP, diastolic arterial pressure

DIC, disseminated intravascular coagulation

ECG, electrocardiogram

EF, ejection fraction

EtCO₂, end-tidal CO₂

GABA, gamma-amino butyric acid

GLU, glutamate

Hb, hemoglobine

HPLC, high-performance liquid chromatography

HR, heart rate

h, hour

hs-cTnT, high sensitivity cardiac troponin T

HT, hematocrit

IL, interleukin

I_{NaL}, late Na⁺ current

LAD, left anterior descending

LV, left ventricle

MAP, mean arterial pressure

Na⁺, sodium ion

NDS, neurological deficit score

NMDA, N-Methyl-D-Aspartate

NO, nitric oxide

NSA, neurological alertness score

NSE, neuron specific enolase

OPC, overall performance category

RAP, right atrium pressure

RMS, root mean square

ROC, receiver operator characteristic

ROS, reactive oxygen species

ROSC, return of spontaneous circulation

SAP, systolic blood pressure

TH, therapeutic hypothermia

TNF α , tumor necrosis factor alpha

TTC, tetrazolium chloride

TTM, targeted temperature management

VF, ventricular fibrillation

Introduction

1 Cardiac arrest: a general overview.

Cardiac arrest (CA) refers to the sudden cessation of cardiac activity with hemodynamic collapse, occurring usually with no premonitory symptoms. Victims of CA become unconscious within seconds to minutes because of insufficient cerebral blood flow. CA usually causes death if it is not treated within minutes.

CA is often due to a cardiac arrhythmia and ventricular fibrillation (VF) appear to be responsible for 22.2% of patients (*Gräsner 2016*); however, the incidence of VF is probably higher. This could be explained by the fact that the exact mechanism of collapse is often difficult to establish because cardiac activity is not being monitored at the time of collapse, and asystole is often the first rhythm observed. However, asystole correlates with the duration of the arrest and may be the result of VF that has been present for several minutes and then leads to the loss of all electrical activity because of hypoxia, acidosis, and death of myocardial tissue (*Tovar 2000*).

The most common cause of CA is coronary heart disease, occurring in 71% of the patients, of whom 48% had an occluded coronary artery (*Deo 2012, Spaulding 1997*). Among the main cardiac causes of CA, there are other types of structural heart disease (e.g., congenital coronary artery anomalies, myocarditis, hypertrophic cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy) and arrhythmias occurring in the absence of structural heart disease (Long QT syndrome, Brugada syndrome). Fifteen to 25 percent of CA are non-cardiac in origin. The causes include trauma, bleeding, drug intoxication, intracranial hemorrhage, pulmonary embolism, drowning and central airway obstruction (*Eckart 2004*). All the causes of CA are summarized in table 1.

Coronary artery disease	Ischemia secondary to arterosclerotic heart disease Anomalous coronary Coronary vasospasm
Cardiomyopathies	Ischemic cardiomyopathy Nonischemic/idiopathic dilated cardiomyopathy Hypertrophic cardiomyopathy Takotsubo cardiomyopathy Infiltrative sarcoid heart disease Infiltrative amyloid heart disease Arrhythmogenic right ventricular dysplasia/ cardiomyopathy Left ventricular noncompaction Myocarditis Valvular heart disease Congenital heart disease
Electrophysiological	Long QT syndrome Short QT syndrome Brugada syndrome Catecholaminergic polymorphic ventricular tachycardia Idiopathic ventricular fibrillation Ventricular pre-excitation
Metabolic	Hyper/hypokalemia Hypomagnesemia Hypocalcemia Severe acidosis
Noncardiac	Intracranial hemorrhage Pulmonary embolus Epileptic seizure

Table 1. Causes of CA. From *Yousuf 2015*.

The cornerstones of survival from CA are early recognition and treatment, specifically, immediate initiation of good quality cardiopulmonary resuscitation (CPR), including chest compression and ventilation, and early defibrillation (DF). Chest compressions with minimized interruptions are the most important element of CPR, maintaining adequate coronary perfusion pressure (CPP) while the DF is performed. CPP is the aortic- right atrium gradient during the relaxation phase of cardiac cycle and it represents a predictor of ROSC (*Paradis 1990*). DF in patients with ventricular fibrillation (VF) represents the single most important intervention to reset cardiac rhythm and restore spontaneous circulation (*Perkins 2015*).

Despite the development of CPR, electrical DF and the implementation of resuscitative techniques over the last 50 years, the overall percentage of return of spontaneous circulation (ROSC) is low, with 28.6% of successful resuscitated victims with extreme variability between different European countries (from 10 to 50% according to the differences in EMS structures and CPR practices in different countries). For this reason, CA is still considered one of the major causes of death in Europe, with an incidence rate of 84 per 100.000 population (*Gräsner 2016*). Therefore, improvement of CA treatment is a clinical priority. This represents the overall purpose of this thesis work.

2 Post-CA syndrome.

Although successful ROSC is the first step of cardiac resuscitation, most of resuscitated patients die within 72 h. Indeed, only 33% of the patients admitted to hospital survived to 30 days or to hospital discharge, with a variability from less than 5.0% to 30.0% due to differences in emergency system structures and CPR practices patients (*Gräsner 2016*). This is due to the complex pathophysiological processes that occur following whole-body ischemia during CA and the subsequent reperfusion response during CPR. This condition has been termed the post CA syndrome. The reflow after the whole-body ischemia might in fact activate detrimental pathways during reperfusion, causing tissue injury, particularly to the brain (*Neumar 2008*). Investigating the effect of a specific treatment option for post-CA syndrome represents the focus of the experimental studies described in this thesis work.

In the early 1970s, Dr. Vladimir Negovsky firstly described the pathology caused by complete, whole-body ischemia and reperfusion as “a second, more complex phase of resuscitation beginning when patients regain spontaneous circulation after CA”. Negovsky named this state “post resuscitation disease” (*Negovsky 1972 and 1988 and 1995*). However, the term “resuscitation” is now used broadly to include treatment of various shock states in addition to CA, in which circulation has not ceased. So, in 2008 Neumar and Nolan proposed the modern term “post CA syndrome”, that sounds more appropriate to identify patients who regain spontaneous circulation after CA (*Neumar 2008*).

The 3 key components of this syndrome are: 1) post CA brain injury; 2) post CA myocardial dysfunction; 3) systemic ischemia/reperfusion response. This state is often complicated by a fourth component: the unresolved pathological process that caused the CA (*Neumar 2008, Nolan 2008*). Pathophysiology, clinical manifestations and potential treatments of the post CA syndrome are summarized in Table 2. Investigating the effect of Argon as a potential treatment option for post-CA brain injury represents the focus of the experimental studies described in this thesis work.

Syndrome	Pathophysiology	Clinical manifestation	Potential treatments
Post-cardiac arrest brain injury	<ul style="list-style-type: none"> • Impaired cerebrovascular autoregulation • Cerebral oedema (limited) • Postischaemic neurodegeneration 	<ul style="list-style-type: none"> • Coma • Seizures • Myoclonus • Cognitive dysfunction • Persistent vegetative state • Secondary Parkinsonism • Cortical stroke • Spinal stroke • Brain death 	<ul style="list-style-type: none"> • Therapeutic hypothermia • Early haemodynamic optimization • Airway protection and mechanical ventilation • Seizure control • Controlled reoxygenation (SaO₂ 94%-96%) • Supportive care
Post-cardiac arrest myocardial dysfunction	<ul style="list-style-type: none"> • Global hypokinesia (myocardial stunning) • Reduced cardiac output • ACS 	<ul style="list-style-type: none"> • Early revascularization of AMI • Hypotension • Dysrhythmias • Cardiovascular collapse 	<ul style="list-style-type: none"> • Early haemodynamic optimization • Intravenous fluid • Inotropes • IABP • LVAD • ECMO
Systemic ischaemia/reperfusion response	<ul style="list-style-type: none"> • Systemic inflammatory response syndrome • Impaired vasoregulation • Increased coagulation • Adrenal suppression • Impaired tissue oxygen delivery and utilisation • Impaired resistance to infection 	<ul style="list-style-type: none"> • Ongoing tissue hypoxia/ischaemia • Hypotension • Cardiovascular collapse • Pyrexia (fever) • Hyperglycaemia 	<ul style="list-style-type: none"> • Early haemodynamic optimization • Intravenous fluid • Vasopressors • High-volume haemofiltration • Temperature control
Persistent precipitating pathology	<ul style="list-style-type: none"> • Cardiovascular disease (AMI/ACS, cardiomyopathy) • Pulmonary disease (COPD, asthma) • CNS disease (CVA) • Thromboembolic disease (PE) • Toxicologic (overdose, poisoning) • Infection (sepsis, pneumonia) • Hypovolaemia (haemorrhage, dehydration) 	<ul style="list-style-type: none"> • Multiorgan failure • Infection • Specific to aetiology, but complicated by concomitant PCAS 	<ul style="list-style-type: none"> • Glucose control • Antibiotics for documented infection • Disease-specific interventions guided by patient condition concomitant PCAS

ACS indicates acute coronary syndrome; AMI, acute myocardial infarction; IABP, intra-aortic balloon pump; LVAD, left ventricular assist device; EMCO, extracorporeal membrane oxygenation; COPD, chronic obstructive pulmonary disease; CNS, central nervous system; CVA, cerebrovascular accident PE, pulmonary embolism; and PCAS, post-cardiac arrest syndrome.

Table 2. Post CA syndrome: pathophysiology, clinical manifestations, and potential treatments. From Nolan 2008.

Post CA syndrome would be described also by a time-based definition of 4 phases, with a more treatment-orientated approach (Figure 1). Indeed, 4 phases are recognized: 1) the *immediate* post-arrest phase, including the first 20 min after ROSC; 2) the *early* post-arrest phase, defined as the period between 20 min and 6-12 hr after ROSC, when early interventions might be most effective; 3) the *intermediate*, between 6-12 and 72 hr, when injury pathways are still active and aggressive treatment is typically instituted; and 4) the period beyond 3 days after ROSC, considered the *recovery* phase when prognostication becomes more reliable and ultimate outcomes are more predictable.

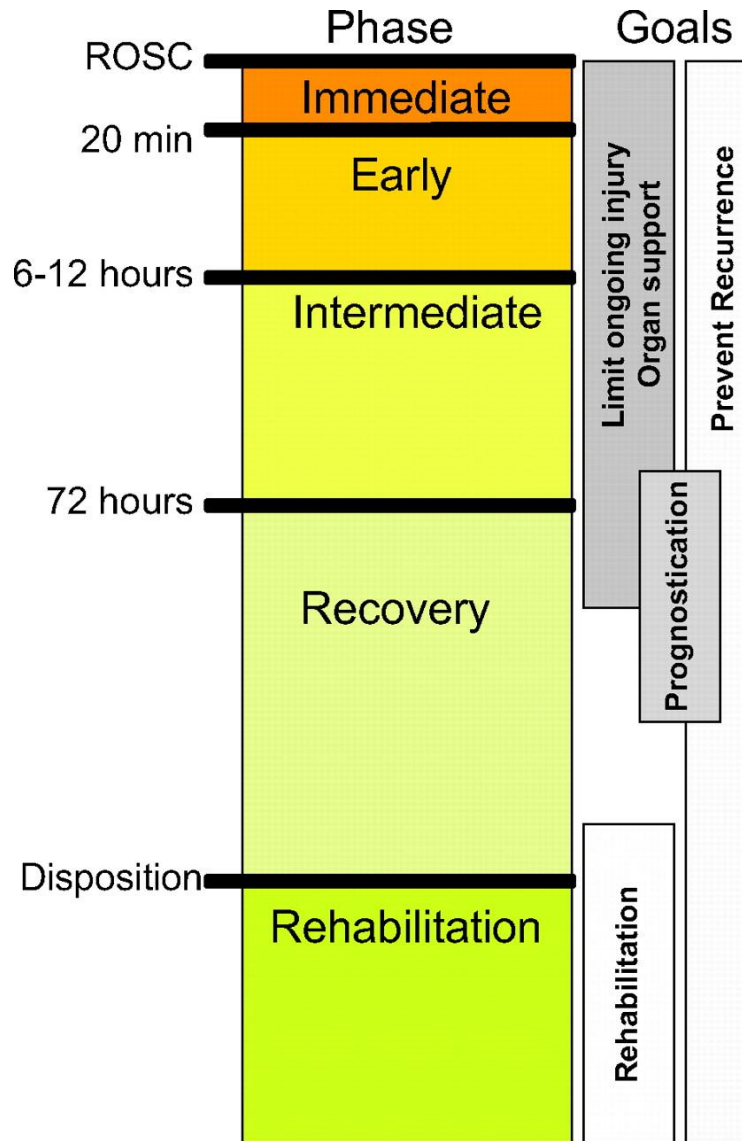


Figure 1. Temporal phases of the post-CA syndrome. From *Neumar 2008*.

Within the first 24 hr post-arrest, a microcirculatory dysfunction from the multifocal ischemia/reperfusion leads to rapid release of toxic enzymes and free radicals into the cerebrospinal fluid and blood. Over the next 1-3 days, cardiac and systemic functions improve, but intestinal permeability increases, predisposing the patient to sepsis and the multiple organ dysfunction syndrome. During the subsequent days, a serious infection may occur causing rapid clinical deterioration. The patient either dies of a complication or of the primary disease that caused the CA, or undergoes a partial or complete recovery.

The mechanisms responsible for post CA myocardial and cerebral injury are not well understood, although several events have been described.

At cellular level, the CA-induced whole-body ischemia causes hypoxia, conversion to anaerobic metabolism, intracellular acidosis, adenosine triphosphate (ATP) depletion, ion pump failure, intracellular calcium ions (Ca^{2+}) accumulation, and cellular and mitochondrial edema (*Chalkias A, 2012, Adrie 2004, Neumar 2008, Reis 2017*). At systemic level, release of catecholamines, tumor necrosis factor alpha ($\text{TNF}\alpha$), interleukin (IL) 1 β , complement and polymorphonuclear (PMN) leukocyte activation, endothelial damage with increased microvascular permeability, coagulation cascade activation, and PMN release of reactive oxygen species (ROS) and cytokines (*Chalkias A, 2012, Adrie 2004, Neumar 2008, Reis 2017, Lindner 1992*). CPR results in partial restoration of organ perfusion and triggering of reperfusion injury mechanisms. Global reperfusion injury is further amplified by the subsequent ROSC. At cellular level, reperfusion injury (*Madathil 2016, Yellon 2007*), causes enhanced ROS production, intracellular nitric oxide (NO) depletion, myocardial contracture and interstitial edema, excessive brain glutamate release, activation of intracellular proteases and phospholipases, cell membrane damage, impaired glucose utilization, opening of the mitochondrial permeability transition pore, and activation of apoptotic pathways. At systemic level, activated PMNs and platelets interacting with an injured and dysfunctional endothelium induce widespread microvascular plugging termed as the “no-reflow” phenomenon (*Chalkias 2012*). Activated PMNs further boost the cytokine storm and infiltrate damaged tissues causing cell death (*Adams 2006*). The ongoing endothelial injury further impairs microvascular permeability contributing to intravascular volume depletion, while the concurrent, immune cells’ inducible NO synthase-related overproduction of NO promotes peripheral vasodilation (*Adrie 2002*). The inflammatory cascade is amplified by nuclear factor kappa β activation. Furthermore, a potential ischemia/reperfusion injury-associated disruption of the intestinal mucosal barrier (*Chalkias 2016*) may cause acute increases of pathogen-associated molecular patterns such as endotoxins in the systemic circulation, with further amplification of the systemic

inflammation, and predisposition to multiple organ failure and “endogenous immunosuppression” (Kakihana 2016).

2.1 Post CA brain injury

Post CA brain injury manifests as coma, seizures, myoclonus, varying degrees of neurocognitive impairment and brain death. It is the responsible of death of the majority of patients after out-of-hospital CA (Lemiale 2013).

The unique vulnerability of the brain is attributed to its limited tolerance to ischemia as well as its unique response to reperfusion. The mechanisms of brain injury triggered by CA and resuscitation are complex and include excitotoxicity, disrupted Ca^{2+} homeostasis, free radical formation, pathological protease cascades, and activation of cell death signaling pathways (Lipton 1999, Neumar 2000 and 2008, Nolan 2008, Polderman 2009).

Levels of high-energy metabolites such as ATP and phosphocreatine decrease within seconds when oxygen supply to the brain is interrupted (Small 1999, Cavus 2006). Once the storage of ATP is completely consumed, the switch of intracellular metabolism to anaerobic glycolysis leads to an increase in intracellular levels of inorganic phosphate, lactate, and hydrogen ion, resulting in both intra- and extracellular acidosis (Figure 2).

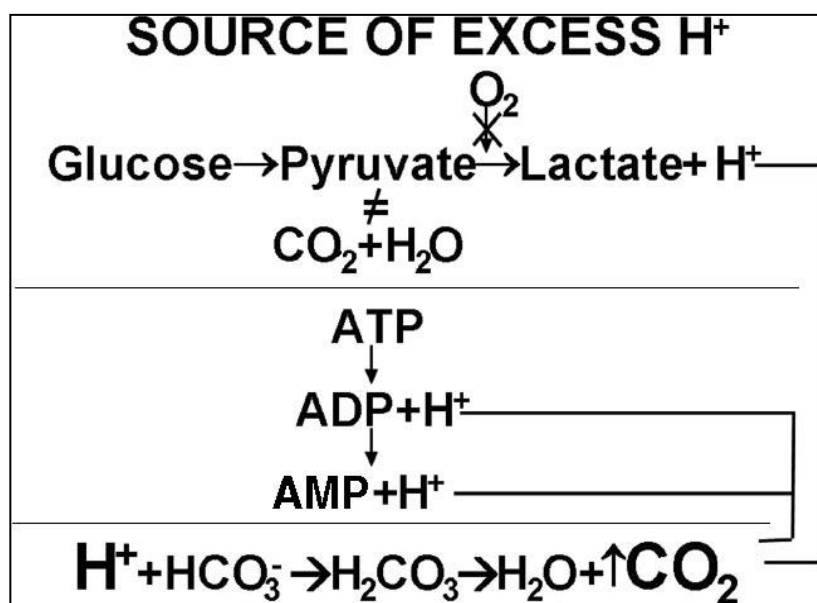


Figure 2. Source of hydrogen ions (H^+) during low-flow states. H^+ derived from the anaerobic oxidation of glucose and from the hydrolysis of high-energy phosphates. ATP, adenosine 5' triphosphate; ADP, adenosine 5' diphosphate; AMP, adenosine monophosphate. From Ristagno 2006.

Consequently, the blood-brain barrier (BBB) of different brain areas breaks down, allowing for serum proteins to enter the brain microfluid environment (*Sharma 2011*).

The passage of large molecules through the BBB enhances the passage of sodium ions (Na^+) and water from blood to brain compartment, altering the osmolality between them. These water and electrolyte changes following cell membrane damage and BBB breakdown result in cell swelling and brain edema formation. Brain edema contributes to the development of post CA intracranial hypertension, which further augments brain damage.

Ischemia/reperfusion can also lead to significant disruptions in the BBB, which can facilitate the subsequent development of brain edema (*Chi 2001, Huang 1999*). In fact, inflammatory cytokines and vascular endothelial growth factor (*Kaur 2008*), via release of NO (*Fischer 1999*) mediate decreased fluidity and integrity of cell membranes and increased vascular permeability of microvascular endothelial cells in the brain. More specifically, NO can interact with ROS producing peroxynitrite radicals, which activate matrix metalloproteinases (MMPs) that ultimately disrupt BBB junctions. Recently, perivascular pool of aquaporin-4 has been suggested as a key mechanism because this brain predominant water channel controls the rate limit of water influx during cerebral edema formation and it is the regulatory site of osmotic agents for brain water efflux (*Zeng 2012*).

The disruption of Ca^{2+} homeostasis due to a progressive increase in cell membrane permeability and to the derangements of intracellular electrolytes represents another important mechanism of brain damage. First, intracellular Ca^{2+} exerts cytotoxic effects leading to free radical production. Second, a massive cytosol Ca^{2+} accumulation is followed by the release of intracellular excitatory amino acids such as glutamate. When activated by glutamate, N-methyl-D-aspartate (NMDA) receptors activate Ca^{2+} ion channels and further increase Ca^{2+} conductance into the intracellular space (*Siesjo 1995, Polderman 2009*). In addition, the excess of in Ca^{2+} induces mitochondrial dysfunction, overwhelming the endogenous mitochondrial scavenging systems leading to mitochondrial

dysfunction and cell death. Indeed, mitochondrial Ca^{2+} overload causes the induction of inner mitochondrial membrane permeability transition, which then leads to disruption of mitochondrial membrane integrity, irreversible oxidative damage, and the loss of ATP production, finally resulting in cell death (*Polderman 2009*).

Post CA brain injury can also be followed by failure of cerebral microcirculation. During CA, in response to the stress of global ischemia, various cytokines are synthesized and released, resulting in activation of blood coagulation, platelets activation and decreased regional blood flow. Furthermore, with the onset of CPR, marked activation of blood coagulation occurs, which can lead to microthrombi, while the activated neutrophils and platelets accumulate in the microvasculature. This impaired reflow can cause persistent ischemia and small infarctions in some brain regions. The cerebral microvascular occlusion that causes no-reflow may further be compromised by the α 1- adrenergic agonist of endogenous or exogenous adrenaline, which reduces capillary blood flow (*Ristagno 2009*). Other phenomena may be involved in cerebral perfusion disturbances, which include not only no-reflow events but also hyperemia episodes following ischemia: increased blood viscosity and perivascular edema, as well as possible down-regulation of NO synthesis, expression of endothelial adhesion molecules and generation of free radicals (*Bottiger 1997, Donadello 2011, Hossmann 1993, Liachenko 2001, van Genderen 2012*).

Histologically, both neuronal necrosis and apoptosis have been reported after CA. Selectively vulnerable neuron subpopulations in the hippocampus, cortex, cerebellum, corpus striatum, and thalamus, degenerate over hours to days (*Brierley 1973, Blomqvist 1985, Hossmann 2001, Neumar 2000, Nolan 2008, Polderman 2009, Pulsinelli 1985, Taraszewska 2002*).

2.2 Post CA myocardial dysfunction

The myocardial dysfunction observed after CA is a transient phenomenon known as myocardial stunning initially described 28 years ago by Braunwald and Kloner (*Patel 1988*).

Post CA myocardial dysfunction contributes to the early deaths after resuscitation from CA (*Lemiale 2013, Herlitz 1995, Laurent 2002, Laver 2004, Neumar 2008, Nolan 2008*). However, it

represents areversible dysfunction, responsive to therapy, characterized by normal or near-normal coronary flow (Ruiz-Bailén 2005) and usually spontaneously disappears within the following 2-3 days (Tang 2006, Neumar 2008, Lemiale 2013).

Knowledge regarding the myocardial dysfunction after CA has grown dramatically over the years thanks to preclinical and clinical studies. Development of contracted LV, namely “stone heart”, is characterized by reduced LV end-diastolic volume, stroke volume and increased LV wall thickness (Figure 1) (Klouche 2000 and 2002). After the onset of contracture, successful DF becomes unlikely.

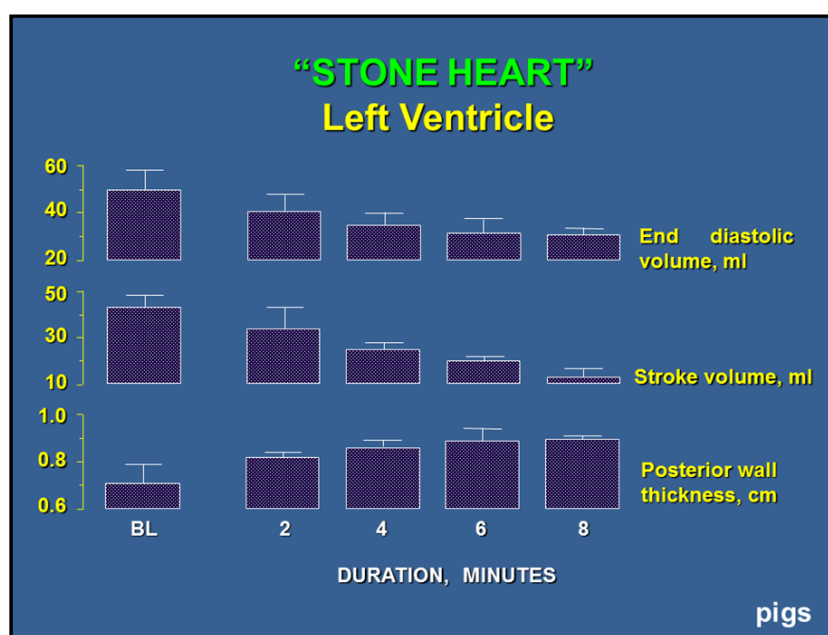


Figure 3. Development of the stone heart with reduced LV end-diastolic volume, stroke volume and increased LV wall thickness over 8 min of cardiac arrest in pigs. Modified from Klouche 2002.

In a model of CA and CPR in rats, significant reductions in post CA left ventricle ejection fraction (LVEF) was observed, at 2 and 4 h following ROSC, with a subsequent recovery 72 h later. A strong relationship between LVEF and circulating high sensitivity cardiac troponin T (hs-cTnT) was confirmed at different time points following ROSC. Normal LV function at 72 h post ROSC indicates that myocardial viability was preserved even in the presence of previous hs-cTnT release. This suggests that post-resuscitation myocardial dysfunction is largely reversible (Hackenhaar 2014).

In a pig model of myocardial infarction and CA, LV EF was deeply depressed during the first 4 h post-resuscitation (*Babini 2017*).

Repetitive defibrillations are known to increase the severity of post-resuscitation myocardial dysfunction (*Tang 2006*). It has been reported that the longer was the duration of no-flow, the greater was the number of defibrillation attempts and such a number of electrical countershocks was significantly related with the systolic dysfunction, represented by the LVEF impairment, and with the myocardial injury (i.e. hs-cTnT release), (*Babini 2017*).

Immediately after ROSC, heart rate (HR) and blood pressure are extremely variable and this is mainly caused by a transient increase in myocardial and circulating catecholamine concentrations (*Prengel 1992, Rivers 1994*). Premature ventricular beats and episodes of ventricular tachycardia and VF commonly occur during the early minutes after resuscitation and account for early death. Furthermore, an overall condition of severe myocardial dysfunction, including variable degrees of systolic and diastolic dysfunction, is present.

In one series of 148 patients who underwent coronary angiography after CA, 49% of subjects had myocardial dysfunction manifested by tachycardia and elevated LV end-diastolic pressure, followed approximately 6 hr later by hypotension and low cardiac output (CO) (*Kern 1996, Laurent 2002*). This global dysfunction was transient, and full recovery occurred. In a swine model with no antecedent coronary or other LV dysfunction features, the time to recovery appeared to range between 24 and 48 hr. Several case series have described transient myocardial dysfunction after human CA. Cardiac index values reached their nadir at 8 hr after resuscitation, improved substantially by 24 hr, and almost uniformly returned to normal by 72 hr in patients who survived out-of-hospital CA. This trend in arterial pressure, cardiac index and other hemodynamic parameters are described in Table 3 from Dr. Laurent's work (*Laurent 2002*), where clinical data over the 72 h post CA are reported.

Hemodynamic Data During the First 72 Hours							
Hemodynamic Parameters	Interval From Onset of Cardiac Arrest to Measurement (h)						p Value§
	ICU Admission: 3.0 (2.0-3.6)	Time 0: 6.8 (4.3-7.3)	Time 1: 8.0 (7.0-9.0)	Time 2: 12.0 (11.0-13.5)	Time 3: 24.0 (23.0-25.7)	Time 4: 67.0 (52.0-72.0)	
Temperature (°C)	36.0 (35.4-36.7)	—	36.6* (35.8-37.5)	37.3† (36.7-38.1)	37.6† (37.0-38.2)	37.8† (37.3-38.3)	< 0.001
Epinephrine perfusion (mg/h)	0	0	1.0‡ (0-2.2)	1.3‡ (0-2.0)	1.5‡ (0-2.7)	0.4‡ (0-1.6)	0.042
HR (beats/min)	110 (89-123)	111 (91-124)	108 (97-125)	111 (98-128)	112 (101-125)	101* (94-120)	0.215
MAP (mm Hg)	87 (75-103)	62† (46-71)	79‡ (69-102)	76‡ (69-87)	80 (71-89)	80 (73-88)	0.04
MPAP (mm Hg)	—	—	28 (22-32)	24‡ (20-28)	24† (20-27)	28 (24-32)	0.005
RAP (mm Hg)	—	—	11 (8-15)	10 (8-13)	11 (8-13)	12 (9-15)	0.189
POAP (mm Hg)	—	—	14 (11-18)	12‡ (10-15)	13 (10-16)	14 (10-18)	0.248
CI (l/min per m ²)	—	—	2.05 (1.43-2.90)	2.61‡ (1.90-3.46)	3.19‡ (2.67-4.20)	3.69* (2.92-4.49)	< 0.001
SVRI (dynes/cm ⁵ ·m ²)	—	—	2,908 (1,946-4,658)	1,936† (1,493-2,951)	1,672† (1,300-2,034)	1,518† (1,153-1,852)	< 0.001
PVRI (dynes/cm ⁵ ·m ²)	—	—	438 (339-593)	363† (221-488)	261† (183-346)	274* (206-371)	< 0.001
SI (ml/m ²)	—	—	20.0 (15.0-23.8)	22.5‡ (18.4-32.1)	29.3‡ (24.8-37.4)	35.3‡ (28.5-42.1)	< 0.001
LVSWS (gm/m ²)	—	—	23.8 (19.3-31.0)	24.5 (19.8-34.9)	33.3† (25.0-43.1)	41.1‡ (31.4-50.0)	< 0.001

*p < 0.05, †p < 0.001, ‡p < 0.01 for all tests performed versus baseline. Baseline values were at intensive care unit (ICU) admission for temperature, epinephrine infusion, and heart rate; time 0 for mean arterial pressure (MAP); and time 1 for the other values. Mean arterial pressure at admission and time 0 was determined by noninvasive methods, and MAP at times 1, 2, 3, and 4 by invasive monitoring. §The p value in the last column refers to nonparametric analysis of variance (Kruskal-Wallis test). Data are presented as the median value (interquartile range).
CI = cardiac index; HR = heart rate; LVSWS = left ventricular stroke work; MPAP = mean pulmonary artery pressure; POAP = pulmonary occlusion arterial pressure; PVRI = pulmonary vascular resistance index; RAP = right arterial pressure; SI = stroke index; SVRI = systemic vascular resistance index.

Table 3. Hemodynamic during the first 72 h after CA. Reproduced from Laurent 2002.

Among the mechanisms underlying early post-resuscitation arrhythmia and myocardial dysfunction, cytosolic and mitochondrial Ca²⁺ overload following CA and CPR has been recognized as a determinant in several preclinical studies (rats and pigs) (Ayoub 2008, Gazmuri 2012). Ca²⁺ overload after CA is related to myocyte Na⁺ content alteration. Under normoxic conditions the late Na⁺ current (I_{NaL}) contributes very little to the total Na⁺ content of the myocardial cell. However, during ischemia the I_{NaL} channel does not close properly. Under this condition, the influx of Na⁺ becomes substantial (Kloner 2011). Indeed, Na⁺ influx through the late Na⁺ channel appears to be the major contributor to the rise of cardiomyocyte intracellular Na⁺ concentration observed during ischemia (Zaza 2008). It has been shown, in fact, that ischemia increases the amplitude of I_{NaL} in rat ventricular myocytes, from 50–100 pA up to 180–205 pA. Furthermore, following reperfusion, production of ROS is known to further increase I_{NaL} (Ma 2005, Slezak 1995, Song 2006).

In the setting of CA and CPR, main routes for cardiomyocyte Na⁺ entry include the Na⁺-hydrogen exchanger isoform-1, the voltage-gated Na⁺ channel, and the Na⁺-bicarbonate co-transporter. This cytosolic Na⁺ accumulation, further augmented by the concurrent ischemia-induced Na⁺-K⁺-ATPase inability to extrude Na⁺, represents an important pathophysiological mechanism responsible for cell injury (Wang 2007). Cytosolic Na⁺

causes, in fact, a subsequent increase in myocyte intracellular Ca^{2+} via the activity of Na^{+} - Ca^{2+} exchanger (Figure 8) (Ayoub 2008).

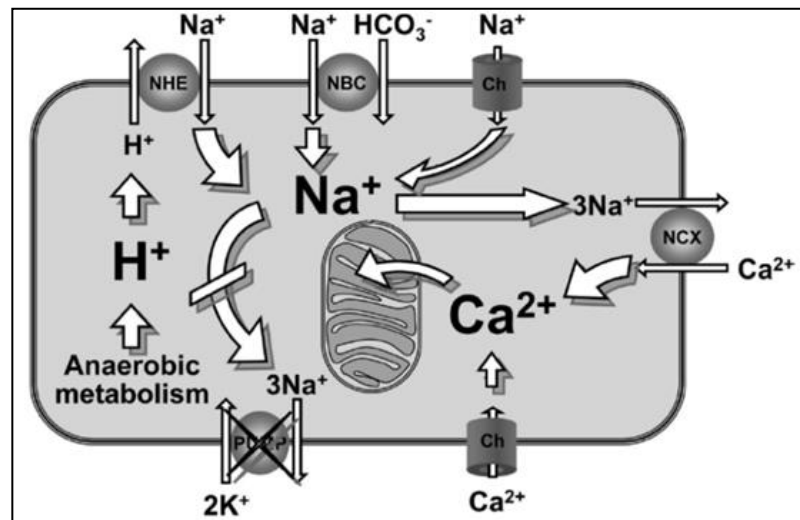


Figure 4. Events leading to cardiomyocyte Ca^{2+} overload during ischemia/reperfusion. NHE = $\text{Na}^{+}/\text{H}^{+}$ exchanger; NBC = $\text{Na}^{+}/\text{HCO}_3^{-}$ exchanger; NCX = $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger; Ch = channel. From Ayoub 2008.

Post-resuscitation cytosolic Ca^{2+} accumulation in cardiomyocytes has, indeed, several deleterious consequences, including: electrical instability with ventricular arrhythmias early after resuscitation, i.e. premature ventricular complexes, VT and VF; varying degrees of mechanical LV dysfunction that can compromise hemodynamic function, i.e. reduced contractility and increased diastolic tension; and mitochondrial dysfunction (Ayoub 2008 and 2010, Gralinski 1996).

Mitochondria are the major contributors in many cellular as well as extracellular regulatory functions that affect survival following an ischemic insult. In the setting of CA, mitochondria are thought to be progressively damaged during ischemia and further injured when reperfusion resumes. This leads to important alterations of the function of this organelle. Specifically, the dysfunction has been shown to be mainly associated with impaired complex activities that begin to deteriorate within the initial 15 min of ischemia. Nevertheless, ultrastructural changes of mitochondria during ischemia, including swelling, loss of matrix density and cristae disintegration, have been also demonstrated to correlate

well with the duration of CA (*Yeh 2009, Xu 2010*). More specifically, within the first hours during the early recovery phase, a greater decrease in respiratory control ratio has been observed. In addition to its bioenergetic function, mitochondria also participate in processes leading to cell death via necrosis or apoptosis. Various distinctive mechanisms have been identified including opening of the so called mitochondrial permeability transition pore (leading to collapse of proton motive force and uncoupling of respiration) (*Halestrap 2004*) and release of various pro-apoptotic proteins, including cytochrome c, apoptosis-inducing factor, Smac/DIABLO, endonuclease G, and a serine protease Omi/HtrA2 (*Cai 1998, Green 1998, Radhakrishnan 2007 and 2009*).

2.3 Post CA systemic inflammatory response

The whole-body ischemia and reperfusion occurring during CA and CPR triggers a systemic inflammatory response, which increase the risk of multiple organ failure and infection (*Adams 2006, Cerchairi 1993, Esmon 2003*). More specifically, this inflammatory response includes endothelial activation, plasma cytokine elevation with deregulated cytokine production, presence of endotoxin in plasma, coagulation abnormalities, and adrenal dysfunction (*Adrie 2002, Ristagno 2014, Nolan 2008, Peberdy 2010*). Accordingly, this systemic inflammatory response brings evident similarities with sepsis and septic shock, and is generally described as a “sepsis-like” syndrome (*Adrie 2002*).

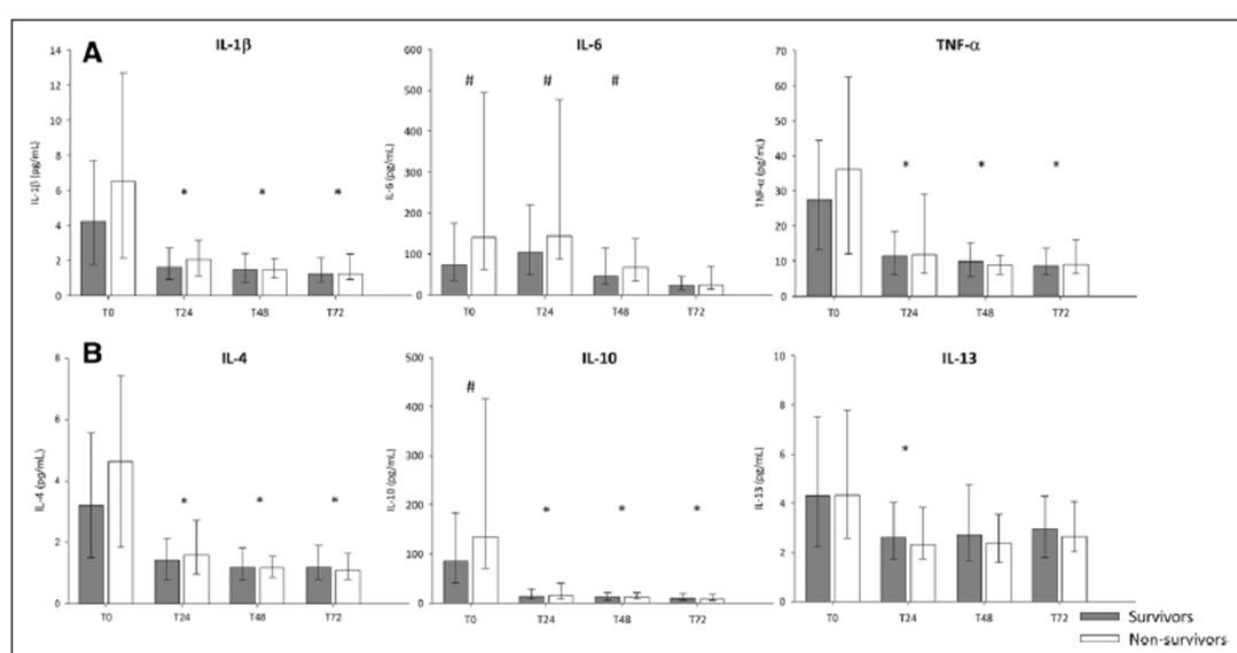
Soluble intercellular adhesion molecule-1, soluble vascular-cell adhesion molecule-1, and P- and E-selectins are increased during and after CPR, suggesting leucocyte activation and endothelial injury (*Adrie 2002 and 2004, Geppert 2000, Gando 2000*).

In addition, high levels of circulating cytokines, dysregulated production of cytokines found in CA patients resemble the immunological profile of patients with sepsis (*Adrie 2002 and 2004*).

Specifically, levels of IL-6 and IL-10 at baseline were significantly higher in non-survivors compared with survivors, whereas baseline levels of IL-1 β , IL-2, IL-4, IL-5, IL-9, IL-12,

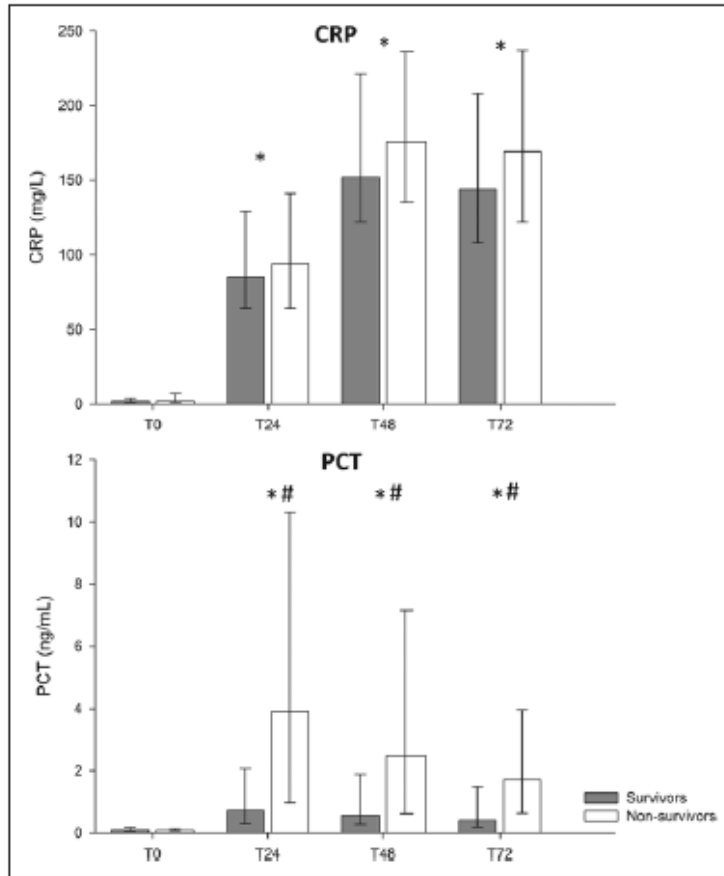
IL-13, TNF- α , interferon- γ , C reactive protein, and procalcitonin were similar in survivors and non-survivors at 30 days, (Fig. 5 and 6), (Bro-Jeppesen 2015).

As early as 3 hours after CA, endotoxin increase (figure 7) was observed. The hypo-responsiveness of circulating leucocytes to endotoxin increase named “endotoxin tolerance”, as observed in severe sepsis (Munoz 1991). Endotoxin tolerance after CA may protect against an overwhelming proinflammatory process, but may induce immunosuppression with increased risk of nosocomial infection (Adrie 2002, Figure 7).



Plasma levels (pg/mL) within 72 hr after out-of-hospital cardiac arrest of selected proinflammatory cytokines—interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α (A)—and anti-inflammatory cytokines—IL-4, IL-10, IL-13 (B)—in survivors and nonsurvivors after 30 days. Bars represent median value, with error bars representing interquartile range. Levels of inflammatory markers were compared by repeated measures models with outcome group and time as fixed effects. #Significant difference between outcome group at each time point was assessed by the interaction of outcome group and time, $p < 0.05$. *Significant difference compared to baseline values, $p < 0.0001$.

Figure 5. From Bro- Jeppesen 2015.



Plasma levels of C-reactive protein (CRP) (mg/L) and procalcitonin (PCT) ($\mu\text{g/mL}$) within 72 hr after out-of-hospital cardiac arrest in survivors and nonsurvivors after 30 days. Bars represent median value, with error bars representing interquartile range. Levels of inflammatory markers were compared by repeated measures models with outcome group and time as fixed effects. #Significant difference between outcome groups at each time point assessed by the interaction of outcome group and time, $p < 0.05$. *Significant difference compared to baseline values, $p < 0.0001$.

Figure 6. From Bro-Jeppesen 2015.

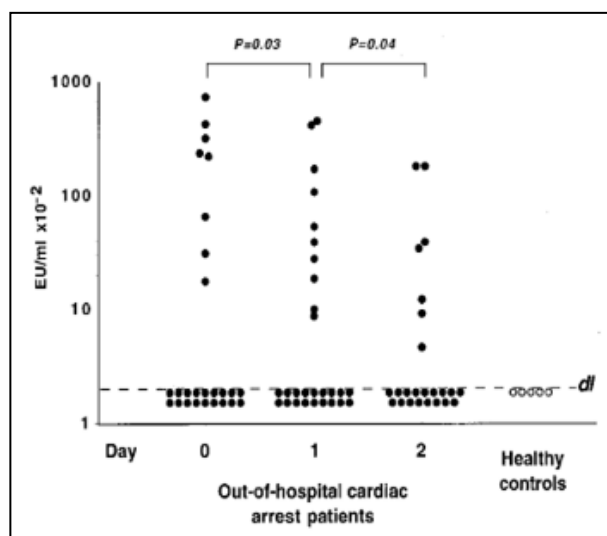


Figure 7. Measurement of endotoxin levels (endotoxin units [EU]/mL) in plasma of 35 resuscitated OHCA patients on admission (day 0) and the 2 days after (days 1 and 2). dl indicates detection limit (0.02 EU/mL). From Adrie 2002.

Activation of blood coagulation is an important pathophysiological mechanism that may contribute to microcirculatory reperfusion disorders (*Adrie 2005, Böttiger 1995*). Coagulation profile in CA patients undergone to CPR has been addressed by a number of studies over the last decade. Gando et al. was among the first studying coagulation during and after CPR, finding massive fibrin generation especially in the first 24 hours in 63 OHCA patients (*Gando 1997*). Similarly, increased levels of thrombin-antithrombin complexes were observed by Hostler et al. in patients affected by OHCA (*Hostler 2007*). Increased activation of blood coagulation without inadequate activation of endogenous fibrinolysis represents one of the main pathophysiological mechanism involved in post CA syndrome. Indeed, this leads to disseminated intravascular coagulation (DIC), which clinically manifests as obstruction of microcirculation and multiple organ dysfunction. In particular, thrombotic occlusion in the brain vessels due to DIC causes the “no-reflow phenomenon” responsible of the microcirculatory failure occurring after CA and CPR. Altered coagulation in patients with post CA syndrome is characterized by tissue factor-dependent coagulation, which is accelerated by impaired anticoagulant mechanisms, including antithrombin, protein C, thrombomodulin, and tissue factor pathway inhibitor (*Wada 2017*).

Finally, the stress of total body ischemia/reperfusion affects adrenal function. Relative adrenal insufficiency is common following cardiac arrest and seems to be related to the severity of the ischemic insult (*Kim 2011*). Although an increased plasma cortisol level occurs in many patients after out-of-hospital CA, relative adrenal insufficiency, defined as failure to respond to corticotrophin, is common (*Hekimian 2004, Schultz 1993*).

3 Circulating biomarkers in CA

Circulating biomarkers have become an increasingly important tool in clinical practice, useful for diagnosis, risk stratification, monitoring, and prognosis of patients. Predicting survival and especially the neurological outcome after ROSC in victims of cardiac arrest remains a difficult challenge. The decision to continue, limit or stop intensive care is a major problem for deep ethical implications and for resources allocation. Accurate prediction of the neurologic outcome of comatose patients following cardiac arrest is therefore essential to establish the model of care (*Thenayan 2008*). Prognostication of outcome in cardiac arrest is of importance because it could help physicians to make decisions and enhance therapeutic efforts in patients with predictable overall good recovery and to re-address the overall management or ultimately to make a decision on withdrawal of care in those with expected poor outcome (*Bouwes 2012*). A procedure used to support no-treatment decisions, however, should ideally have no false-positive results (*Wijdicks 2006, Zandbergen 2001*) reaching a specificity of 100% for poor outcome. A false prediction of a poor outcome may cause the patient to be denied life-supporting treatment. On the other hand, a falsely optimistic prediction, although less serious from an ethical point of view, may lead to unnecessary prolongation of costly therapy. With the help of the most sophisticated and highly accurate methods measuring biomarkers, high serum concentrations of biomarkers are associated with false positive rates close to 0% in cardiac arrest patients; however, the thresholds used in the reports are inconsistent. This is partly because measuring techniques vary among studies, and it is an obstacle to widespread clinical implementation of biomarkers for prognostication (*Nolan 2017*).

Due to the heterogeneity of cardiac arrest population and scenarios, no single factor has been identified as a reliable predictor of outcome. In addition, with recent advances in the use of therapeutic hypothermia (TH), prediction of patients' outcome has become even more challenging. In fact, there is evidence that TH makes the well-established tests in normothermic patients less reliable. Early assessment of patient brain damage remains

quite difficult in the intensive care unit (ICU), and could be actually reachable only through the integration of the answer given by clinical examination, neuro-functional and neuro-imaging evaluation, and biochemical markers dosing (*Sandroni 2014*). This evaluation should be carried out after the initial 72 h post-ROSC. Normothermic and hypothermic patients with a favorable prognosis predominantly regain consciousness within 3 days (*Fugate 2011*). A reliable prediction in TH is, however, quite difficult. Likewise, a residual effect of sedative and analgesic substances must be excluded, due to longer drugs' half-life during TH. Indeed, a significantly delayed awakening in patients treated with TH could be observed (*Lurie 2011*). For this reason, the early termination of life support 72 hr after re-warming needs to be reconsidered critically and is not indicated.

The current clinically used biomarkers to predict outcome of cardiac arrest are cTn and creatine kinases (CKs) for the heart, and NSE and S-100b, for the brain (*Peberdy 2010, Scolletta 2012*). Nevertheless, evidence does not support the use of these serum biomarkers alone as predictors of outcome in the setting of cardiac arrest (*Neumar 2010, Nolan 2010*). Furthermore, the use of the above markers is depending from conditions of “localized” myocardial or brain ischemic insults, while cardiac arrest is a wider and more complex event, involving the whole body. Accordingly, research in this domain extends from localized to generalized ischemic injury, a condition of “global” ischemia and reperfusion (*Becker 2002*).

Experimentally, both brain and cardiac injury biomarkers mainly allow evaluating the severity of the injury. Indeed, biomarkers plasma levels correlate with the extent of anoxic-ischaemic neurological and cardiac injury from cardiac arrest. Furthermore, biomarkers are able to provide feedback regarding the effects of treatment, and the progress of the disease. Finally, biomarkers level adds information about mechanisms of injury and preservation. Up to date cardiac, neurological and inflammatory biomarkers, as potential predictors of outcome after cardiac arrest, are summarized in Supplemental Table 1 (see appendix 2), (*Scolletta 2012*).

3.1 Circulating biomarkers of cardiac injury

Within the last decade a broad range of circulating markers associated with an increased risk for death and cardiovascular endpoints have been identified (Supplemental Table 2, Appendix 2). Adding to markers of cell necrosis are markers of ischemia, inflammation, plaque destabilization or rupture, myocardial dysfunction, and stress. Most of these markers have demonstrated at least some prognostic value (*Hochholzer 2010*).

For patients resuscitated from cardiac arrest, it is important to determine a definite cause, that is, myocardial ischemia vs. primary ventricular arrhythmia, owing to the risk of recurrence and the different therapeutic approaches available. Acute myocardial infarction (AMI) may account for up to 50% of out-of-hospital cardiac arrest, ischemic heart disease without AMI for one-third of cases and non-ischemic cardiomyopathy for nearly 10% of all arrests (*de Vreede-Swagemakers 1998, Voicu 2012*).

Troponins are the most used biomarkers of myocardial injury/dysfunction. Troponin I, C, and T form a complex that regulates the Ca²⁺-modulated interaction of actin and myosin in striated muscle. Among the cardiac markers, cTn I and T are sensitive and specific markers of myocardial injury and are used routinely for the diagnosis of acute coronary syndromes. They provide prognostic information and are of great value for risk stratification of patients (*Antman 1996, Apple 2005, Aviles 2002, Donnelly 1998, Lindahl 2000*). Elevated cTn blood levels have been reported in several cohorts of patients with heart failure, and the magnitude of elevation has been correlated with the severity of the disease and with adverse outcomes (*Ishii 2002, Setsuta 1999, Sato 2001*). Because of their high cardiac specificity, elevated blood cTn may suggest ongoing myocardial damage and may serve as a marker for the progression of disease during the post reperfusion recovery. Currently, for every patient who has suffered a myocardial ischemic event or who has been resuscitated from cardiac arrest, cTn T or I are considered the first-line test (*Williams 2005*). Cardiac troponin T has been investigated extensively and has been found to be a sensitive marker of myocardial necrosis (*Katus 1991 and 1992, Mair 1992*). The presence of elevated levels of cTnT

in the general population has a prevalence of less than 1% and this condition is commonly associated with an underlying cardiovascular disease or high-risk phenotypes for cardiac accidents, especially in persons with chronic heart failure. Currently, new highly sensitive assays for determination of cTn are available and have shown that cTnT retains a prognostic value at previously undetectable concentrations (*Latini 2007*). When cTnT levels were investigated in more than 4.000 patients with a LV ejection fraction (EF) of <40% using both the standard assay and the high sensitivity assay, cTnT detection increased from approximately 10% of the population to more than 90%. The circulating concentration of this highly sensitive (hs) cTnT showed even greater prognostic accuracy in association with increases of another biomarker, namely brain natriuretic peptide (BNP). In 658 patients who presented with BNP above the normal median concentration and cTnT below, mortality was 14%. However, in an additional 632 patients with BNP below the median and cTnT above, mortality was 20%. Finally, in the 1.331 patients with both markers above their respective median concentrations, mortality increased to 32% (*Latini 2007*). A continuous, slow release of cTn from the myocardium might reflect an ongoing cardiac myocyte cell death. This condition has been associated with the condition of LV dysfunction following myocardial ischemia in both animal models and humans patients suffering with chronic heart failure (*Narula 1999, Olivetti 1997*). If ongoing cardiac damage at a very low rate is the determinant of these circulating troponins, other mechanisms may, however, account for this phenomenon, such as stretching of cardiac myocytes with transient loss of cell membrane integrity. There are few investigations on the role of cTn for the differential diagnosis of AMI in patients successfully resuscitated after out-of-hospital cardiac arrest. Early studies reported a substantial lack of sensitivity and specificity (*Müllner 1996*). This might have been because procedures used for CPR (CC or DF) or persistent circulatory shock might have directly provoked myocardial damage and the release of cardiac markers, in the absence of a coronary artery occlusion (*Müllner 1998*). Troponin elevation in patients resuscitated from cardiac arrest but who do not have AMI is

however lower and normalizes faster than after AMI (*Oh 2012*). In a recent, single-centre study, 163 patients resuscitated from cardiac arrest were assessed with coronary angiography on admission for AMI, that was diagnosed in 37% of the cases (*Voicu 2012*). High circulating cTn concentrations were measured very early after cardiac arrest, even in patients with normal angiograms (median cTnI was 0.6 ng/mL in the latter group), indicative of non-ischemic myocardial injury during CC. However, combined with ST-elevation on ECG, elevated cTn concentration on admission (cTnI > 2.5 ng/mL) showed a good performance to exclude the diagnosis of AMI (sensitivity 93%, negative predictive value 94%). The specificity remained low even in combination with ST-elevation on ECG (64%).

International guidelines recommend considering emergent coronary angiography and percutaneous coronary intervention in cardiac arrest patients after ROSC, even in the absence of ST elevated myocardial infarction (STEMI) (*Peberdy 2010*). Since clinical findings such as chest pain are often lacking and the predictive value of ECG for AMI is poor, cTn testing may provide a simple and objective selection of post-resuscitation patient candidate for immediate coronary angiography. This triage strategy has recently been tested in 422 cardiac arrest survivors without obvious extra-cardiac causes (*Dumas 2012*). In this large study, a coronary angiography was systematically performed and cTn measured on admission. However, even if independently associated with coronary occlusion, elevation of cTn levels had a poor accuracy to identify a recent coronary lesion, precluding its use as the sole criteria for the decision to perform or not early coronary angiography in these patients (Supplemental Figure 1).

Acute coronary artery occlusion is therefore difficult to diagnose in survivors of cardiac arrest using circulating markers alone, especially in case of ambiguous ECG. There are no data available to date on the diagnostic performance of cTn after cardiac arrest, when using high sensitivity assays that may readily detect concentrations in the order of few nanograms per liter, ten to hundred times lower than the conventional assays. However,

one may speculate that specificity will become an even more critical issue since the recent introduction of this new generation of high sensitivity reagents has led to a substantial increase in the proportion of detectable cTn levels attributable to conditions distinct from acute coronary syndromes (*de Lemos 2013*). Moreover, the diagnostic value of cTn could be altered by CPR. Increased cTn levels were, in fact, reported in approximately 40% of cardiac arrest patients, when samples were drawn during CPR (*Lai 2004*).

3.2 Circulating biomarkers of brain injury

Biochemical markers of brain injury are logical choices as prognostic markers of neurological outcome. Of these biomarkers neuron specific enolase (NSE) and S-100b are among the most widely studied and used. Considering the different characteristics of these two biomarkers, a possible rationale for analyzing both molecules in patients after cardiac arrest is their different distribution within the grey (NSE) and white (S-100b) matter.

NSE is a neuron-derived enzyme, which is released after stroke and cardiac arrest and can be detected in the blood. Its level in serum correlates with the extent of the neurological damage. NSE is a 78-kDa with a half-life of 24 hr, intracellular enzyme found in neurons and other cells of neuroectodermal origin. Elevation of serum NSE 1–3 days after cardiac arrest is regarded as a severity marker of post-anoxic neuronal injury. The American Academy of Neurology, which considered studies from the pre-hypothermia era, suggested a cutoff of > 33 µg/L at days 1–3 after ROSC as a robust predictor for poor outcome (*Wijdicks 2006*); the reported false positive rate (FPR) varied from 0 to 3%. Cerebral hypoxia causes death of neuronal cells as well as damage to the BBB, resulting in an elevation of the serum levels of this and other markers. The serum levels often correlated with the extent of brain damage and in some studies with the prognosis of the patients. The predictive value of NSE in patients with ROSC has been shown in several trials prior to the use of TH (*Reisinger 2007, Zandbergen 2006*). An elevation of NSE was associated with poor outcome for comatose patients after cardiac arrest (*Auer 2006, Grubb 2007, Meynaar 2005, Roine 1989*). Fogel et al. (*Fogel 1997*) reported that NSE concentrations exceeding 33 µg/l at any

time within a week since arrest had a sensitivity of 80% and specificity of 100% to predict persistent coma. In addition, Zandbergen et al. (*Zandbergen 2006*) found in a large cohort of resuscitated patients that NSE concentrations above 33 µg/l within 72 hr after arrest predicted poor outcome with a FPR of 0%. However, a subgroup analysis of the Hypothermia after Cardiac Arrest trial (*Tiainen 2003*) showed a decreased prognostic value of NSE in patients treated with TH. Since the introduction of the 2010 guidelines, various studies have dealt with NSE during TH. Although an elevated NSE predicts a poor outcome, there are case reports of good neurological survival despite extremely high NSE levels in serum (*Grubb 2007, Krumnikl 2002*). In a prospective observational study on 97 patients, patients with poor outcome at 3 months had significantly higher NSE levels at 24 and at 72 hr than those with good neurological. A peak NSE level of 47 µg/l had the highest specificity (84%) and sensitivity (72%) to predict poor outcome, with a positive predictive value (PPV) of 93%. A cutoff value for NSE of 97 µg/l predicted a poor neurological outcome with a specificity of 100% and a sensitivity of 49% (*Daubin 2011*), NSE levels >33 µg/l resulted in a rate of false prediction of poor outcome ranging from 7% for measurements taken 48 hr after arrest to 10% if NSE was assessed during TH (*Bouwes 2012*). Other cutoffs have been also reported, widely ranging from 9 to 91 µg/l (*Reisinger 2007, Zingler 2003*). It is therefore difficult to identify a specific cut off predictive of neurological outcome.

In addition, there is also evidence for a constant turnover of NSE in blood, making changes specifically associated with brain damage in serum levels difficult to evaluate. For example, hemolysis caused by invasive procedures might produce a false rise in NSE (*Zandbergen 2001*). Moreover, it is well known that platelets are markedly activated and hemolysis may occur during early reperfusion after cardiac arrest. Therefore, even though at least a proportion of NSE is released from neurons, the determination of NSE, particularly during early reperfusion after cardiac arrest, may not be brain-specific. More

recently, increases of NSE levels over time, i.e. 2 to 6.4 ug/L during the initial 24 and 48 hr after ROSC, have shown better predictivity for poor outcome (*Oksanen 2009, Rundgren 2009*).

4 Post CA syndrome current treatments

The treatment that patients receive during the post resuscitation period depends on the severity of the post CA syndrome, as well as on the cause of the arrest, and influences significantly the quality of life (*Nolan 2015*). Specifically, during years, it has become clearer that the management of these patients once ROSC is achieved can affect substantially their ultimate neurological outcome. For this reason, great emphasis has been given to the first European Guidelines on post-resuscitation care, produced in 2015 and resulting from a collaboration between the European Resuscitation Council (ERC) and the European Society of Intensive Care Medicine (ESICM), where the key interventions required to optimize outcome for these patients are set out.

The management of the post-CA patients is complex and must address multiple major problems simultaneously. The main critical interventions in post CA management are highlighted below.

Immediately following ROSC, the earliest threat to survival is cardiovascular collapse. Interventions to optimize blood pressure and maintain end-organ perfusion (fluids, vasopressors and inotropes) helps to avoid hypotension. Additional goals in the short-term post resuscitation care include optimizing oxygenation and ventilation. Determination of the etiology of CA and the initiation of relevant treatments are performed concurrently in order to prevent recurrent arrest and to optimize outcomes. Finally, evidence supports the use of targeted temperature management (TTM) to minimize brain injury, together with seizures prevention and control and glucose control.

4.1 Hemodynamic management

Post-resuscitation myocardial dysfunction causes hemodynamic instability, which manifests as hypotension, low cardiac index and arrhythmias (*Laurent 2015*). For this reason, an adequate blood pressure must be maintained in the post CA patients. However, the optimal targets for mean arterial pressure and/or systolic arterial pressure remain unknown.

In general, when determining blood pressure goals, clinicians must balance the metabolic needs of the ischemic brain with the potential of the decompensated heart. So, mean arterial pressure (MAP) should be above 65 mmHg to maintain the end-organ perfusion (*Kern 1997*), and preferably 80 to 100 mmHg to optimize cerebral perfusion and optimize neurological outcome (*Kilgannon 2014*). Inotropic and vasopressor support can mitigate the myocardial dysfunction during the first hours after CA. Inotropic support (i.e. dobutamine) is often required, at least transiently, even if tachycardia may be caused by dobutamine and was associated with bad outcome in one retrospective study (*Torgersen 2013*). Inotropic agents are usually administered in association to vasoactive drugs (i.e. noradrenaline). Indeed, the systemic inflammatory response that occurs frequently in post CA patients may also cause vasoplegia and severe vasodilation (*Laurent 2002*). Infusion of relatively large volumes of fluid is tolerated remarkably well by patients with post-cardiac arrest syndrome (*Gaieski 2009*). Immediately after CA, there is typically a period of hyperkalaemia. Subsequent endogenous catecholamine release and correction of metabolic and respiratory acidosis promotes intracellular transportation of potassium, causing hypokalemia. Hypokalemia may predispose to ventricular arrhythmias. Potassium administration to maintain the serum potassium concentration between 4.0 and 4.5 mmol l⁻¹ may be helpful (*Nolan 2015*).

4.2 Oxygen and carbon dioxide target

Guidelines indicates to maintain oxygen saturation > 94%, avoiding hyperoxia which has been associated with worse outcomes. Indeed, one of the major events characterizing the ischemia/reperfusion injury is the generation of oxygen free radicals that are known to injure cells. Supplementary oxygen increases their production. However, the importance of avoiding hyperoxia in the post arrest patients remains an area of debate. Whereas animal studies always supports the hypothesis that hyperoxia is harmful, differing conclusions emerged from clinical studies comparing hyperoxia with normoxia in post CA patients.

Several observational studies showed an association between hyperoxia and poor outcome (Janz 2012). In contrast, an observational study of over 12,000 post-cardiac arrest patients showed that after adjustment for sickness severity, hyperoxia was no longer associated with mortality (Bellomo 2011). The heterogeneity across studies could be because, in the animal studies, the relationship between hyperoxia and worse neurological outcome after CA have generally evaluated in the first hour after ROSC. Clinically, there are significant practical challenges with the titration of inspired oxygen concentration immediately after ROSC, particularly in the out-of hospital setting. The only prospective clinical study to compare oxygen titrated to a target range (in this case 90–94 % oxygen saturation) versus giving 100% oxygen after out of hospital cardiac arrest was stopped because of unreliable pulse oximetry readings (Young 2014). Further studies involving titration of oxygen to a target oxygen saturation of 94 % as soon as possible after ROSC are advocated. Hypocapnia causes cerebral vasoconstriction and a decreased cerebral blood flow (Menon 2004). After CA, hypocapnia induced by hyperventilation may exacerbate cerebral ischemia and impair neurological outcome (Bouzat 2013, Buunk 1998). In contrast, two observational studies have documented an association with mild hypercapnia and better neurological outcome among post-CA patients in the ICU (Schneider 2013, Vaahersalo 2014). Until prospective data are available, it is reasonable to adjust ventilation to achieve normocapnia.

4.3 Temperature control

Mild induced hypothermia initiated within minutes to hours after ROSC and maintaining a temperature range of 32–34°C for 12–24 h was recommended from 2003 as the unique effective neuroprotective strategy improving outcome of CA. In fact, cooling suppresses many of the pathways leading to delayed cell death, including apoptosis (programmed cell death) and reduces the release of excitatory amino acids and free radicals (Gunn 2006, McCullough 1999). Furthermore, hypothermia blocks the intracellular consequences of excitotoxin exposure (high calcium and glutamate concentrations) and reduces the

inflammatory response associated with the post-CA syndrome (*Bro-Jeppesen 2014*). At that time, the supporting evidence was based on experimental animal data, non-randomized clinical trials and two small randomized trials. Furthermore, hypothermia presented several well-recognized physiological side effects, that need to be taken into consideration clinically. First, despite shivering is a sign of a normal physiological response and its presence is associated with a good neurological outcome, it will increase metabolic and heat production, thus reducing cooling rates. Second, mild induced hypothermia increases systemic vascular resistance and causes bradycardia that may be beneficial; it reduces diastolic dysfunction and its occurrence has been associated with good neurological outcome. Moreover, mild induced hypothermia causes a diuresis and electrolyte abnormalities (i.e. hypophosphataemia, hypokalaemia, hypomagnesaemia and hypocalcaemia), decreases insulin sensitivity and insulin secretion, and causes hyperglycaemia, which will need treatment with insulin. Importantly, hypothermia can impair the immune system and increase infection rates. It is associated with an increased incidence of pneumonia even if this seems to have no impact on outcome (*Nolan 2008*).

Studies supporting the use of mild therapeutic hypothermia presented several limitations and data are extremely difficult to interpret because of other changes in post cardiac arrest care that occurred simultaneously. Specifically, it was not clear if the potential benefits of cooling on brain injury due to circulatory arrest would be the same irrespective of the cause of arrest. In addition, the most beneficial target temperature for therapeutic hypothermia remained unknown. The recommended temperature of 32° to 34°C has been extrapolated from experiments in animals but similar results have been observed with milder cooling.

In the Targeted Temperature Management (TTM) trial, 950 all-rhythm OHCA patients were randomized to 36 h of temperature control (comprising 28 h at the target temperature followed by slow rewarm) at either 33 or 36 °C (*Nielsen 2013*). There was no difference in

all-cause mortality, and neurological outcome at 6 months was also similar. Importantly, patients in both arms of this trial had their temperature well controlled so that fever was prevented in both groups.

After the neutral results of the TTM Trial, current recommendations have been modified to include a broader range of target temperature (*Nolan 2015*).

Optimum timing and duration of for mild induced hypothermia and TTM are still unknown but some trials are ongoing. Both are currently most commonly used for 24 h.

Prior recommendations suggest that cooling should be initiated as soon as possible after ROSC, but this recommendation was based only on preclinical data indicating that earlier cooling after ROSC produces better outcomes (*Kuboyama 1993, Colbourne 1995*). Observational studies are confounded by the fact that there is an association between patients who cool faster spontaneously and worse neurological outcome (*Haugk 2011, Benz-Woerner 2012, Perman 2015*). It is hypothesized that those with the most severe neurological injury lose their ability to control body temperature easier.

TTM can be induced and maintained using cold intravenous fluids after ROSC (*Kim 2007, Kamarainen 2009, Bernard 2010, Kim 2014*) or during resuscitation (*Debaty 2014*); however, in one prehospital randomized controlled trial this intervention was associated with increased pulmonary edema and an increased rate of re-arrest during transport to hospital (*Kim 2014*). Based on this evidence, prehospital cooling using a rapid infusion of large volumes of cold intravenous fluid immediately after ROSC is not recommended. Additional methods of inducing and/or maintaining TTM include ice packs and/or wet towels application, water or air circulating blankets, water circulating gel-coated pads, transnasal evaporative cooling to obtain local cooling of the brain, intravascular heat exchanger, placed usually in the femoral or subclavian veins and extracorporeal circulation (e.g. cardiopulmonary

bypass, ECMO). There are no data indicating that any specific cooling technique increases survival when compared with any other cooling technique.

To date, neuroprotective treatments, pharmacological or not, other than hypothermia, used alone or in association, failed to improve neurologically intact survival when clinically tested.

The overall scenarios makes the the use of inhaled noble gases (Argon, helium and particularly xenon) a promising research option, wich need a consistent rigorous testing in different animal experimental models, providing sufficient informations on the efficacy and safety aspect needed to proceed to clinical application.

5 Medical uses of noble gases

The inert or noble gases helium, neon, Argon, krypton and xenon exist as monatomic gases with low chemical reactivity due to the full electron valence shell which avoids covalent bindings with other elements. However, the noble gases cannot be defined as a “biologically” inert gas, despite their chemical profile. The potential for noble gases to produce a biological effect was firstly suggested 70 years ago by Behnke and Yarbrough, who reported the development of progressive “narcosis” in United States Navy divers exposed to 80% Argon or krypton in 20% oxygen under hyperbaric conditions (10 atm), (*Behnke 1939*). The narcotic effect was not observed for helium and neon, that appeared both chemically and biologically inactive, at least at tolerable pressures (*Miller 1967, Koblin 1998*). Radon instead might be predicted to be an anesthetic; it is radioactive, however, and exposure to radon – even at very low levels – is a health risk (*Gray 2009*). Particular attention has been focused on the potential use of xenon as a general anesthetic. Indeed, xenon in contrast to Argon and krypton, exerts anesthetic and analgesic effects under normal (as opposed to hyperbaric) atmospheric pressure conditions. Its physical properties and clinical characteristics suggest that xenon may be especially useful as an inhaled anesthetic; in fact, xenon produces rapid induction of and emergence from anesthesia, does not cause teratogenic effects or undergo biotransformation, and is essentially devoid of cardiovascular effects (*Lockwood 2006, Wappler 2007*).

At molecular level, xenon anesthetic effect is mediated by the NMDA; Franks et al. in the 1998 demonstrated firstly that xenon inhibited NMDA-evoked currents in cultured hippocampal neurons by ~60% at a clinically relevant concentration of 80% xenon (*Franks 1998*).

The discovery that xenon is an NMDA-receptor antagonist led to the idea that xenon may be neuroprotective. Indeed, it is well-known that one of the mechanisms of injury activated under conditions of energy substrate depletion (i.e. after different kinds of ischemic injuries) is the glutamate excitotoxicity, exerted by the excessive activation of NMDA

receptors. Xenon exerts a NMDA receptor antagonism at non-anesthetic concentrations avoiding or reducing adverse side effects and potential neurotoxicity associated with the NMDA receptor stimulation (*Lipton 2004, Palmer 2000*).

The purpose of the majority of the studies involving xenon was the neuroprotection after different kinds of ischemic injuries. Nevertheless, the effects as cardioprotective and nephroprotective agent have been investigated in animal and human studies as well.

Among the noble gases, xenon has been the most widely studied either in preclinical and clinical models. For this reason, the organ- protective properties of xenon will be addressed in the following section.

Thereby, the evidence for the organ-protective properties of xenon has prompted interest in investigating whether other inert gases have similar potential in ischemia reperfusion injury.

Briefly, neon was only explored in myocardial ischemia reperfusion injury in rabbits and showed a reduced infarct size when administered as preconditioning (*Pagel 2007*).

The organ protection by helium was explored in different model of ischemia reperfusion injury. More specifically, helium was shown to be neuroprotective in rodents subjected to cerebral ischemia (*Liu 2011, Pan 2011, David 2009, Pan 2007, Zhuang 2012*), and cardioprotective in rabbit model of myocardial infarction (*Pagel 2009 and 2008*). These studies explored three windows of protection (pre-, post- and reconditioning), and all these administration regimens have been demonstrated to be effective. Furthermore, there were few data on renal and hepatic ischemia reperfusion injury, where helium administration did not improve outcomes significantly (*Voss 1970, Braun 1973, Braun 2014*).

The organ protection by Argon will be discussed separately.

5.1 Organ protection by xenon

In order to evaluate its neuroprotective effects, xenon was administered either as precondition, recondition and postcondition treatment, to evaluate the cerebral damage *in vitro* and *in vivo* after ischemic stroke, asphyxia (both neonatal and intrauterine),

cardiopulmonary bypass and cardiac arrest. Rodent models were employed very frequently, both adult and neonatal. Swine model were employed as well, but the results were less straightforward at least in models of postconditioning after cardiac arrest. Interestingly, attempts to administer Xenon through echogenic liposomes were reported and the results were encouraging (*Britton 2010, Peng 2013*).

Overall, the results were in favor of the administration of Xenon as neuroprotective agent and the beneficial effect seemed to be equally exerted to both genders. Optimal timing and concentration of Xenon have not been determined yet although the neuroprotective effect seemed to be dose – dependent, with a more pronounced effect when administered within 8 hours after the insult (*Ma 2005, David 2008, Peng 2013*).

Models of neonatal asphyxia in rats to induce brain damage were employed in different experiments. Xenon at a concentration of 70% was evaluated as preconditioning before the right common carotid artery ligation followed by hypoxia (90 minutes): while a 2-h exposure before injury had no effects on ischemic brain injury, exposure 4-, 8-, or 24- h before ischemia reduced infarct volumes. A time point (4 h before hypoxia–ischemia) was chosen to investigate whether the morphologic improvement by xenon preconditioning extended to a long-term improvement in neurologic function. Authors found that, 30 days after the injury, the impairments in neurologic motor function and coordination were both significantly improved by xenon preconditioning. The same group performed a dose-response study and found that higher Xenon doses were more effective, as 75% xenon but not 30% reduced infarct size. Postconditioning with Xenon after both moderate and severe hypoxia–ischemic brain injury provides neuroprotection. Moreover, postconditioning with hypothermia were tested, as described in the dedicated section (*Hobbs 2008*). Another group used the same model of 90-minute hypoxic insult after unilateral carotid ligation in 7 days-old rats. Rats were then randomized to breathe 1 of 2 gas mixtures for 3 hours: 50% Xenon/30% O₂/20% N₂ or 30% O₂/70% N₂. One week after hypoxic-ischemic survival,

significant global protection was seen in the Xenon group (*Dingley 2006*). The neurologic improvement was seen even 10 weeks after the injury (*Hobbs 2008*). The effects of xenon on the neonatal hypoxic-ischemic encephalopathy was evaluated in a model of intrauterine perinatal asphyxia, where the treatment of the mother with subanesthetic doses of xenon (35%) provided both analgesia and neuroprotection, less neurologic deficit, neural loss, cellular disorganization, and cell shrinkage. A clinical application of these favorable preclinical evidences in birth asphyxia has been recently published. In this multicenter randomized, open-label, parallel-group trial. Infants (92) were included if: 36-43 weeks of gestational age, had signs of moderate to severe encephalopathy and moderately or severely abnormal background activity for at least 30 min or seizures and had one of the following: Apgar score of 5 or less 10 min after birth, continued need for resuscitation 10 min after birth, or acidosis within 1 h of birth. Participants were randomized within 12 h of birth to cooling at 33.5°C for 72 h (standard treatment) or to cooling in combination with 30% inhaled xenon for 24 h started immediately after randomization. This study demonstrated how the administration of xenon is feasible and apparently safe, but unfortunately is unlikely to enhance the neuroprotective effect of cooling after birth asphyxia (*Azzopardi 2016*).

Transient middle cerebral artery occlusion in mice was used to evaluate the treatment with Xenon in the setting transient focal cerebral ischemia. 70% Xenon administration during the injury improved both functional and histologic outcomes. In rats with transient brain ischemia through middle cerebral artery occlusion, exposure to Xenon at subanesthetic doses (50%) provided global neuroprotection up to at least 2 h after induction of ischemia. However, at a higher concentration (75%), Xenon exhibits potentially neurotoxic effects (*David 2008 and 2003*). Using a similar model of transient focal ischemia, authors couldn't confirm the benefit at 28 days when a subanesthetic Xenon concentration was used. A better outcome was also described in models of intracerebral hemorrhage.

In a global cerebral ischemia through bilateral common carotid artery occlusion in rats, 50 % Xenon administration for 45 minutes was neuroprotective 1 hour after the injury.

Studies on neuroprotection after cardiac arrest, mainly performed in swine models, paved the way to a study in the clinical scenario (*De Deken 2016*). Models of cardiac arrest of 8 to 10 minutes were employed to determine the effects of Xenon postconditioning. 70% Xenon administered 1 hour after resuscitation improved short-term neurologic and neurocognitive recovery whereas ventilation with Xenon 10 minutes after the cardiac arrest did not improve neurologic dysfunction. The combination of either a short (1 hr) or a long (18–24 hr) period of xenon administration and mild hypothermia immediately after resuscitation improved functional recovery and histologic injury.

The feasibility and cardiac safety of inhaled Xenon treatment in a clinical setting were firstly investigated through an open controlled and randomized single-centre clinical trial involving 36 out-of-hospital cardiac arrest patients, with ventricular fibrillation or pulseless ventricular tachycardia as initial cardiac rhythm. Patients were randomly assigned to receive either mild therapeutic hypothermia treatment with target temperature of 33°C alone or in combination with 40% Xenon for 24 hours. The frequency of serious adverse events, including in-hospital mortality, status epilepticus, and acute kidney injury, was similar in both groups and there were no unexpected serious adverse reactions to xenon during hospital stay. In addition, xenon did not induce significant conduction, repolarization, or rhythm abnormalities. Xenon treatment in combination with hypothermia showed favorable cardiac features in survivors, as median dose of norepinephrine during hypothermia was significantly lower, heart rate was significantly lower and post hospital arrival incremental change in troponin-T at 72 hours was significantly less in the Xenon + mild therapeutic hypothermia group.

A randomized single-blind phase 2 clinical trial randomized 110 comatose patients who had experienced out-of-hospital cardiac arrest to receive either inhaled Xenon (40% end-

tidal concentration) combined with hypothermia (33°C) for 24 hours or hypothermia treatment alone. The effect of inhaled Xenon on ischemic white matter damage was assessed with magnetic resonance imaging between 36 and 52 hours after cardiac arrest (by fractional anisotropy from diffusion tensor magnetic resonance imaging). Inhaled Xenon combined with hypothermia compared with hypothermia alone resulted in less white matter damage but no statistically significant difference in neurological outcomes or mortality at 6 months was demonstrated. Later, the same group assessed the effect of inhaled Xenon on myocardial ischemic damage in the study population (Arola 2017). Myocardial ischemic damage was assessed by Troponin-T levels at hospital admission, and at 24 h, 48 h, and 72 h post-cardiac arrest. After adjustments for age, sex, study site, primary coronary percutaneous intervention (PCI), and norepinephrine dose, the post-arrival incremental change of the ln-transformed troponin-T at 72 h was significantly reduced in the group treated with Xenon. The effect of Xenon on the change in the troponin-T values did not differ in patients with or without PCI or in those with a diagnosis of ST-segment elevation myocardial infarction (Arola 2017).

Based on evidences of cardioprotection in a variety of preclinical models, (rat, ex-vivo rabbit heart, pig, and dog) a recent randomized trial evaluated the effects of Xenon anaesthesia in 492 low-risk elective, on-pump coronary artery bypass graft surgery patients in 17 university hospitals in Europe. Patients were randomized to receive Xenon, sevoflurane, or propofol-based total intravenous anesthesia for anesthesia maintenance. Authors found that in postoperative cardiac troponin I release, Xenon was non-inferior to sevoflurane and that cardiac troponin I release was less than with total intravenous anesthesia. Moreover, Xenon anesthesia appeared safe and feasible, as previously demonstrated for other surgical settings.

Experiments on nephroprotection showed improved kidney function with Xenon administration, in animal models of rat transplant with Xenon preconditioning,

postconditioning, and reconditioning of the kidney graft, stored in a Xenon-saturated preservation solution (*De Deken 2016*).

Surgical patients undergoing partial nephrectomy were included in a small randomized trial to receive xenon or isoflurane, with the aim to explore whether Xenon anesthesia could reduce renal damage and to gather pilot data of possible nephroprotection. Xenon anesthesia was feasible and safe with regard to postoperative renal function, but no significant effect on early renal function were found (*Stevanovic 2017*).

The combination of Xenon and hypothermia was tested in many preclinical models. The majority of results suggest that the administration of these two treatments, both simultaneously or delayed, potentiate the neuroprotection provided by Xenon even if a low concentration is used; this additive effect seem to be conserved either in case of short or long period of xenon administration (*Ma 2005 Hobbs 2008, Sheng 2012, Martin 2007, Fries 2012, Chakkarapani 2010, Faulkner 2011, Schwiebert 2010*). Given these preclinical evidences, the association of Xenon and hypothermia has been evaluated as potentially beneficial in a population of out-of-hospital cardiac arrest patients in two recent trials (*Arola 2017, Laitio 2016*) and in infants affected by birth asphyxia.

Anesthetic properties of Xenon were discovered more than 60 years ago, and since then, a variety of clinical experiences demonstrated that it is a safe drug. Thanks to its small blood-gas partition, Xenon offers a rapid onset and offset of its action. It can produce profound analgesia and hypnosis without producing hemodynamic instability as described in various clinical experiences involving different surgical settings, either non-cardiac or cardiac surgery, and in patients with impaired cardiac function (*Arola 2017, Laitio 2016*).

Xenon production is highly energy-consuming requiring a fractional distillation process of liquid air. Moreover, a special equipment is required to deliver Xenon. As a result, Xenon treatment is very expensive, from 3 to 10 folds more than other anesthetic agents

(sevoflurane, isoflurane, propofol) and 100 folds more than Argon. As a future perspective, the use of microbubbles and liposomes to deliver Xenon could allow a cheaper and more efficient way to treat selected patients.

5.2 Argon

Argon belongs to the noble gas group, discovered by the British physicist Lord Rayleigh and the chemist Sir Ramsay, in 1894 (*Ramsay 1984*). Argon is colorless, tasteless, odorless, noncorrosive, noninflammable and nontoxic (*Material Safety Data Sheet Gaseous Argon, Universal Industrial Gases, Inc. Retrieved 14 October 2013. http://www.uigi.com/MSDS_gaseous_Ar.html*). It is the most abundant noble gas, and the third major component of the air, with a concentration of 0.93%. From a physical point of view, Argon's density is 38% higher than air's one, the solubility in water and plasma is 24-fold lesser than that of carbon dioxide (CO₂), but 5-fold greater in oil compared to water (*Roberts 1997*).

Like the other noble gases, Argon has a full electron valence shell which avoids covalent bindings with other elements; for this it is usually considered a non-reactive chemical gas, as pinpointed by its Greek name “αργός”, meaning “inert” (*Ulbrich 2016*). But, as mentioned above, starting from the evidence of the narcotic effects of Argon in divers in 1939 (*Behnke 1939*), it became evident that it is able to exert biological effects. Further confirmatory evidences of such a Argon's property were added over time (*Taylor 1976, Sauter 1979, Smith 2010, Rostain 2011, Clark 2015*).

Argon showed narcotic effects only under hyperbaric conditions (*Balon 2002, Fowler 1972*). Different theories have been proposed to explain this property (*Rostain 2011*). Most likely, the increase of the inert gas pressure induces modification on cytoplasmic membrane and/or receptor condition, subsequently modifying the neurotransmission. Indeed, when rats were exposed to Argon at ≈ 19 atm for 2 h, dopamine release from striatum decreased by approximately 10% compared to animals that were not exposed to the gas (*Balon 2002*). Since the striatum coordinates different aspects of motor planning and motivation, a

subsequent study investigated the relationship between motor activity and dopamine release in rats exposed to Argon in a pressurized chamber for 100 min. During the compression period, a transient phase of hyperactivity was observed, followed by a decrease in the motor activity. More specifically, exposure to Argon at a pressure of ≈ 10 atm, showed motor hyperactivity, probably related both to the higher pressure and to the narcotic potency of the gas, dependent on its lipid solubility. When the pressure raised to ≈ 20 atm, loss of righting reflex appeared, indicating the onset of the anesthetic effect. This subsequent decrease of behavioral activity could be ascribed to the decrease of dopamine release from the striatum that occurred when the 20 atm pressure was achieved and was kept constant for at least 10 min (*Balon 2003*).

Another study concerning Argon anesthetic properties analyzed the contribution of GABA transmission as a possible mechanism of action for narcosis. Rats were treated with different GABA receptor antagonists (i.e. gabazine and flumazenil, as GABA_A antagonists, and 2-idrossisaclofene, as GABA_B antagonist) and then exposed to Argon in a pressurized chamber. Pretreatment with GABA_A inhibitors increased significantly the Argon threshold pressure for the loss of righting reflex. Instead, pretreatment with GABA_B inhibitor showed no significant effects on narcosis (*Abraini 2003*)

Besides narcosis, evidence of neuroprotection played by this noble gas has been arising during the last 20 years, since the first Russian report in a rodent model of hypoxic brain injury, in 1998 (*Soldatov 1998*). Recently, Argon's protective effects after ischemia became more and more evident preclinically.

5.2.1 *In vitro* studies

The protective effects of Argon on cell damage were investigated in several *in vitro* studies together with potential pathways activated after Argon exposure and involved in cell protection. These studies were performed in different cell types (i.e. rodent cardiomyocytes, rat neurons or hippocampal slices, human neuroblastoma, kidney, airway

epithelium, and osteosarcoma cell sand in whole blood from rodents) and the models for inducing cell damage were different, including oxygen-glucose deprivation (OGD); hypoxia/reoxygenation injury (H/R); peroxide-induced oxidative stress; and mechanical means to simulate a traumatic brain injury. In these studies, cells and/or tissues were incubated in chambers containing Argon atmosphere in a concentration varying from 25% to 75% in air and/or O₂. The average Argon exposure was 90 min, ranging from a minimum of 5 min in the H/R study (Lemoine 2017) up to 72 hrs in the traumatic brain injury simulation (Loetscher 2009). In the majority of the studies, Argon was applied as a potential treatment and thus administered either immediately after the onset of the insult or delayed, up to 3 hrs later. In only 3 studies, targeting specific protective pathways, Argon was administered before the insult, as a preconditioning agent (Mayer 2016, Hafner 2016, Ulbrich 2016)

Overall, the *in vitro* studies showed improved cell survival and viability when Argon was used both before and after the insult. More specifically, Argon appeared to be capable to reduce apoptosis in neuronal, tubular kidney, and airway epithelial cells (Hafner 2016, Ulbrich 2016).

5.2.2 *In vivo* studies

The majority of *in vivo* studies are focused on neuroprotection after a variety of brain damages; from these studies, combining different neurological, behavioral, and behavioral tests with histological and biochemical assays, evidence in favor of a beneficial effect of Argon treatment emerged. In all these studies, Argon was administered by inhalation, at a concentration varying from 25% up to 88% in air or in a mixture of Nitrogen (N₂)/O₂. Focusing on the use of Argon as neuroprotective agent, 4 studies (David 2012, Höllig 2016, Zhuang 2012, Ryang 2011) evaluated the post-insult neurological recovery and in half of them (Zhuang 2012, Ryang 2011) there was evidence for a better outcome after inhalation of this noble gas when compared to the control ventilation.

All the investigations performed a histological evaluation, including various essays in different brain areas. Overall, there was evidence for a mitigation of the brain tissue injury as represented by increased cell viability and reduced neuronal necrosis and apoptosis and infarct size. In one study on a rat model of MCAO (David 2012). Argon had contrasting effects, with a reduced infarct size in the cortical area, but an increased injury in the subcortical ones, compared to sham animals. However, in this study, animals treated with Argon had a significantly higher body temperature (average of 38.5°C), which could explain the greater brain damage compared to the controls (David 2012).

The cardioprotective effects of Argon were investigated in three *in vivo* studies and are commented in details in the Discussion as well as studies focused on neurological injury following resuscitation from CA.

Overall, both the *in vitro* and *in vivo* studies provided evidence in support of a protective role of Argon, independently of the disease model and of the duration and onset of exposure to the treatment (i.e. pre- or post-acute event).

5.2.3 Clinical uses of Argon

Since the Sixties, Argon was applied clinically for the measurement of lung volumes, i.e. total lung capacity (TLC) (Black 1984), functional residual capacity (FRC) (Ozanne 1981), and closing volumes (McCarthy 1972). For these measurements, the re-breathing/gas-dilution technique was used. Healthy volunteers were exposed to a known volume of Argon 50% in O₂, that was re-breathed in a closed system. After a few breaths the new Argon concentration was detected and lung volumes derived (Black 1984, Ozanne 1981). A single breath method, instead, was applied to measure the lung closing volumes (McCarthy 1972). Considering the non-invasive estimation of CO, Argon 6-7 %, as inert and insoluble gas, has been used as part of a mixture along with other inert but soluble gases, i.e freon-22 (or acetylene), O₂ and N₂. This mixture was re-breathed by the patient through a re-breathing bag and, at the same time, a mass spectrometer measured the

concentration curve of the inert gases and calculated the wash-out rate, which is proportional to the CO (Henrghan1981, Bonde-Petersen 1980). Another use of Argon inhalation in humans was the estimation of global myocardial blood flow (MBF) through the measure of the difference of Ar concentration in the arterial and the coronary sinus blood during inhalation of a mixture of Ar 75% in O₂ (Kotzerke 2001). In these studies, focused on evaluating lung and cardiac functions, subjects were exposed to a single or a few breaths of an isobaric mixture of Argon; therefore, no conclusions on effects of Argon or potentials safety issues may be extrapolated.

Of different value were instead the studies conducted on divers with the intent to investigate the feasibility of Argon use in the diving mixture in place of N₂. In these studies, divers breathed Argon mixtures for long periods and in hyperbaric conditions. In 1939, motor and cognitive impairment, such as slowed mental activity, inability to perform efficient manual work and emotional disturbances, which might lead to loss of consciousness, were described in divers breathing a mixture of 69% Argon, 20% O₂ and 11% N₂ from 1 to 10 atm. It was the first report on Argon's narcotic properties under hyperbaric conditions (Behnke 1939). In another study, 10 subjects performed mental arithmetic tasks while exposed to Argon 80% in O₂ at 1, 4 and 7 atm. Again, Argon under hyperbaric condition showed more narcotic effects (Fowler 1972).

The longest exposure to Argon under hyperbaric condition was in 1998 (Pavlov 1999). Four male volunteers participated in a simulated diving at 10 meters for 7 days to determine effects on physiological variables of to the following gas mixture: O₂ 0.2 ± 0.005 kg/cm², N₂ 0.8 ± 0.01 kg/cm² and Argon 1.0 ± 0.01 kg/cm². No effects on central nervous system electrophysiology and functional test, cardiopulmonary system, i.e. ECG, oxihemometry, and biochemical analysis of urine, were detected. All measured data did not exceed physiological range. Thus, the prolonged normoxic exposure to Argon was safe. Under hyperbaric pressure a workload of 100 Wt was performed 62% higher when

breathing Ar 15% in O₂ than N₂ 15% in O₂. Moreover, the exposure to a hypoxic Argon-N₂ mixture containing O₂ 0.15 ± 0.005 kg/cm², instead of O₂ 0.2 ± 0.005 kg/cm², determined an increase in the performed work volume, showing that Argon causes a positive effect on organism adaptation to hypoxia.

Another study performed in 8 human volunteers investigated the possible adverse effects from breathing isobaric mixture of 80% Argon and 20% O₂ for 30 min to determine if Argon might cause neurological impairment or embolism. The authors did not report narcosis or coagulation and fibrinolytic abnormalities, which would have been present in case of gas embolism (*Horrigan 1979*).

More recently, Argon inhalation has been proposed as a new method to measure the global cerebral blood flow (CBF), by a modification of the Kety-Schmidt inert gas saturation technique. Patients were exposed to Argon 70% in O₂ and during a 10-min wash-in period, blood samples from the arterial and the jugular bulb catheter were withdrawn simultaneously at a constant rate. Then, Argon concentration in the samples was measured through gas chromatography and CBF was calculated (*Mielck 1999*). To establish this new method, the presence of any potential influence of Argon inhalation on cerebrovascular and cerebro-metabolic variables needed to be excluded. Thus, additional 30 anesthetized patients undergoing cardiovascular surgery were subjected to ventilation with 70% Ar in O₂ for 15 min, prior to the surgical procedure (*Grüne 2017*). Argon ventilation did not show any influence on cerebrovascular circulation or metabolism, evaluated by transcranial Doppler sonography nor difference in content between arterial and jugular-venous lactate, glucose and oxygen, respectively.

6 Aims and hypothesis

The aim of my PhD studies was to investigate the neuroprotective and cardioprotective effects of the noble gas Argon using preclinical models of CA and CPR.

The overall aim of my studies was to identify a novel treatment option and provide solid efficacy and safety data, requested for future clinical investigation. The consequent research programme included complementary experiments with specific aims, which I have detailed in the experimental design section.

The overarching hypothesis was that Argon treatment would ameliorate post-resuscitation neurological dysfunction in different animal models of CA and CPR.

Material and methods

1 Experimental design

Procedures involving animals and their care were performed in accordance with the institutional guidelines in compliance with the national and international law and policies. The protocols were reviewed and approved by the Animal Care and Use Committee of the IRCCS – Istituto di Ricerche Farmacologiche “Mario Negri”, Milano and by the Italian Health Ministry (Legislative Decree n° 76/2014- B).

The thesis work includes a spectrum of different experiments with specific aims, performed in three different animal models of both CA and CPR and myocardial ischemia reperfusion injury in order to investigate the potential protective neuro- and myocardial effect of Argon.

The experimental design, together with the experimental groups are schematically detailed in the table 4.

The overall aim of my studies was to identify a novel treatment option and provide solid efficacy and safety data, requested for future clinical investigation. The consequent research programme included complementary experiments both in pig (efficacy and safety) and rats (mechanism of action).

Specifically, the effects of post-resuscitation treatment with inhaled Argon on outcome of CPR were initially investigated in a porcine model of short duration of no flow time (i.e. 6 minutes of ventricular fibrillation) and CPR (*Experiment 1*). The protective effects of observed in experiment 1 were subsequently validated in the more severe model of CA (i.e. 12 minutes of ventricular fibrillation) and CPR in pigs (Experiment 2). A model of prolonged untreated CA was used to obtain greater ischemic injury and worse outcome. In addition, the experiment 2 aimed to optimize the Argon treatment in the same model of

severe CA, by testing the effects of different Argon concentrations in the inhaled mixture on brain injury.

Finally, in order to investigate the safety of Argon ventilation, anesthetized domestic healthy pigs were ventilated with Argon and potentially harmful adverse effects were evaluated (*Experiment 3*).

The effect of different no-flow durations on post-resuscitation myocardial and neurological injury and survival in a pig model have been investigated with the aim to identify an optimal duration that adequately reflects the clinical scenario. This experiment is fully described in the Appendix 3. A duration of untreated CA of 12-13 min may be an optimal choice for clinically relevant CA models.

National laws about experimental research are changed over time. For this reason anesthesia and drugs administration during experiments changed among different experiments. All the doses of anesthetic and analgesic drugs are due to minimize pain and are imposed by the national restrictive policy regulating experimental research in animals.

Experiment	Experimental model	Argon treatment duration	Observation	Experimental groups	Endpoint
1	Pigs, CA 8 mins and CPR 5 mins	4 h	72 h	70% N ₂ - 30% O ₂ 70% Ar- 30% O ₂	Efficacy
2	Pigs, CA 12 mins and CPR 5 mins	4 h	96 h	70% N ₂ - 30% O ₂ 50% Ar- 30% O ₂ - 20% N ₂ 70% Ar- 30% O ₂	Efficacy
3	Healthy pigs	4 h	72 h	70% Ar- 30% O ₂	Safety
4	Rats, CA 7 mins and CPR 5 mins	2 h	2 h	70% N ₂ - 30% O ₂ 70% Ar- 30% O ₂	Mechanism of action-brain injury
5	Rats, Coronary artery occlusion 30 mins and reperfusion	1 h	6 h or 24 h	70% N ₂ - 30% O ₂ 70% Ar- 30% O ₂	Mechanism of action-myocardial injury

Table 4. Experimental design. Ar, Argon. N₂, nitrogen. O₂, oxygen. CA, cardiac arrest. CPR, cardiopulmonary resuscitation. H, hours.

2 Pig model of short duration CA and CPR- Experiment 1

2.1 Animal preparation

Twelve male domestic pigs (38 ± 1 kg) were supplied by a single-source breeder and were housed in a conditioned room with a 12-h light-dark cycle. Pigs were fasted the night before experiment except for free access to water. Anesthesia was induced by intramuscular injection of ketamine (20 mg/kg) and completed by ear vein injection of sodium pentobarbital (30 mg/kg). Additional doses of pentobarbital (8 mg/kg) were administered at intervals of approximately 1 hr or when necessary (if response to painful stimuli or awakening signs were present) to maintain anesthesia. Animals were placed on a V-board and secured with rope before starting the surgical procedures. Animals received 500 ml of fluids before the induction of VF and 500 ml after ROSC. A cuffed tracheal tube was inserted and animals were mechanically ventilated with a volume-controlled ventilator (Servo 300, Siemens, Sweden), with a tidal volume of 15 mL/kg, peak flow of 40 L/min, and FiO₂ of 0.21. End-tidal CO₂ (EtCO₂) was monitored with an infrared capnometer (MRx, Philips Medical Systems, Andover, MA). Respiratory frequency was adjusted to maintain EtCO₂ between 35-40 mmHg prior to inducing cardiac arrest and post-resuscitation (*Ristagno 2007 and 2008*). No positive end expiration pressure (PEEP) was applied.

A fluid filled 7-Fr catheter (Edwards Lifesciences, Irvine, CA) was advanced from the right femoral artery into the thoracic aorta to measure aortic pressure. For measurements of right atrial pressure (RAP), core temperature, and thermodilution CO, a 7-Fr, 5-lumen, thermodilution-tipped catheter was advanced from the right femoral vein into the pulmonary artery. Conventional pressure transducers were used (Medex TranStar, Monsey, NY). Myocardial infarction was induced in a closed-chest preparation by intraluminal occlusion of the left anterior descending (LAD) coronary artery between the first and second diagonal branches. Briefly, a 6-Fr balloon-tipped catheter was inserted from the

right common carotid artery and advanced into the LAD; its position was confirmed with contrast medium (Renografin-76, Squibb Diagnostics, New Brunswick, NJ). For inducing VF, a 5-Fr pacing catheter (EP Technologies, Inc., Mountain View, CA) was advanced from the right subclavian vein into the right ventricle (*Ristagno 2008*). The position of all catheters was confirmed by characteristic pressure morphology and/or fluoroscopy before and after CPR. For recording the frontal plane ECG, three adhesive electrodes were applied to the skin of the proximal right and left upper and lower limbs.

Experimental procedures

Fifteen min prior to inducing cardiac arrest, the animals were allocated into one of the two study groups: 1) “Argon treatment” (n=6), in which animals were mechanically ventilated with an experimental inhalation mixture of 70% Argon – 30% oxygen for 4 hr post-resuscitation; or 2) “Control treatment” (n=6), in which animals were mechanically ventilated with a control inhalation mixture of 70% nitrogen – 30% oxygen for 4 hr post-resuscitation.

The balloon of the LAD catheter was then inflated with 0.7 mL of air to occlude the flow. If VF did not occur spontaneously after 10 min of LAD occlusion, it was induced with 1–2 mA alternating current delivered to the right ventricle endocardium. Ventilation was discontinued after onset of VF. After 8 min of untreated VF, CPR was initiated. Precordial compressions were performed with the LUCAS 2 compressor (PhysioControl Inc., Sweden). Coincident with the start of precordial compressions, the animals were ventilated with a tidal volume of 500 mL, 10 breaths/min, and FiO₂ of 1.0. After 2 min of CPR, adrenaline (30 µg/kg) was administered via the right atrium. After 5 min of CPR, defibrillation was attempted with a single biphasic 150-J shock, using a MRx defibrillator (Philips Medical Systems). During CPR manoeuvres, pigs were stabilized using a custom made V- board. If resuscitation was not achieved, CPR was immediately resumed and continued for 1 min prior to a subsequent defibrillation. Pads with latero-lateral placement were used for defibrillation. Additional dose of adrenaline was given after 7 min of CPR.

Successful resuscitation was defined as restoration of an organized cardiac rhythm with a mean arterial pressure (MAP) of more than 60 mmHg, which persisted for more than 1 min. If a recurrent VF occurred after successful resuscitation, it was treated with immediate delivery of biphasic 150-J shocks.

Immediately after ROSC, the LAD catheter placement was reconfirmed by fluoroscopy and then the experimental or control inhalation was started. Anesthesia was maintained and animals were invasively monitored during the 4-h treatment. Forty-five min after resuscitation, the balloon was deflated and the LAD catheter withdrawn. Temperature of the animals was maintained at $38^{\circ}\text{C} \pm 0.5^{\circ}\text{C}$ during the whole experiment, with the aid of infrared lamps. After 4 hr of treatment, catheters were removed, wounds were repaired surgically, and the animals were extubated and returned to their cages. Analgesia with butorphanol (0.1 mg/kg) was administered by intramuscular injection. At the end of the 72 hr post-resuscitation observation, animals were reanesthetized reanesthetized as described before (ketamine and pentobarbital) for echocardiographic examination and blood sample withdrawn. Animals were then sacrificed painlessly with an intravenous injection of 150 mg/kg pentobarbital, before heart and brain were harvested. Autopsy was performed routinely for documentation of potential injuries during CPR or due to obfuscating disease.

2.2 *Measurements*

Hemodynamics, EtCO₂, and ECG were recorded continuously on a PC-based acquisition system (CODAS hardware/software). The coronary perfusion pressure (CPP) was computed from the differences in time-coincident diastolic aortic pressure (DAP) and RAP. CO was measured by thermodilution technique after injection of 5 mL of saline at 0-5 °C (COM-2, Baxter, IL). Echocardiography was performed at baseline and at 2, 4, and 72 hr post-resuscitation using a phase-array multifrequency 2.5-5 MHz probe (MyLab 30Gold, Esaote S.p.A., Italy). Parasternal long-axis, short axis at base, mid papillary and apical level, and 2&4 apical chamber views were obtained. LV end-systolic, end-diastolic volumes and EF were calculated by the Simpson's method (*Ristagno 2007*).

Arterial blood gases were assessed with i-STAT System (Abbott Laboratories, IL, USA), at baseline, and at 2 and 4 hr post-resuscitation. Plasma hs-cTnT was measured with an electrochemiluminescence assay (ECLIA, Elecsys 2010 analyzer, Roche Diagnostics, Germany), at baseline and 2, 4, and 72 hr post-resuscitation. Serum neuron specific enolase (NSE) was also measured with an electrochemiluminescence assay (Roche Diagnostics), at baseline and 72 hr post-resuscitation.

The neurological alertness score (NAS) was used for evaluating neurological recovery at 24, 48, and 72 hr post-resuscitation. Briefly, the test consisted of five items: level of consciousness, respiration, posture, feeding, and self care behavior. Each of the items was graded by severity contributing to a final score ranging from 100 (normal) to 0 (brain death). The swine neurologic deficit score (NDS) that included evaluation of consciousness, breathing, motor/sensory function, and behavior, was also assessed. A score of 0 was considered normal and a score of 400 was brain death. Finally, 72 hr performance was evaluated according to overall performance categories (OPC) as follows: 1) normal (able to walk and eat); 2) slight disability (able to sit but not stand or walk); 3) severe disability (unaware of surroundings, withdraws to pain); 4) coma (some reflexes or pathologic movements, but no response to pain); and 5) brain death or death. Scores were assessed by veterinarian doctors blinded to treatment. No inter-rater test was performed.

At sacrifice, the brains were carefully removed from the skulls and fixed in 4% buffered formalin. Standardized 5-mm coronal slices were taken. The hippocampus was chosen as region of interest and was paraffin embedded. Five μm -thick sections were then obtained and stained with hematoxylin-eosin. An 8-mm tract of the CA1, including the layer of pyramidal cells, was considered. The proportion of neuronal loss and degeneration (shrunken neurons with deeply acidophilic cytoplasm and pyknotic nucleus) was quantified as absent (0), rare (1), few (2), and numerous (3), in relation to the total amount of cells. Immunohistochemistry with antibody against microglia-specific ionized calcium binding adaptor molecule-1 (Iba1) was used to detect reactive microglia activation. Iba1 stained

sections were analyzed with ImageJ software (<http://rsb.info.nih.gov/ij/>). The extent of reactive microglia was quantified and expressed as percentage of the layer of pyramidal cells involved by reactive microglial activation. An experienced pathologist, blinded to treatment, performed the assessments.

Myocardial infarct was assessed by tetrazolium chloride (TTC) staining. The LV was sliced into 5-mm thick transverse sections which were incubated (20 min) in a solution of TTC and then transferred to 4% formalin overnight before image analysis (Analytical Imaging Station 3.0, Imaging Research, Canada). The pale area (TTC-negative) was considered dead tissue. Infarct size was reported as percentage of TTC-negative area relative to LV area.

2.3 Statistical analyses

For comparisons between time-based measurements within the two groups, Repeated Measures Analysis of Variance (ANOVA), which enabled time and treatment effects and their interaction to be evaluated, was used. The same analysis allowed for within-subjects comparison between two subsequent times. Non parametric Mann-Whitney U-test was used for variables that were not normally distributed. For comparisons between groups at the given time points, one-way ANOVA was used. When the dependent variable was categorical, χ^2 test was performed. Statistical analysis was performed using SPSS 16.0 software (SPSS, Inc., Chicago, IL). A value of $p < 0.05$ was regarded as statistically significant.

3 Pig model of prolonged CA and CPR- Experiment 2

3.1 Animal preparation

Thirty-six male domestic pigs (39 ± 2 kg) were fasted the night before experiment except for free access to water. Anesthesia was induced by intramuscular injection of ketamine (20 mg/kg) followed by intravenous administration of propofol (2 mg/kg) and sufentanyl (0.3 μ g/kg) through an ear vein access. Anesthesia was then maintained by continuous intravenous infusion of propofol (4-8 mg/kg/h) and sufentanyl (0.3 μ g/kg/h). A cuffed tracheal tube was placed, and animals were mechanically ventilated with a tidal volume of 15 mL/kg and FiO_2 of 0.21. Respiratory frequency was adjusted to maintain the $EtCO_2$ between 35 and 40 mmHg, monitored with an infrared capnometer (*Ristagno 2014*). No PEEP was applied. For measurement of aortic pressure, a fluid-filled 7F catheter was advanced from the right femoral artery into the thoracic aorta. For measurements of RAP, core temperature, and CO, a 7F pentalum thermodilution catheter was advanced from the right femoral vein into the pulmonary artery. Conventional pressure transducers were used (MedexTransStar, Monsey, NY). Myocardial infarction was induced in a closed-chest preparation by intraluminal occlusion of the LAD coronary artery. More in details, a 6F balloon-tipped catheter was inserted from the right common carotid artery and advanced into the aorta and then into the LAD, beyond the first diagonal branch, with the aid of image intensification and confirmed by injection of radiographic contrast media. A 5F pacing catheter was advanced from the right subclavian vein into the right ventricle to induce VF (*Babini 2017*). The position of all catheters was confirmed by characteristic pressure morphology and/or fluoroscopy. Frontal plane electrocardiogram was recorded.

3.2 Experimental procedures

Fifteen minutes before inducing CA, the animals were allocated using the envelop method into one of the following study groups: (1) *Control*: post- resuscitation 4 hours ventilation with 70% nitrogen- 30% oxygen (n=12); (2) *Argon 50%*: post- resuscitation 4 hours ventilation with 50% Argon- 50% oxygen (n=12); (3) *Argon 70%*: post- resuscitation 4

hours ventilation with 70% Argon- 30% oxygen (n=12). Argon or control treatments were initiated within 5 min following resuscitation, after hemodynamic stabilization.

The balloon of the LAD coronary artery catheter was then inflated with 0.7 mL of air to occlude the flow. Occlusion was confirmed by the rapid occurrence of progressive electrocardiographic ST segment elevation. If VF did not occur spontaneously, after 10 min it was induced with 1 to 2 mA AC current delivered to the right ventricular endocardium. Ventilation was discontinued after onset of VF. After 12 min of untreated VF, CPR, including chest compressions with the LUCAS 2 (PhysioControl Inc, Lund, Sweden) and ventilation with oxygen (tidal volume of 500 mL, 10 breaths/min), was initiated. After 5 min of CPR, defibrillation was attempted with a single biphasic 150-J shock, using an MRx defibrillator (Philips Medical Systems, Andover, MA). If resuscitation was not achieved, CPR was resumed and continued for 1 min before a subsequent defibrillation. Adrenaline (30 µg/kg) was administered via the right atrium after 2 and 7 min of CPR. Successful resuscitation was defined as restoration of an organized cardiac rhythm with a MAP of more than 60 mmHg, which persisted for more than 1 min. After that, if VF reoccurred, it was treated by immediate defibrillation. During CPR, the LAD occlusion balloon was deflated to avoid possible rupture during mechanical chest compression, but the catheter was left in place in order to maintain a partial occlusion of the LAD, which was approximately 75% of the internal lumen (*Babini 2017*). After successful resuscitation, anesthesia was maintained, and animals were monitored during the 4-h treatment. Forty-five minutes after resuscitation, the LAD coronary artery catheter was withdrawn. Temperature of the animals was maintained at 38°C ± 0.5°C during the whole experiment. After 4 h of treatment, catheters were removed, wounds were repaired, and the animals were extubated and returned to their cages. Analgesia with butorphanol (0.1 mg/kg) was administered by intramuscular injection. At the end of the post-resuscitation observation period, up to 96 h, animals were re-anesthetized as described

before (ketamine and propofol) for echocardiographic examination and blood sample withdrawn. Animals were then sacrificed painlessly with an intravenous injection of 150 mg/kg tiopental, and heart and brain were harvested. Autopsy was performed routinely for potential injuries due to CPR or obfuscating disease.

3.3 Measurements

Hemodynamics, EtCO₂, and electrocardiogram were recorded continuously on a personal computer-based acquisition system (WinDaq DATAQ Instruments Inc, Akron, OH). The coronary perfusion pressure was computed from the differences in time-coincident diastolic aortic pressure and right atrial pressure. CO was measured by thermodilution technique (COM-2; Baxter International Inc, Deerfield, IL). Transthoracic echocardiography was performed using a phase-array multifrequency 2.5- to 5-MHz probe (CX50, Philips, The Netherlands). Two-dimensional apical four chamber view was acquired to determine LV volumes and EF, calculations were computed using the modified single-plane Simpson's rule (*Babini 2017*). Arterial blood gases were assessed with i-STAT System (Abbott Laboratories, Princeton, NJ), at baseline and at 2 and 4 h post-resuscitation. Plasma hs-cTnT at baseline and 2, 4, and 96 h postresuscitation and NSE at baseline and 96 h post- resuscitation were measured with electrochemiluminescence assays (Roche Diagnostics Italia, Monza, Italy).

As previously described (*Ristagno 2014*), neurologic recovery at 24, 48, and 96 h post-resuscitation was assessed with the NAS, ranging from 100 (normal) to 0 (brain death), and with the swine NDS, ranging from 0 (normal) and 400 (brain death). Finally, the functional recovery was evaluated prior to sacrifice according to OPC as follows: 1 = normal, 2 = slight disability, 3 = severe disability, 4 = coma, and 5 = brain death or death. Outcome was defined poor when OPC was ≥ 3 . Scores were assessed by veterinarian doctors blinded to treatment.

Brain and heart histology were evaluated as described above.

3.4 Statistical analysis

One sample Kolmogorov–Smirnov Z test was used to confirm normal distribution of the data. For comparisons of time-based variables, two-way ANOVA with the Tukey's multiple comparisons test was used. Time-based variables not normally distributed were corrected by logarithmic transformation before the analysis. For comparisons between groups at the given time points, one-way ANOVA with Tukey's multiple comparison was used for parametric variables, while Kruskal-Wallis test with Dunn's multiple comparison was used for not normally distributed variables. Parametric variables are expressed as mean \pm SEM, whereas non parametric variables and presented as median [Q1-Q3]. When the dependent variable was categorical, χ^2 test for trend test was performed. Statistical analyses were performed using GraphPad Prism 7.02 (GraphPad Software Inc.). A p value ≤ 0.05 was considered statistically significant.

4 Safety- Experiment 3

4.1 Animal preparation

Five pigs (36±1 kg) were anesthetized by intramuscular injection of ketamine (20 mg/kg) followed by intravenous administration of propofol (2 mg/kg) and sufentanyl (0.3 µg/kg) through an ear vein access. Anesthesia was then maintained by continuous intravenous infusion of propofol (4-8 mg/kg/h) and sufentanyl (0.3 µg/kg/h). Animals were , orotracheally intubated and mechanically ventilated (tidal volume 15 mL/kg, positive end-expiratory pressure (PEEP) 5 cmH₂O, Inspired O₂ fraction (F_IO₂) 0.21, and respiratory frequency adjusted to maintain the EtCO₂ between 35-40 mmHg. An arterial line was introduced in Aorta through the femoral artery to monitor arterial pressure.

4.2 Experimental procedures and measurements

After recording baseline variables, the anesthetized animals were ventilated with a mixture of 70% Argon in O₂ for 4 h. Arterial pressure and ventilatory parameters were continuously monitored and recorded (WinDaq DATAQ Instruments Inc, Akron, OH), together with hourly blood samples for blood gas analysis, including also hemoglobin (Hb), hematocrit (Ht) and electrolytes with i-STAT System (Abbott Laboratories, Princeton, NJ), and assessment of circulating markers of cardiac, liver and renal injury, i.e. plasma hs-cTnT (electrochemiluminescence assay, Roche Diagnostics Italia, Monza, Italy), and serum alanine aminotransferase (ALT) and creatinine (colorimetric assays, Cayman Chemical).

At the end of the 4 hrs of observation, animals were weaned and returned to their cages. They were observed up to 72 hrs, after which another blood sample was obtained and animals were then sacrificed painlessly.

5 Rat model of CA and CPR- Experiment 4

5.1 Animal preparation

Thirty- seven male Sprague Dawley rats (500 g) were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). Additional doses (10 mg/kg) of pentobarbital were administrated at intervals of approximately 1 h or when necessary to maintain anesthesia. The trachea was orally intubated with a 14-gauge cannula as previously reported (*Fumagalli 2014*). A PE-50 catheter was advanced into the descending aorta from the left femoral artery for measurement of arterial pressure and blood sampling. Through the left external jugular vein, another PE-50 catheter was surgically advanced into the right atrium with reference to midchest for measurement of RAP and for the administration of drugs. Aortic and right atrial pressures were measured with reference to the mid-chest with conventional external pressure transducers. A 3-Fr PE catheter was advanced through the right external jugular vein into the right ventricle. A pre-curved guide wire supplied with the catheter was then advanced through the catheter into the right ventricle and confirmed by endocardial electrocardiogram, for inducing VF. All catheters were flushed intermittently with saline containing 2.5 IU/mL of bovine heparin. A conventional lead II ECG was continuously monitored. Temperature was continuously monitored with the aid of a rectal probe and maintained at 37 ± 0.5 °C.

5.2 Experimental procedures

VF was electrically induced with progressive increases in 60-Hz current to a maximum of 4 mA delivered to the right ventricular endocardium. The current flow was maintained for 3 min to prevent spontaneous defibrillation. Precordial compression was begun after 7 min of untreated VF with a pneumatically driven mechanical chest compressor as previously described (*Fumagalli 2014*). Coincident with the start of precordial compression, animals were mechanically ventilated at a frequency of 50/min in order to maintain chest compression/ ventilation ratio of 2:1, and with a tidal volume 0.6 ml/100g and a FiO₂ of 1.0. A single dose of epinephrine (0.02 mg/kg) was injected into the right atrium 2 min

after the start of precordial compression. After 5 min of CPR, resuscitation was attempted with up to three 2 joule defibrillations (CodeMaster XL, Philips Heartstream). Successful resuscitation was defined as the return of supraventricular rhythm with a mean aortic pressure > 50 mmHg for a minimum of 5 min. After resuscitation animals were randomized to a 2 hours ventilation with: (1) an experimental inhalation mixture of 70% Argon - 30% oxygen (n=14); or (2) a control inhalation mixture of 70% nitrogen - 30% oxygen (n=23). The non balance in the number of animals per group is because additional animals were needed in order to set up the experimental procedures.

In vivo micro-dialysis was performed as described below during all the experimental phases and samples of perfusate were collected from the dorsal hippocampus because of its marked susceptibility to ischemic injury. Extracellular concentrations of glutamate (GLU), gamma-amino butyric acid (GABA), as indicators of excitatory or inhibitory neurotransmission, were measured by high performance liquid chromatography (HPLC) as well as lactic acid and piruvic acid concentration as indicators of anaerobic metabolism. Heart rate, arterial and right atrial pressures were invasively monitored and CPP calculated till 2 hrs following ROSC. After the two hours observation period, plasma was collected and hs-cTnT assayed. Animals were then sacrificed.

5.3 *Microdialysis procedure*

Microdialysis is a well-established technique allowing *in vivo* monitoring of extracellular neurotransmitters. The basic principle is the positioning of probe made with a membrane that allows free diffusion of water and low molecular weight solutes between the brain interstitial space and a solution lacking the substance of interest continuously flowing into the probe lumen. The membrane made of Cuprophan (Sorin Biomedica, Italy) acts as a mechanical barrier to turbulence in the fluid flow reducing the mechanical stimulation of the tissue. Furthermore, it acts as a filter against the large molecules and proteins present in the extracellular fluid.

One week before induction of CA, rats were anesthetized with ketamine (80 mg/kg) and xylazine (10 mg/Kg) and a guide cannula for the microdialysis probe was implanted into the brain parenchyma using a stereotaxic frame (model 900, David Kopf, CA), after the surgical exposure of the skull. The guide cannula for the microdialysis probe was secured to the skull with two stainless steel screws and dental cement (Paladur, New Galetti e Rossi, Milan, Italy).

Twenty-four hours before the induction of CA, the dialysis probe was perfused with artificial cerebrospinal fluid (aCSF, composition in mM: NaCl 145, CaCl₂ 1.26, KCl 3, MgCl₂ 1, Na₂HPO₄ 1.4, pH 7.4 with 0.6 M NaH₂PO₄) at a constant flow of 1 µL/min with a microinfusion pump and was lowered slowly into the dorsal hippocampus. The day of CA, the probe was connected by polythene tubing (about 50-60 cm; Portex Ltd., Hythe, UK) to a 2.5 mL syringe (Icogamma plus, Novico spa, Ascoli Piceno, Italy) with a 26-gauge needle, mounted on a CMA/100 microinjection pump (CMA Microdialysis, Stockholm, Sweden) containing aCSF and perfused at a constant flow-rate of 1 µL/min. After 1 h washout, perfusate was collected every 15 min with a microsampler (Univentor, Zeitun, Malta) and samples were stored at -20°C.

GABA and GLU were measured after derivatization with *o*-phthalaldehyde (OPA; Sigma-Aldrich, Milan, Italy) based reagent according to Donzanti and Yamamoto (1988). Stock derivatizing reagent was prepared by dissolving 27 mg OPA in 1 mL methanol, followed by 5 µL β-mercaptoethanol and 9 mL 0.1 M sodium tetraborate buffer (pH 9.3) prepared by dissolving 0.62 g boric acid in about 80 mL ultrapure water (MilliQ, Millipore, USA). pH was adjusted to 9.3 with 2-3 mL 5 M NaOH and the final volume brought to 100 mL with water. Stock reagent solution was maintained at room temperature in a darkened bottle for one week. Derivatizing reagent was prepared by diluting stock solution 1:4 with 0.1 M sodium tetraborate buffer, 24 h before use. Five µL of derivatizing reagent were added to 5 µL sample to measure GLU or GABA, thoroughly mixed and immediately

injected into the HPLC by a refrigerated Midas autosampler (Spark-Holland, Emmen, The Netherlands) set at 4°C.

GABA and GLU were separated through a 4.6 x 80 mm C18 reverse-phase column (HR-80, ESA, Chelmsford, MA). New Guard RP-18 guard column (3.2 x 15 mm; Perkin-Elmer, USA) was used to protect the analytical column. The mobile phase for GABA was as follows: 0.05 M Na₂HPO₄, 35% methanol, pH 6.25 with 85% phosphoric acid, pumped at 1.2 mL/min with a LC10-ADvp HPLC pump (Shimadzu, Milan, Italy).

The mobile phase for GLU separation contained 0.05 M Na₂HPO₄, 28% methanol, pH 6.4 with 85% phosphoric acid at a flow rate of 1 mL/min with a LC20-AD HPLC pump (Shimadzu, Milan, Italy).

GABA and GLU were measured by a fluorescence detector (Jasco SP2020, Tokyo, Japan). Excitation and emission wavelengths were 335 and 450 nm for both aminoacids. Assays were calibrated daily by injecting 0.2, 0.4 and 0.8 pmol/20 µL GABA or 1, 5 and 10 pmol/5 µL GLU, made up freshly in aCSF. Detection limits were 0.025 pmol/20 µL for GABA and 0.1 pmol/5 µL for GLU (signal-to-noise ratio = 2).

Lactate and pyruvate were measured by HPLC associated with ultraviolet detection (wavelength, 220 nm) essentially according to the method described elsewhere (*Biagi 2012*) using a Synergi RP-18 Hydro 250 x 4.6 mm (Phenomenex) column in combination with a mobile phase composed by 30 mM NaH₂PO₄, pH 2.4 with 85% phosphoric acid. Pyruvate and lactate concentrations were determined by comparison to a standard calibration curve freshly prepared daily.

5.4 Statistical analysis

Comparisons between the two groups were analyzed by repeated measure ANOVA with Sidack's or Tuckey's multiple comparisons test and data are reported as mean ± SEM. Comparison between variables at distinct time point were analyzed with Student t-test,

with the exception of hs-c TnT, reported as median and analyzed using non parametric Mann-Whitney U test and interquartile range. When the dependent variable was categorical, Fisher's exact test was performed. Statistical analysis was performed using GraphPad Prism 6.0 software or SPSS for Analytics Software. A value of $p < 0.05$ was regarded as statistically significant.

6 Rat model of myocardial ischemia-reperfusion injury- Experiment 5

6.1 Animal preparation and experimental procedures

Fifty-nine Sprague Dawley rats weighting 305 ± 6 were anesthetized by intraperitoneal injection with ketamine (75 mg/kg) and medetomidine (0.5 mg/kg), intubated and ventilated at a frequency of 98/min with a tidal volume 0.6 ml/100g, positive end expiratory pressure of 2 cmH₂O and a FiO₂ of 0.21. The heart was surgically exposed and a 7.0 silk suture was passed under the left anterior descending coronary artery and tied over two pieces of suture to produce the occlusion. After 30 minutes of ischemia, the occlusion was removed by pulling on the two pieces of suture to allow the reperfusion, under continuous electrocardiographic monitoring. The chest was then closed under negative pressure. Five minutes before reperfusion, animals were randomized to a 1 hour ventilation with: (1) 70% Argon - 30% oxygen; or (2) 70% nitrogen - 30% oxygen. One hour after reperfusion, post surgical analgesia was achieved by buprenorphine (0.1 mg/kg subcutaneous). Rats were weaned from mechanical ventilation and returned to their cages. Six or 24 hours after reperfusion plasma samples were collected for hs-cTnT electrochemiluminescence assay (Roche Diagnostics Italia, Monza, Italy) and animals were sacrificed. Myocardial infarct size was assessed as follow. Six or twenty-four hours after the onset of reperfusion, blue particles (Unisperse Ciba) suspension was injected into the inferior vena cava, after retying the ligature around the coronary artery. The heart was excised, blotted and weighed. The heart was sliced into 1-mm thick transverse sections which were incubated (20 min, 37°C) in a solution of tetrazolium chloride (TTC), then transferred to 4% formalin overnight before image analysis (Analytical Imaging Station, version 3.0, Imaging Research, St. Catherine's, ON, Canada). The area lacking blue particles was considered the AAR. Within the AAR, the pale area (TTC-negative) was considered dead tissue (i.e. infarct) by TTC staining and inflammatory response was evaluated in terms of neutrophil infiltration by naphtol staining (Sigma-Aldrich).

6.2 *Statistical analysis*

Comparisons between the two groups were analyzed by unpaired T test and data reported as mean \pm SEM for variables normally distributed. Non-parametric Mann-Whitney U test was used for variables that were not normally distributed and data are reported as median \pm interquartile range. When the dependent variable was categorical, Fisher's exact test was performed. Statistical analysis was performed using GraphPad Prism 7.0 software. A value of $p < 0.05$ was regarded as statistically significant.

Results

1 Pig model of short duration CA and CPR- Experiment 1

There were no differences in the weight of the animals and in the baseline values of blood gases, heart rate (HR), SAP, MAP, DAP, RAP, EtCO₂, CO, and EF between the two groups (Table 5 and Figure 8).

No statistical differences in the duration of LAD occlusion prior to onset of VF, CPP and amount of adrenaline administered during CPR were observed between the two groups (Table 5). Animals in the Argon group received a greater number of defibrillations in comparison to control animals (Table 5, p not significant).

All the animals were successfully resuscitated. No significant differences in post-resuscitation hemodynamics, including SAP, MAP, DAP, and RAP, EtCO₂ and myocardial function, were observed between the two groups (Table 5 and Fig. 8). Nevertheless, animals receiving Argon showed a trend toward a better post-resuscitation arterial pressure (p not significant, Fig. 8) and 72 h LVEF (p, not significant, Table 5). Animals in the Argon group presented a transient significantly higher HR at 2 hr post-resuscitation in comparison to those in the control group (p < 0.05, Table 1). However, this higher HR was mainly due to two animals in the Argon group that received a large number of defibrillations.

	Control (n=6)	Argon (n=6)
Time from LAD occlusion and onset of VF, min	9 ± 2	9 ± 1
Coronary perfusion pressure, mmHg		
CPR 1 min	33 ± 5	25 ± 4
CPR 3 min	44 ± 4	47 ± 11
CPR 5 min	35 ± 3	42 ± 7
Total dose of adrenaline administered, mg	1.5 ± 0.3	1.3 ± 0.2
Total defibrillations delivered, n	6 ± 2	12 ± 6
Successful Resuscitation, n	6/6	6/6
72 hr survival, n	5/6	6/6
72 hr survival with OPC score = 1, n	2/6 *	6/6
Serum NSE increase from baseline, %	234 #	12
Heart rate, beat/min		
Baseline	106 ± 12	118 ± 11
PR 2 hr	129 ± 6*	156 ± 9
PR 4 hr	136 ± 9	138 ± 6
Right atrial pressure, mmHg		
Baseline	5 ± 1	4 ± 1
PR 2 hr	7 ± 1	5 ± 1
PR 4 hr	6 ± 1	5 ± 1
EtCO₂, mmHg		
Baseline	35 ± 0	36 ± 1
PR 2 hr	36 ± 0	36 ± 0
PR 4 hr	36 ± 1	38 ± 1
LV cardiac output, l/min		
Baseline	4.2 ± 0.6	4.5 ± 0.3
PR 2 hr	3.3 ± 0.4	3.6 ± 0.6
PR 4 hr	3.3 ± 0.3	3.9 ± 0.5
LV ejection fraction, %		
Baseline	69 ± 2	69 ± 2
PR 2 hr	35 ± 6	39 ± 2
PR 4 hr	46 ± 5	48 ± 7
PR 72 hr	61 ± 2	67 ± 5

Table 5. Resuscitation outcome, hemodynamics and myocardial function. CPR, cardiopulmonary resuscitation; LAD, left anterior descending coronary artery; VF, ventricular fibrillation; PR, post resuscitation; OPC, overall performance category; EtCO₂, end tidal PCO₂; LV left ventricle. All data are reported as mean ± S.E.M., except for NSE increase that is reported as median. * p < 0.05 vs. Argon; # p < 0.05 vs. baseline.

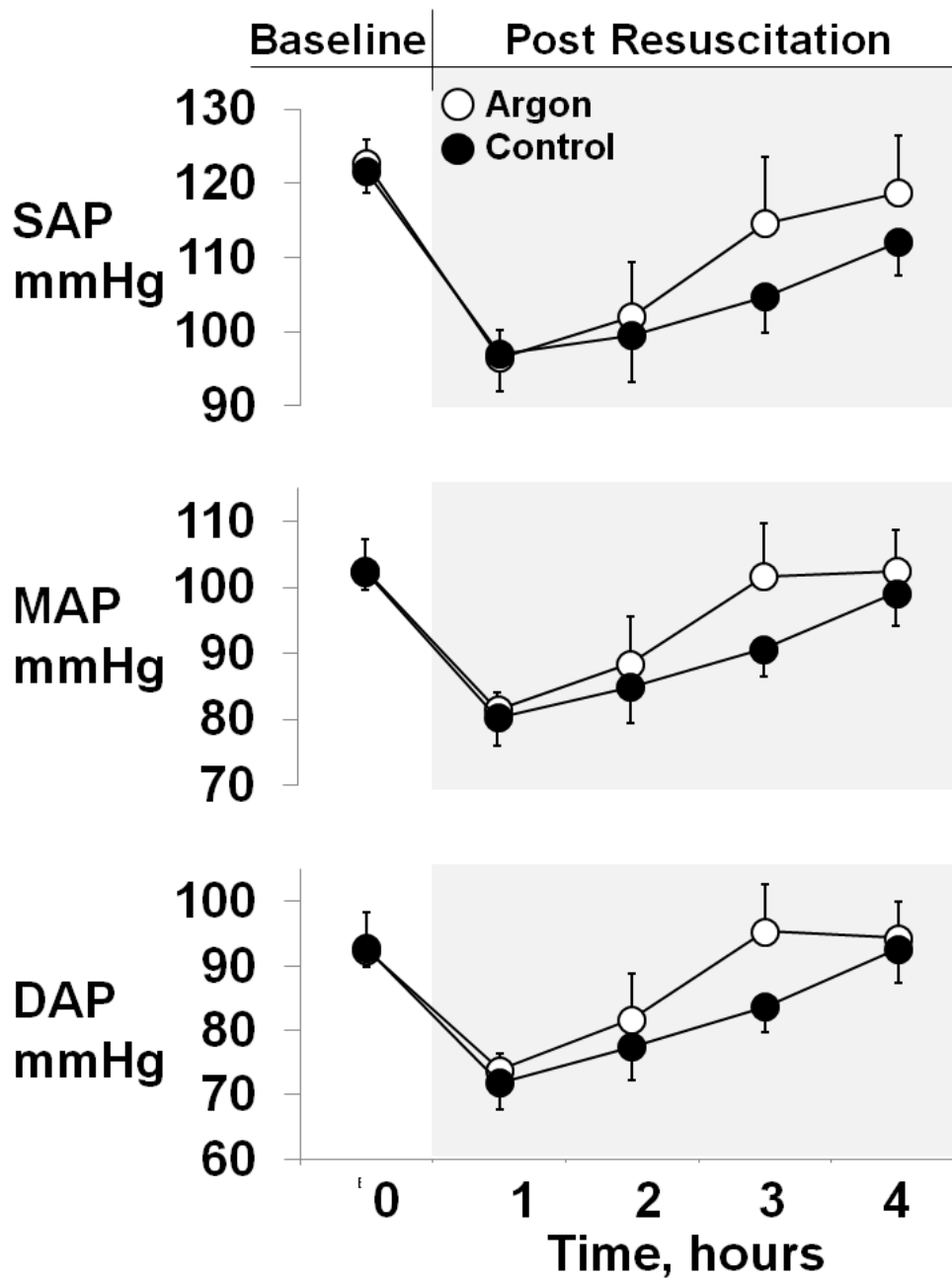


Figure 8. Systolic, mean, and diastolic arterial pressures (SAP, MAP, and DAP) in Argon treated animals and controls, at baseline and during the 4 h post-resuscitation period. Repeated measures ANOVA and unpaired t test for comparison at each time-point. Mean \pm S.E.M.

Ventilation with Argon did not impair respiratory gas exchange and acid-base homeostasis, as reflected by EtCO₂ and arterial blood gases (Tables 5 and 6). No differences in arterial pH, PO₂, PCO₂, HCO₃, and BE, were observed between the two groups during the 4 h post-resuscitation treatment (Table 6).

	Argon (n=6)	Control (n=6)
pH		
Baseline	7.469 ± 0.02	7.509 ± 0.02
PR 2 h	7.458 ± 0.06	7.449 ± 0.03
PR 4 h	7.451 ± 0.02	7.473 ± 0.02
PO₂, mmHg		
Baseline	79 ± 5	82 ± 15
PR 2 h	83 ± 9	90 ± 8
PR 4 h	76 ± 9	88 ± 4
PCO₂, mmHg		
Baseline	38 ± 1	38 ± 2
PR 2 h	39 ± 1	40 ± 2
PR 4 h	39 ± 1	42 ± 1
HCO₃, mmol/L		
Baseline	28.8 ± 1.2	30.4 ± 0.9
PR 2 h	24.8 ± 1.1	27.9 ± 1.1
PR 4 h	27.8 ± 1.5	30.8 ± 1.1
BE, mmol/L		
Baseline	5.3 ± 1.5	7.7 ± 1
PR 2 h	0 ± 1.5	4.2 ± 1.6
PR 4 h	3.8 ± 2	7.3 ± 1.4

Table 6. Arterial blood gases. PR, post-Table 5. Resuscitation outcome, hemodynamics and myocardial function. PR, post resuscitation; PO₂, oxygen partial pressure; PCO₂, carbon dioxide partial pressure; HCO₃, bicarbonate; BE, base excess.

Each of the six resuscitated animals in the Argon group survived for 72 hr and had a complete neurological recovery, represented by an OPC = 1 (Table 5). Five of the six resuscitated animals in the control group survived for 72 hr. However, among them, only two animals achieved a complete neurological recovery, i.e. OPC = 1 ($p < 0.05$ vs. Argon, Table 5). More specifically, animals treated with Argon presented a significantly higher NAS and a significantly lower NDS compared to control animals, already 24 hr after resuscitation ($p < 0.01$, Fig. 9). These differences persisted up to 72 hr post-resuscitation ($p < 0.01$, Fig. 9). Brain histology of the CA1 hippocampal sector showed the presence of lesser, although not statistically significant, neuronal loss and degeneration and microglia activation in animals that received Argon in contrast to those that received the control ventilation (Fig. 11). These functional and histological results were paralleled by the

increases in serum NSE levels at 72 hr post-resuscitation, that were minimal in the Argon treated animals, while were statistically significant in the control ones (Table 5).

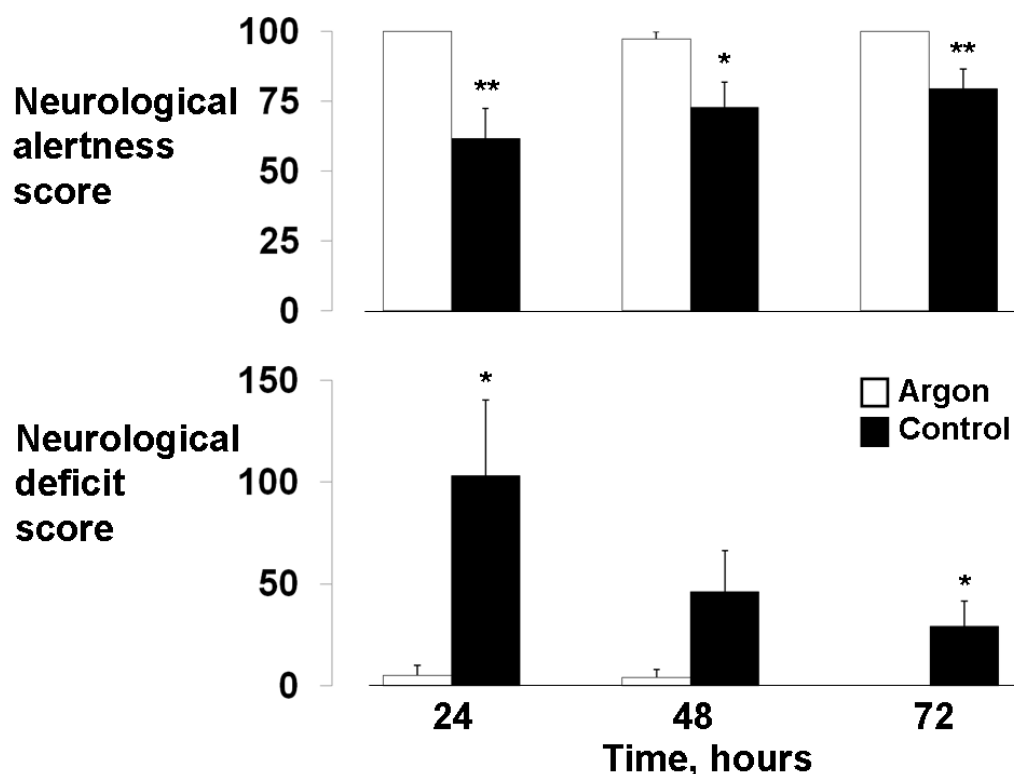


Figure 9. Neurological alertness score and neurological deficit score in Argon treated animals (n=6) and controls (n=5), at 24, 48, and 72 hr post-resuscitation. Repeated measures ANOVA and unpaired t test for comparison at each timepoint. Mean ± S.E.M. * p < 0.05 and ** p < 0.01 vs. Argon treated animals.

Neither the total dose of administered sodium pentobarbital nor the duration of CPR prior to resuscitation affected the above results on neurological function and injury. The average amount of sodium pentobarbital administered to the animals during the whole experimental procedure was equivalent in the two groups (Tab. 5. 1.606 ± 95 mg in the Argon group and 1.613 ± 120 mg in the control one, p NS). The duration of CPR prior to resuscitation was also equivalent in the two groups (Tab 5. 337 ± 24 secs in the Argon group and 353 ± 42 secs in the control one, p not significant).

Heart gross anatomy showed a slightly smaller (p NS) infarct size, reported as percentage of TTC-negative area relative to LV area, in animals that received Argon in comparison to those that received the control ventilation (Fig. 10). These histological findings were

paralleled by a three-fold lower post-resuscitation increases in plasma hs-cTnT in pigs that received Argon in comparison to control animals (p NS, Fig. 10).

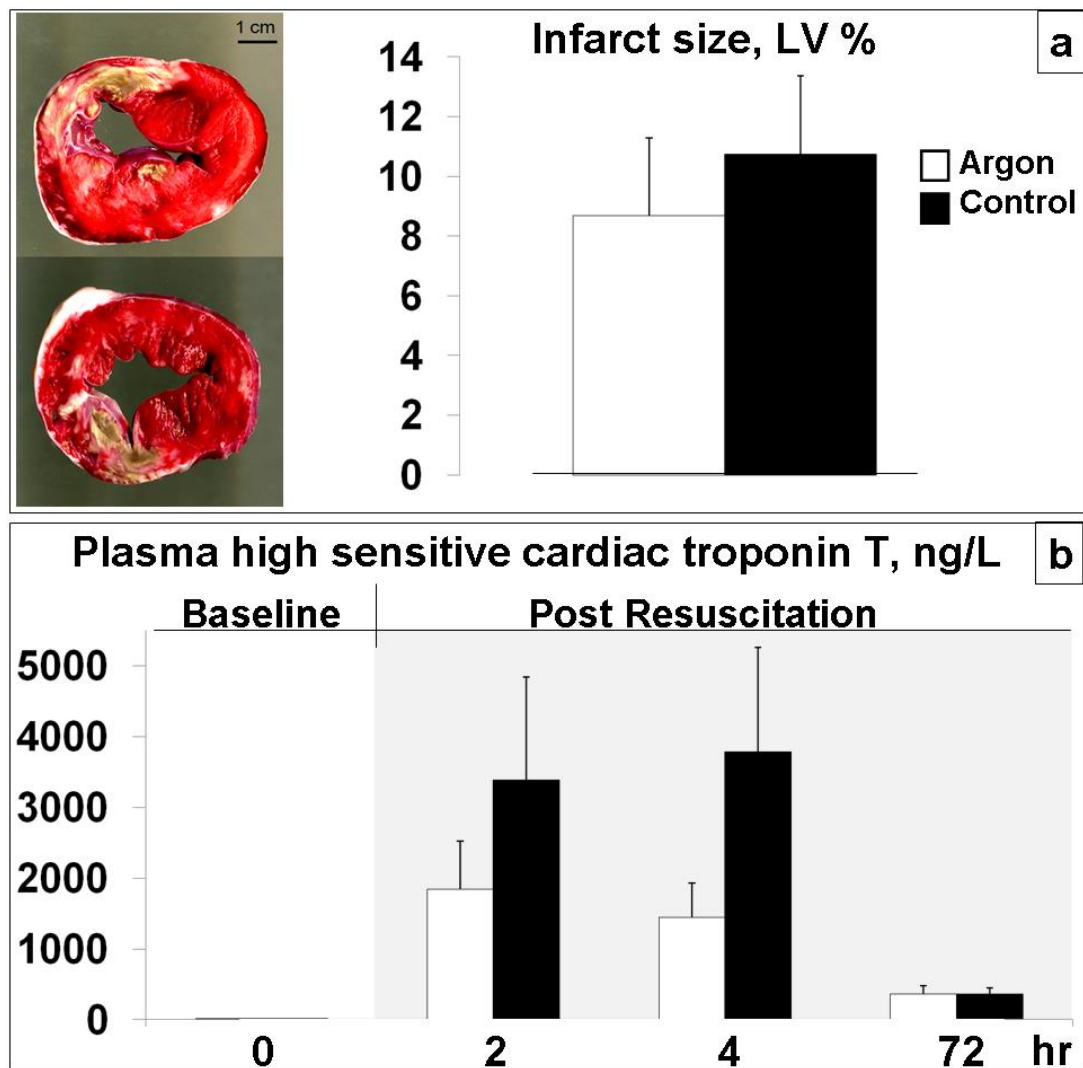


Figure 10. Part a: Left ventricular (LV) infarct size. On the left: 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) staining of the upper and lower faces of one LV slice. Vital myocardium is TTC-positive (red), while infarcted myocardium is TTC-negative (white). On the right: infarct size, reported as percentage of TTC-negative area relative to LV area, in animals treated with Argon in comparison to controls (Mean \pm S.E.M.). Part b: Plasma high sensitive cardiac troponin T in Argon treated animals and controls, at baseline, during the 4 hr post-resuscitation period, and 72 hours later (Repeated measures ANOVA. Mean \pm S.E.M.).

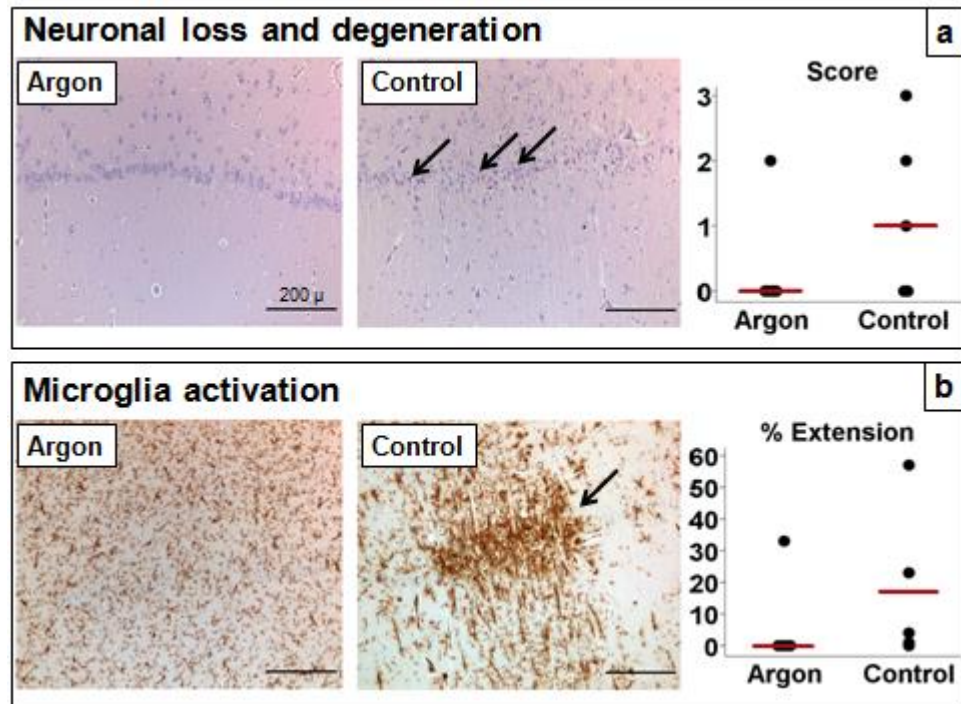


Figure 11. Histology of brain hippocampal CA1 sector. A, Neuronal loss and degeneration. Hematoxylin-eosin staining, original magnification X100, showing intact neurons in an Argon-treated animal, in contrast to ischemically damaged neurons in a control (arrows). On the right, proportion of neuronal loss and degeneration in animals treated with Argon in comparison to controls (Nonparametric Mann-Whitney U test, dot-plot and median). B, Microglia activation. Immunohistochemistry for microglia-specific ionized calcium-binding adaptor molecule 1 (Iba1), original magnification X100, showing morphology typical for normal, resting microglia in an Argon-treated animal, in contrast to activated microglia in a tract of pyramidal cells in a control (arrow). On the right, extension of microglia activation in the brain hippocampal CA1 sector, in animals treated with Argon in comparison to controls (Nonparametric Mann-Whitney U test, dot-plot and median).

2 Pig model of prolonged CA and CPR- Experiment 2

No differences in body weight, HR, hemodynamics, EtCO₂, and myocardial function, arterial blood gases and biomarkers were observed between the three groups at baseline (Table 7 and Fig. 12).

No differences in CPP during CPR (Table 7) were observed among the 3 groups. Ten animals of 12 were successfully resuscitated in each group.

The duration of CPR prior to ROSC and the number of total defibrillations delivered were greater in the Argon treated animals compared to controls (Table 7, $p=0.056$); specifically, the animals treated with Argon 50% presented the longest duration of CPR and the highest number of defibrillations (Table 7).

After resuscitation, animals treated with Argon 70% showed a significantly higher systolic, mean and diastolic arterial pressure compared to controls and to the Argon 50% treated animals ($p=0.008$, $p=0.001$, $p=0.0015$ respectively, Figure 12). Moreover, animals treated with Argon 70% presented a significantly lower post resuscitation heart rate compared to controls ($p<0.0001$, Table 7). No differences were observed in other hemodynamic variables between groups (Table 7).

	Control (n=12)	Argon 50% (n=12)	Argon 70% (n=12)
Body weight, kg	38 ± 0.7	40 ± 0.6	39 ± 0.5
Coronary perfusion pressure, mmHg			
CPR 1 min	19 ± 4	25 ± 6	22 ± 5
CPR 3 min	35 ± 3	39 ± 6	38 ± 5
CPR 5 min	22 ± 4	26 ± 4	27 ± 3
Time to ROSC, s	341[301.0-366.0]	371.5 [325.3-430.3]	301.5 [301.0-358.9]
Total defibrillations delivered, n	5 [2.5-11.7]	18 [7.0-24.8]	11.5 [6.5-15.0]
Successful resuscitation, n	10/12	10/12	10/12
96 h Survival, n	6/10	7/10	9/10
96 h Neuronal Specific Enolase, ng/ml	21.15 [16.21-60.51]	8.26 [1.19-18.07]	16.42 [2.52-22.14]
Heart rate, beats/min			
Baseline	102 ± 9	95 ± 4	82 ± 7
PR 2 h	173 ± 7	154 ± 12	131 ± 5 **
PR 4 h	165 ± 14	135 ± 9	120 ± 9 **
Right atrial pressure, mmHg			
Baseline	5 ± 1	8 ± 1	5 ± 1 †
PR 2 h	7 ± 1	8 ± 1	6 ± 1
PR 4 h	7 ± 1	9 ± 1	7 ± 1
EtCO₂, mmHg			
Baseline	36 ± 0	36 ± 1	36 ± 1
PR 2 h	36 ± 0	35 ± 0	36 ± 0
PR 4 h	36 ± 0	36 ± 0	37 ± 0
Pulmonary arterial pressure, mmHg			
Baseline	19 ± 2	22 ± 1	18 ± 1
PR 2 h	20 ± 1	19 ± 2	19 ± 2
PR 4 h	21 ± 1	21 ± 1	21 ± 2
Pulmonary capillary wedge pressure, mmHg			
Baseline	9 ± 1	11 ± 1	9 ± 0
PR 2 h	11 ± 1	12 ± 1	9 ± 1
PR 4 h	12 ± 1	12 ± 1	10 ± 1
LV cardiac output, L/min			
Baseline	4.4 ± 0.2	3.9 ± 0.2	4.0 ± 0.2
PR 2 h	3.2 ± 0.3	2.9 ± 0.3	3.0 ± 0.3
PR 4 h	3.0 ± 0.2	2.5 ± 0.1	2.6 ± 0.2

Table 7. Resuscitation outcome and hemodynamics. All data are reported as mean ± SEM, except for duration of CPR before resuscitation, total defibrillations delivered, defibrillations to ROSC and NSE levels, reported as median. PR indicates post resuscitation. * p< 0.05, ** p< 0.01 Controls vs. Argon 70%. † p< 0.05, †† p< 0.01 Argon 50% vs. Argon 70%.

The 4-h ventilation with Argon did not impair respiratory gas exchange and acid-base homeostasis regardless to the Argon concentration administered, with the exception of a decreased BE and HCO₃⁻ in animals treated with Argon 50% compared to other groups (p=0.0015 and p=0.0011 respectively, Tables 8).

	Control (n=12)	Argon 50% (n=12)	Argon 70% (n=12)
pH			
BL	7.487 ± 0.02	7.459 ± 0.01	7.500 ± 0.03
PR2	7.373 ± 0.02	7.325 ± 0.03	7.398 ± 0.02
PR4	7.424 ± 0.03	7.408 ± 0.02	7.439 ± 0.02
PCO ₂			
BL	38 ± 1	37 ± 1	36 ± 1
PR2	40 ± 1	38 ± 1	40 ± 1
PR4	40 ± 1	39 ± 1	40 ± 1
PO ₂			
BL	80 ± 4	83 ± 3	88 ± 4
PR2	110 ± 6	113 ± 7	112 ± 8
PR4	103 ± 8	111 ± 8	114 ± 9
BE			
BL	5.4 ± 1.4	2.3 ± 0.7	4.8 ± 1.3
PR2	-1.8 ± 1.6	-6.1 ± 1.3	-0.1 ± 1.4 ††
PR4	2.3 ± 1.5	0 ± 1.2	3.0 ± 1.1
HCO ₃ ⁻			
BL	28.9 ± 1.1	26.5 ± 0.6	28.1 ± 1.0
PR2	23.3 ± 1.3	19.9 ± 1.2	24.9 ± 1.1 ††
PR4	26.4 ± 1.2	24.6 ± 0.9	27.3 ± 0.9

Table 8. Arterial Blood Gases. PR indicates post- resuscitation; PO₂, oxygen partial pressure; PCO₂, carbon dioxide partial pressure; HCO₃, bicarbonate; BE, base excess. Data shown as mean ± SEM. †† < 0.01 Argon 50% vs. Argon 70%.

Post resuscitation myocardial function was significantly impaired in each animal (Table 9), with LV volumes increases and LVEF decreases. Although absolute mean values between groups were not significantly different, animals treated with Argon 70% achieved a faster myocardial function recovery. In fact, as shown in Table 10, LVEF from 4 to 96 h increased more in Argon 70% compared to Argon 50% and to Control animals ($28.5 \pm 6.1\%$ vs 5.3 ± 7.4 and $18.5 \pm 7.0\%$ respectively, $p < 0.01$). Moreover, LV systolic volume at 96 h normalized in animals treated with Argon 70% (Table 10).

	Control (n=8)	Argon 50% (n=9)	Argon 70% (n=9)
LVEF, %			
Baseline	76.6 ± 2.3	69.5 ± 3.9	72.7 ± 3.1
PR 2 h	35.7 ± 3.1	46.8 ± 8.2	34.6 ± 3.3
PR 4 h	36.6 ± 3.5	45.6 ± 7.1	39.0 ± 4.5
PR 96 h	55.1 ± 7.4	50.9 ± 5.9 [†]	67.5 ± 2.6
EDV, mL			
Baseline	33.7 ± 3.0	31.5 ± 1.8	39.5 ± 3.5
PR 2 h	35.4 ± 3.3	27.3 ± 2.3	31.5 ± 2.9
PR 4 h	42.5 ± 5.7	36.6 ± 6.1	39.4 ± 2.6
PR 96 h	41.3 ± 4.3	36.2 ± 2.4	37.1 ± 4.8
ESV, mL			
Baseline	7.8 ± 0.9	9.8 ± 1.4	13.3 ± 1.8
PR 2 h	22.8 ± 2.6	14.3 ± 2.8	20.4 ± 2.4
PR 4 h	26.4 ± 4.2	21.3 ± 5.6	23.0 ± 2.3
PR 96 h	18.2 ± 3.5	17.8 ± 2.3	12.4 ± 2.1

Table 9. Echocardiographic data. Left Ventricle Ejection Fraction, End-Diastolic and End-Systolic Volumes. All data are reported as mean ± SEM. p value from two-way ANOVA. Tukey's multiple groups comparisons: † p < 0.05 Argon 50% vs. Argon 70%.

	Control (n=8)	Argon 50% (n=9)	Argon 70% (n=9)
LVEF, %			
PR2 h vs Baseline	-40.9 ± 6.8**	-22.7 ± 6.3**	-38.1 ± 5.9**
PR 4 h vs Baseline	-40.0 ± 6.5**	-23.9 ± 7.0**	-33.6 ± 5.9**
PR 96 h vs Baseline	-21.5 ± 6.8*	-18.6 ± 6.3*	-5.1 ± 6.1
PR 4 h vs PR 2 h	0.9 ± 7.0	-1.2 ± 7.4	4.4 ± 5.9
PR 96 h vs PR 2 h	19.3 ± 7.3*	4.1 ± 6.7	32.9 ± 6.1**
PR 96 h vs PR 4 h	18.5 ± 7.0*	5.3 ± 7.4	28.5 ± 6.1**
EDV, mL			
PR2 h vs Baseline	1.77 ± 5.1	-4.2 ± 4.6	-8.1 ± 4.6
PR 4 h vs Baseline	8.9 ± 5.1	5.0 ± 5.3	-0.2 ± 4.5
PR 96 h vs Baseline	7.6 ± 5.1	4.7 ± 4.7	-2.4 ± 4.6
PR 4 h vs PR 2 h	7.1 ± 5.5	9.2 ± 5.4	7.9 ± 4.6
PR 96 h vs PR 2 h	5.9 ± 5.5	8.9 ± 4.9	5.6 ± 4.7
PR 96 h vs PR 4 h	-1.3 ± 5.3	-0.3 ± 5.5	-2.3 ± 4.6
ESV, mL			
PR2 h vs Baseline	15.0 ± 3.8**	4.6 ± 3.4	7.1 ± 3.4
PR 4 h vs Baseline	18.6 ± 3.8**	11.5 ± 3.9*	9.8 ± 3.3*
PR 96 h vs Baseline	10.4 ± 3.8*	8.0 ± 3.5	-0.9 ± 3.4
PR 4 h vs PR 2 h	3.6 ± 4.0	6.9 ± 4.0	2.6 ± 2.1*
PR 96 h vs PR 2 h	-4.6 ± 4.0	3.5 ± 3.6	-8.0 ± 3.5
PR 96 h vs PR 4 h	-8.2 ± 4.0	-3.5 ± 4.1	-10.7 ± 3.4

Table 10. Echocardiographic data. Left Ventricle Ejection Fraction, End-Diastolic and End-Systolic Volumes. All data are reported as mean ± SEM. p value from two-way ANOVA. Tukey's multiple comparisons within each group: * p< 0.05; ** p< 0.01.

Animals treated with both Argon concentrations presented a significantly higher NAS and a significantly lower NDS compared with control animals, markedly at 72 hrs after resuscitation (p=0.028 and p=0.021 respectively, Figure 13).

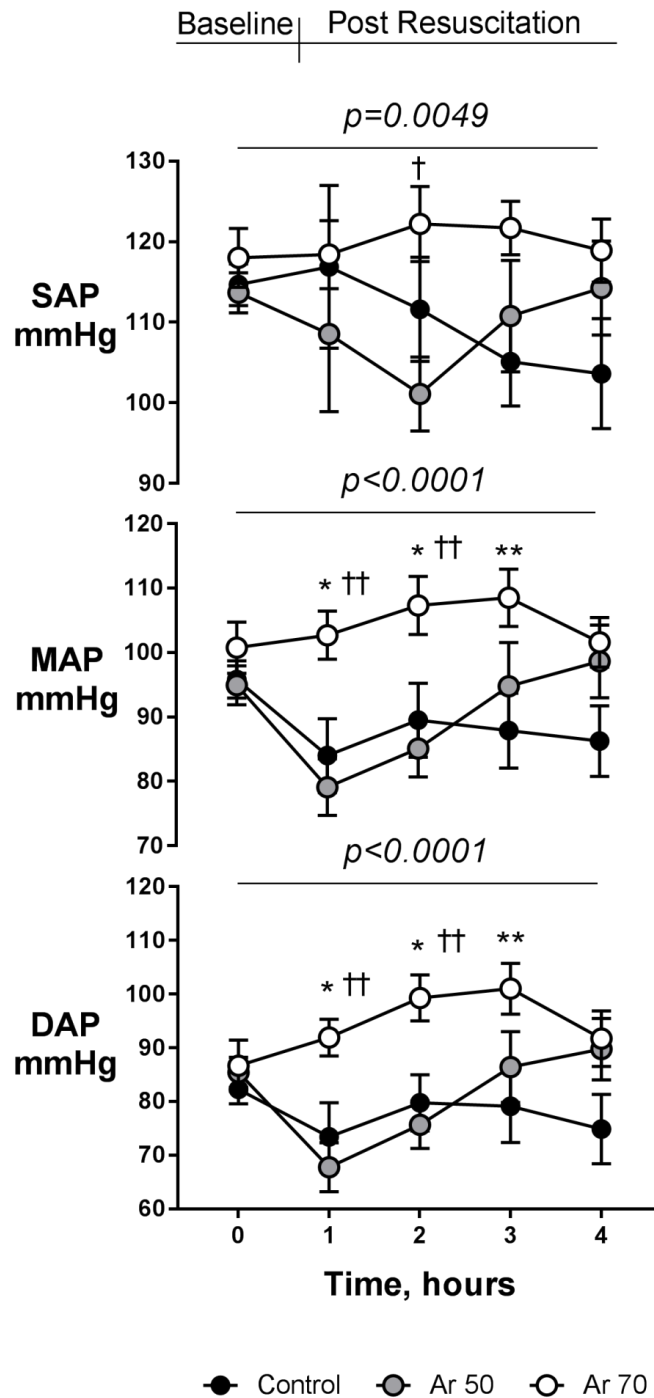


Figure 12. SAP, MAP, and DAP in Argon-treated animals and controls, at baseline and during the 4-h post- resuscitation period. Ordinary two-way ANOVA and Tuckey's multiple comparison test. Mean \pm SEM. * $p<0.05$, ** $p<0.01$ Ar 70% vs Control; † $p<0.05$, †† $p<0.01$, Ar 70% vs Ar 50%.

There was no statistically significant difference in the number of the resuscitated animals surviving up to 96 h ($p=0.3$, Table 7). Respectively 6 (60 %) and 8 (80 %) resuscitated animals in the Argon 50% and Argon 70% groups achieved a complete neurological recovery (OPC=1 or 2), whereas only 3 (30%) animals survived up to 96 hours with a

complete neurological recovery in the Control group (Figure 14, $p < 0.0001$). The better neurological condition of the treated animals at 96 hrs is accompanied by a trend towards lower plasma levels of NSE at the same time- point in these two groups of treated animals compared to controls ($p = 0.21$, Table 7).

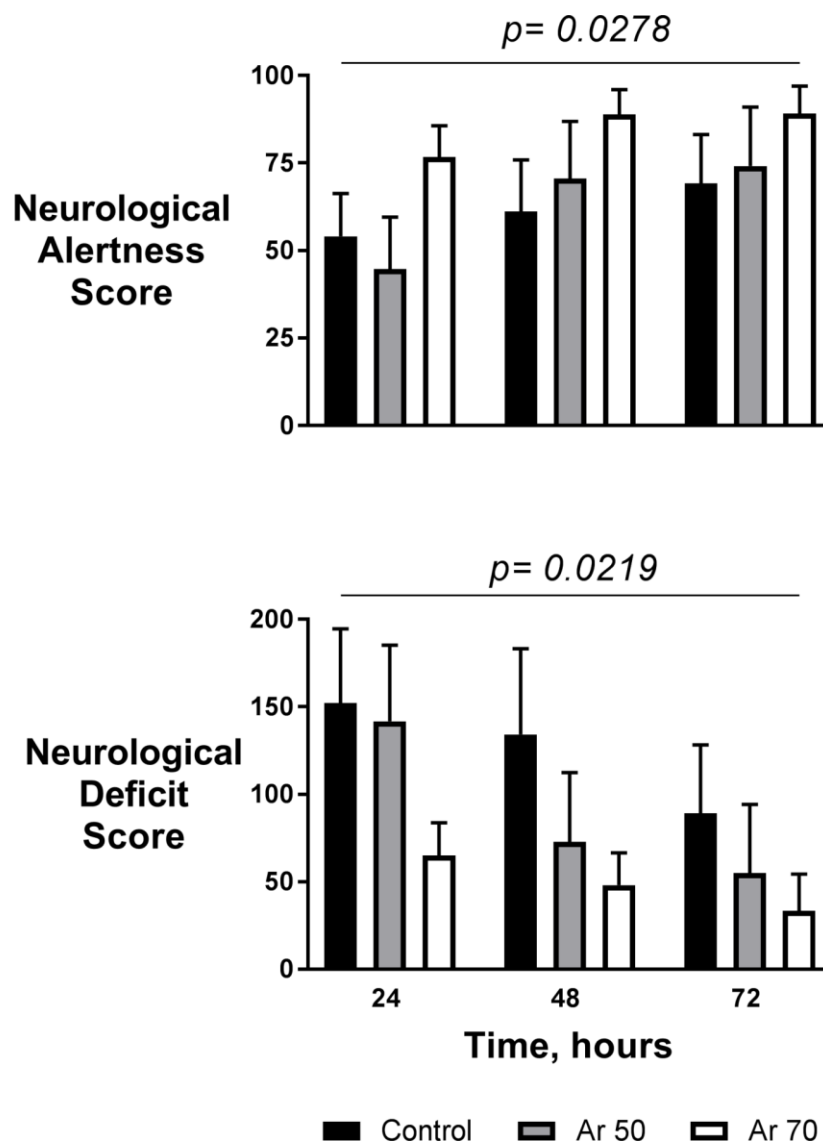


Figure 13. Neurologic alertness score and neurologic deficit score in Argon-treated animals and controls, at 24, 48, and 72 h post- resuscitation. Ordinary two-way ANOVA and Tuckey's multiple comparison test. Data shown as mean with SEM.

96 hrs Survival with OPC

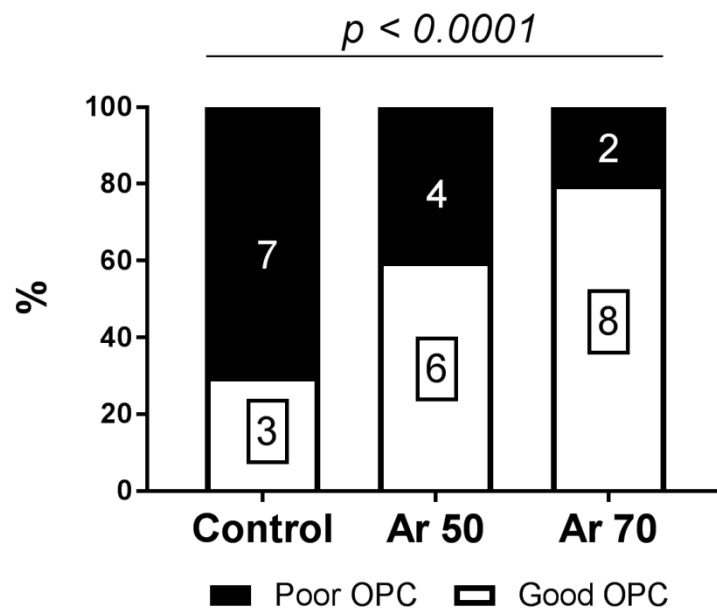


Figure 14. 96 hrs survival with Overall Performance Category (OPC).

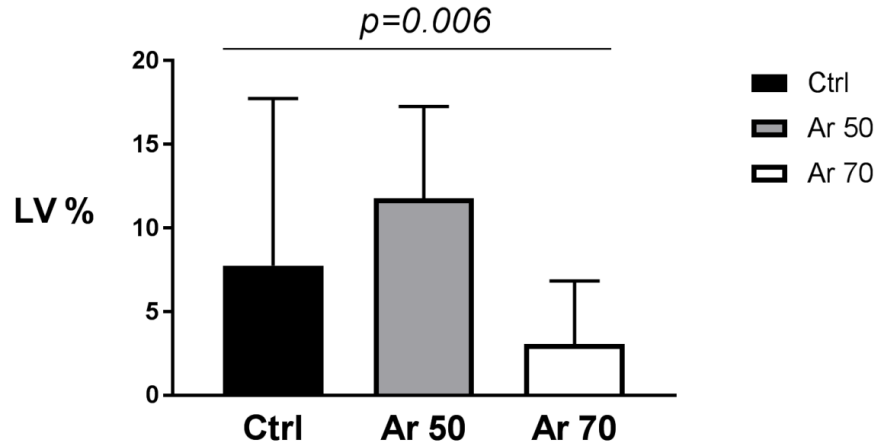
	Control (n=6)		Argon 50% (n=7)		Argon 70% (n=9)	
	Cortex	Hippocampus	Cortex	Hippocampus	Cortex	Hippocampus
Perivascular cuffing, score	0.25[0-1]	0.75[0.5-1]	0[0-0]	0[0-0]*	0[0-0]*	0[0-1]
Neuronal degeneration, score	1[0.3-1]	2.5[2-3]	0[0-0]*	3[0.5-3]	0[0-1]	2[2-2]
Microglia activation, % μm^2	-	24.3[20-25.5]	-	13.1[5.9-14-6]**	-	23.1[18.1-23.6]

Table 11. Histology of brain cortex and hippocampal CA1 sector. Perivascular cuffing, neuronal degeneration and microglia activation in animals treated with Argon in comparison to controls. Data shown as median [Q1-Q3]. * p < 0.05, ** p < 0.01 Argon 70% or Argon 50% vs Controls.

Brain histology showed reduced neuronal loss and degeneration, perivascular cuffing and microglia activation in animals that received Argon in contrast to controls. These lesions were found both in cortex and in hippocampus (Table 11).

Heart gross anatomy showed no statistically significant differences in infarct size between groups. However, there was a trend towards to a reduction in infarct size in animals that received Argon 70% in comparison to Controls and an increased infarct size in animals treated with Ar 50% (p=0.064, Figure15. A). The reduction in the infarct size in Argon 70% treated group is reflected in a statistically significant lower 96-h post-resuscitation plasma level of hs-cTnT observed in pigs that received Argon 70% in comparison to controls (p=0.001, Figure 15.B).

A. Infarct Size



B. Plasma high sensitive cardiac troponin T

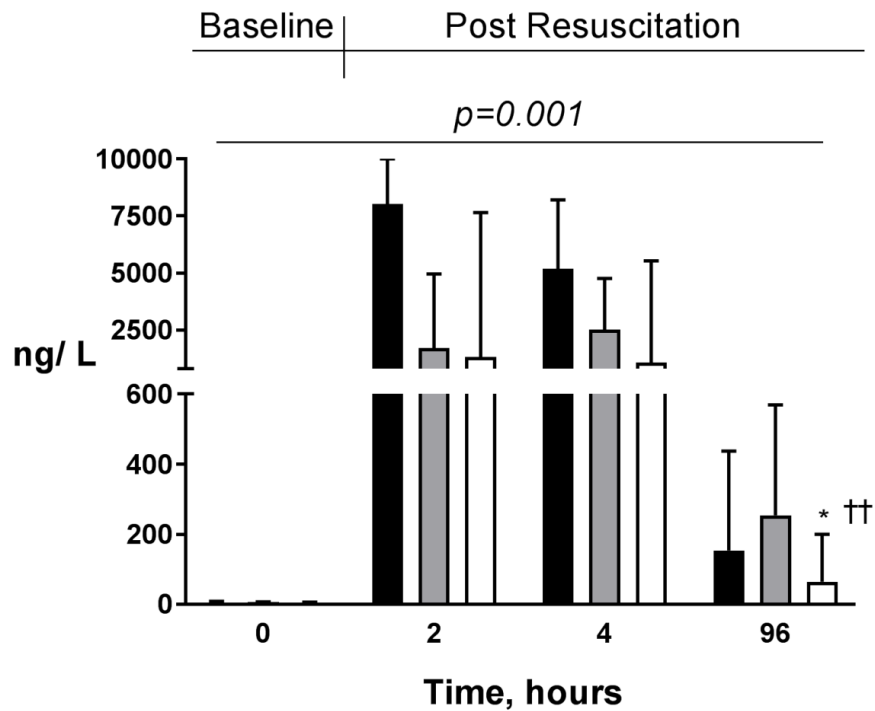


Figure 15. A. Left ventricular infarct size, reported as percentage of TTC-negative area relative to LV area in animals treated with Argon in comparison to controls. Data shown as median and interquartile range. Kruskal-Wallis test, $p = 0.0637$. B, Plasma hs-cTnT in Argon-treated animals and controls, at baseline, during the 4-h post- resuscitation period, and 96 h later. Data shown as median and interquartile range. Two- ways ANOVA and Tuckey's multiple comparisons test on values corrected by logarithmic transformation. * $p < 0.05$ Ar 70% vs Control; †† $p < 0.01$, Ar 70% vs Ar 50%.

3 Safety- Experiment 3

All the animals survived without any apparent behavioral changes and/or impairment over the 3 days of observation. Overall, no adverse events during and after Argon ventilation were reported. No significant changes in hemodynamics were observed; similarly, blood gas variables, Hb, Ht, and serum electrolytes remained in the physiological range (Figure 16, Table 12). Hs-cTnT, ALT, and creatinine values were also stable during the whole period of observation (Table 12). Of interest, it was noticed that airways peak pressure (Ppeak) value after 4 h of Argon ventilation was comparable to baseline.

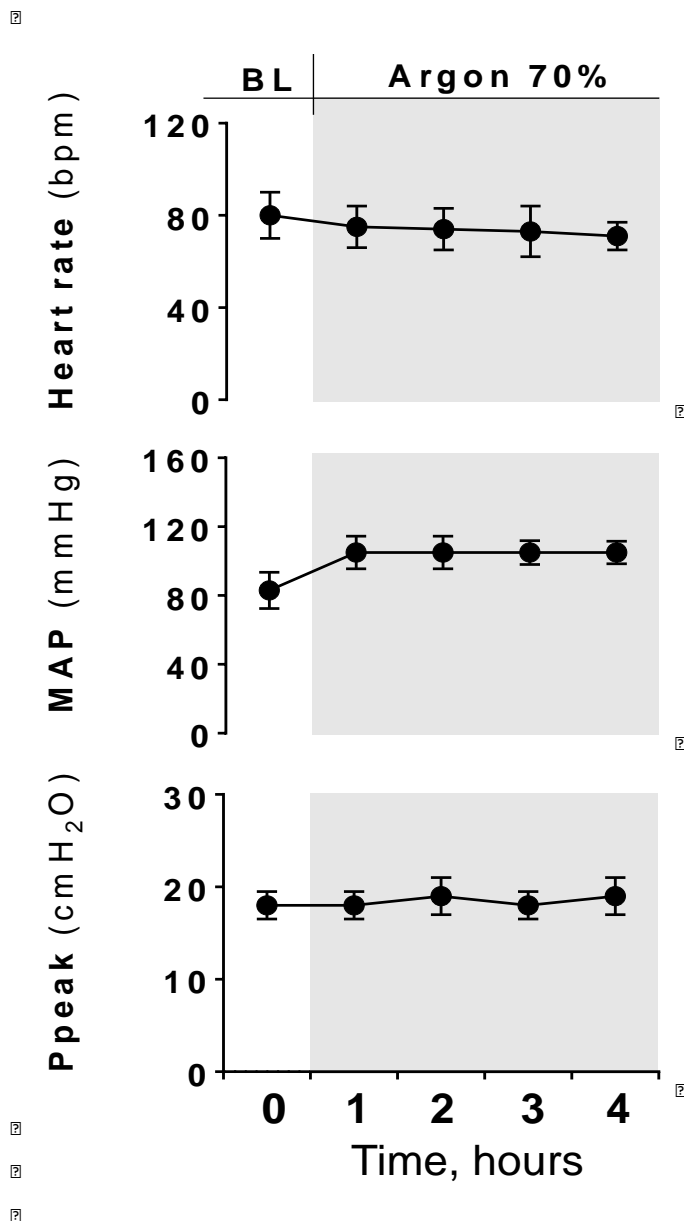


Figure 16. Heart rate (HR), mean arterial pressure (MAP) and peak airways pressure (Ppeak), after a 4-h ventilation with Argon 70%. BL, baseline; Mean \pm SEM.

	Baseline	Argon 70% - Oxygen 30%				Sacrifice
	0 h	1 h	2 h	3 h	4 h	72 h
HR (bpm)	80 ±10	75 ±9	75 ±9	73 ±11	71 ±6	n.a.
MAP (mmHg)	83 ±10	105 ±9	105 ±8	105 ±5	104 ±4	n.a.
Ppeak (cmH ₂ O)	18 ±0	18 ±0	19 ±0	19 ±0	19 ±0	n.a.
pH	7.511 ± 0.021	7.521 ± 0.006	7.538 ± 0.010	7.532 ± 0.011	7.530 ± 0.007	n.a.
PaO₂ (mmHg)	80 ± 3	133 ± 4	130 ± 8	121 ± 8	123 ± 9	n.a.
SaO₂ (%)	97 ± 0	99 ± 0	99 ± 0	99 ± 0	99 ± 0	n.a.
PaCO₂ (mmHg)	34.9 ± 0.5	35.5 ± 0.3	35.3 ± 0.7	36.3 ± 1.0	36.1 ± 0.4	n.a.
HCO₃⁻ (mmol/L)	28.1 ± 1.5	29.2 ± 0.4	30.2 ± 0.4	30.2 ± 0.6	30.1 ± 0.5	n.a.
Hb (g/dL)	9.0 ± 0.3	9.1 ± 0.4	9.1 ± 0.3	9.3 ± 0.4	8.8 ± 0.2	n.a.
Ht	26 ± 1	27 ± 1	27 ± 1	27 ± 1	26 ± 0	n.a.

(%)						
Na⁺ (mmol/L)	138 ± 1	138 ± 1	137 ± 1	137 ± 1	137 ± 1	n.a.
K⁺ (mmol/L)	3.7 ± 0.1	4.0 ± 0.1	4.3 ± 0.1	4.4 ± 0.2	4.5 ± 0.2	n.a.
Lac (mmol/L)	2.48 ± 0.88	1.81 ± 0.63	0.95 ± 0.10	0.84 ± 0.07	0.80 ± 0.14	n.a.
ALT (U/L)	29 ± 7	26 ± 1	32 ± 2	24 ± 2	23 ± 2	26 ± 2
Creatinine (mg/dL)	3.36 ± 0.42	2.58 ± 0.29	2.41 ± 0.47	2.52 ± 0.39	2.87 ± 0.16	2.85 ± 0.94
hs-cTnT (ng/L)	8.23 [7.76-8.7]	7.5 [7.76-10.92]	6.65 [6.47-7.66]	7.24 [5.59-10.02]	6.54 [5.98-8.26]	6.33 [4.41-6.55]
NSE (ng/L)	0.62 [0.53-6.62]	0.56 [0.55-0.69]	0.48 [0.42-0.76]	0.61 [0.46-0.62]	0.55 [0.42-0.59]	0.41 [0.31-0.43]

Table 12. Arterial blood gases; hemoglobin; electrolytes; lactates; liver, renal, and cardiac biomarkers after a 4-hr ventilation with Ar 70%. PaO₂, partial arterial oxygen pressure; SaO₂, arterial oxygen saturation; PaCO₂, partial arterial carbon dioxide pressure; HCO₃⁻, serum bicarbonates; Hb, hemoglobin; Ht hematocrit; Lac, serum lactates; ALT, alanine aminotransferase; hs-cTnT, high sensitivity cardiac troponin T. Mean ± SEM except for hs-cTnT and NSE, shows as median and interquartile range.

4 Rat model of CA and CPR- Experiment 4

More than 85% of the rats were successfully resuscitated and no differences were observed in the CPR procedures between experimental groups (Table 13, p not significant).

	Vehicle	Argon
Number of animals with CA and CPR	18	9
Body Weight (g)	476 ± 29	489 ± 31
ROSC	85%	90%
Number of Shocks	2.6 ± 2.8	1.9 ± 1.6

Table 13. Resuscitation outcome. CA, cardiac arrest; CPR, cardiopulmonary resuscitation; ROSC, return of spontaneous circulation. All data are reported as mean ± SEM, except for ROSC rate reported as percentage.

Ventilation with Argon had no detrimental effects on HR, MAP and CPP during the 2-h observation period. Furthermore, animals treated with Argon showed a faster increase in MAP and CPP compared to controls (Repeated measurement ANOVA, effect of treatment: $p < 0.0001$ (Figure 17, a, b and c).

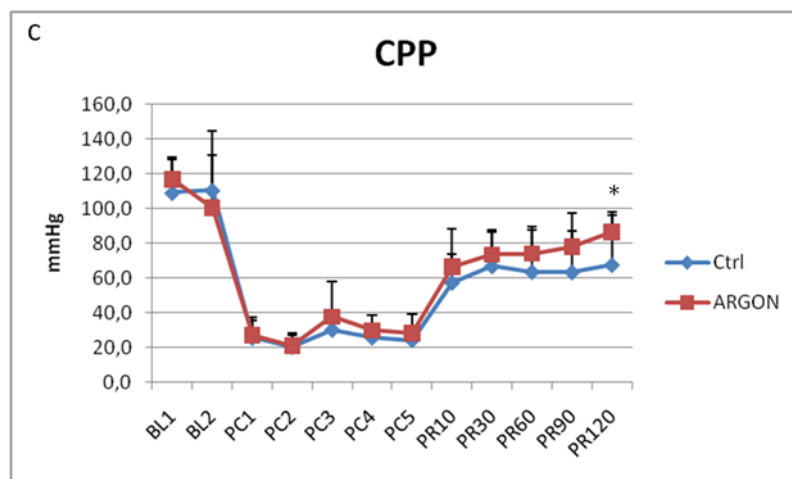
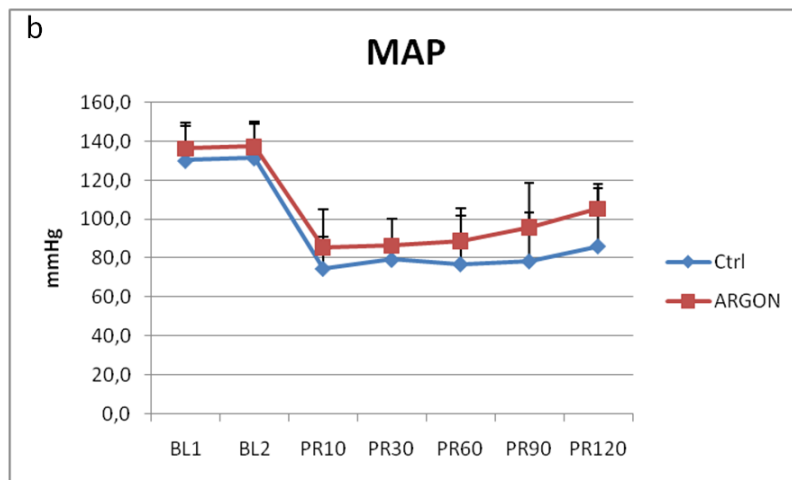
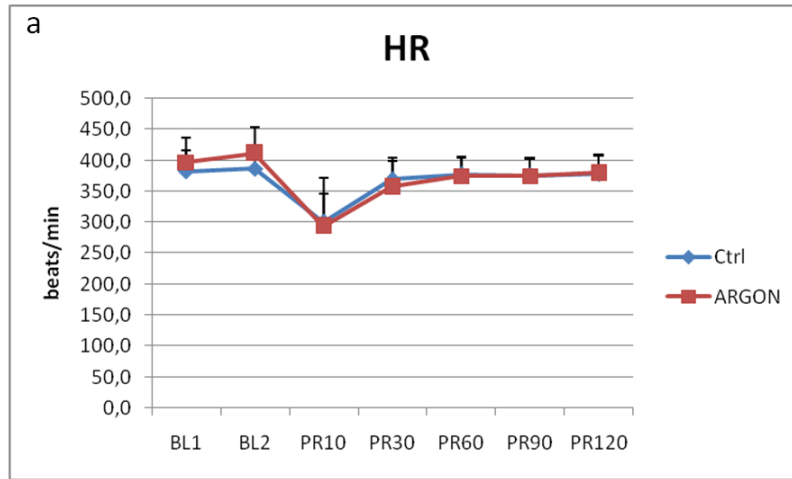


Figure 17. (a) HR, heart rate; (b) MAP, mean arterial pressure; (c) CPP, coronary perfusion pressure in Argon treated animals (n=9) and controls (n=14), at baseline and during the 4 hr post-resuscitation period (PR). Data are reported as mean \pm SEM. Repeated measurement ANOVA with Sidak's multiple comparisons test, * $p < 0.05$.

Figure 18 shows the extracellular levels of GLU and GABA in the dorsal hippocampus at baseline, during and after cardiac arrest in the two experimental groups. As shown, cardiac arrest did not affect GLU extracellular levels (Repeated measurement ANOVA, effect of time $p=0.16$), but it determined the increase in GABA from 0.6-0.7 to 4.3-6.5 pmol/20 μ L (Fig. 18, Repeated measurement ANOVA, effect of time: $p<0.0001$). However, ventilation with Argon did not affect the extracellular levels of GLU and GABA (Fig 18) Repeated measurement ANOVA, effect of treatment: $p=0.39$ and $p=0.38$ respectively).

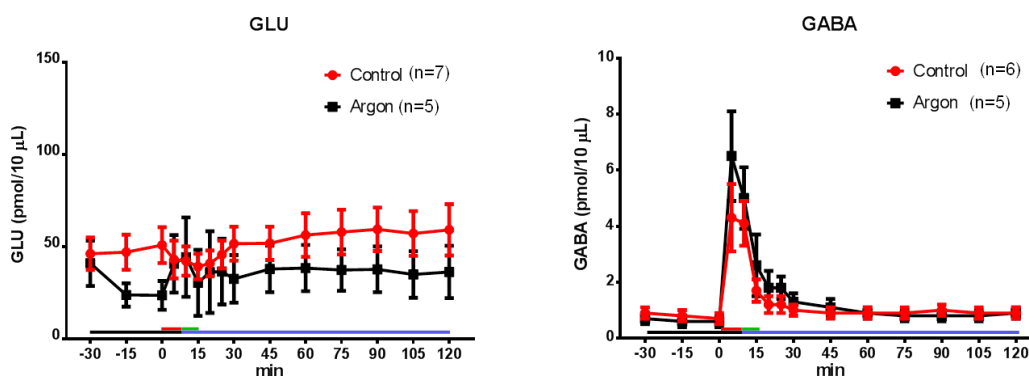


Figure 18. Extracellular levels of GLU and GABA in the dorsal hippocampus at baseline, during CA (red line) and CPR (green line), and after cardiac arrest. The black and the blue line over the X axis indicates the duration of ventilation with room air (at baseline) and the Argon or control ventilation (post cardiac arrest). Data shown as mean and standard error.

The extracellular levels of lactic acid in the dorsal hippocampus increased significantly at 30 min after CA compared to the baseline levels (Fig 19. Repeated measurement ANOVA, effect of time: $p=0.0003$), as expected after an ischemic insult. Lactic acid levels return to baseline levels 2 h after CA induction. The increase in lactic acid levels after CA was more consistent in control ventilation group than in the Argon ventilation one, even if the difference was not statistically significant (Figure 19 Repeated measurement ANOVA, effect of treatment: $p=0.5$).

The extracellular levels of pyruvic acid in the control group decreased during the first 30 min after CA, compared to the baseline levels, then increased 1 h after cardiac arrest and remained higher than baseline levels till the end of the experiment. The decrease in pyruvic acid levels early after CA was not observed in the Argon treated animals, where levels of

pyruvic acid increase starting from the induction of CA (Figure 19. Repeated measurement ANOVA, effect of time: $p=0.0006$). Treatment does not affect pyruvic acid levels during CPR and after resuscitation (Figure 19. Repeated measurement ANOVA, effect of treatment: $p=0.52$).

The ratios between the lactate and pyruvate concentration are shown over time to highlight the effect of the Argon ventilation on anaerobic metabolism. The rise in ratio was significantly attenuated in rats ventilated with Argon during the first hour after cardiac arrest compared to controls (Figure 19. Two-way ANOVA, effect of time: $p<0.0001$, effect of treatment: $p=0.001$).

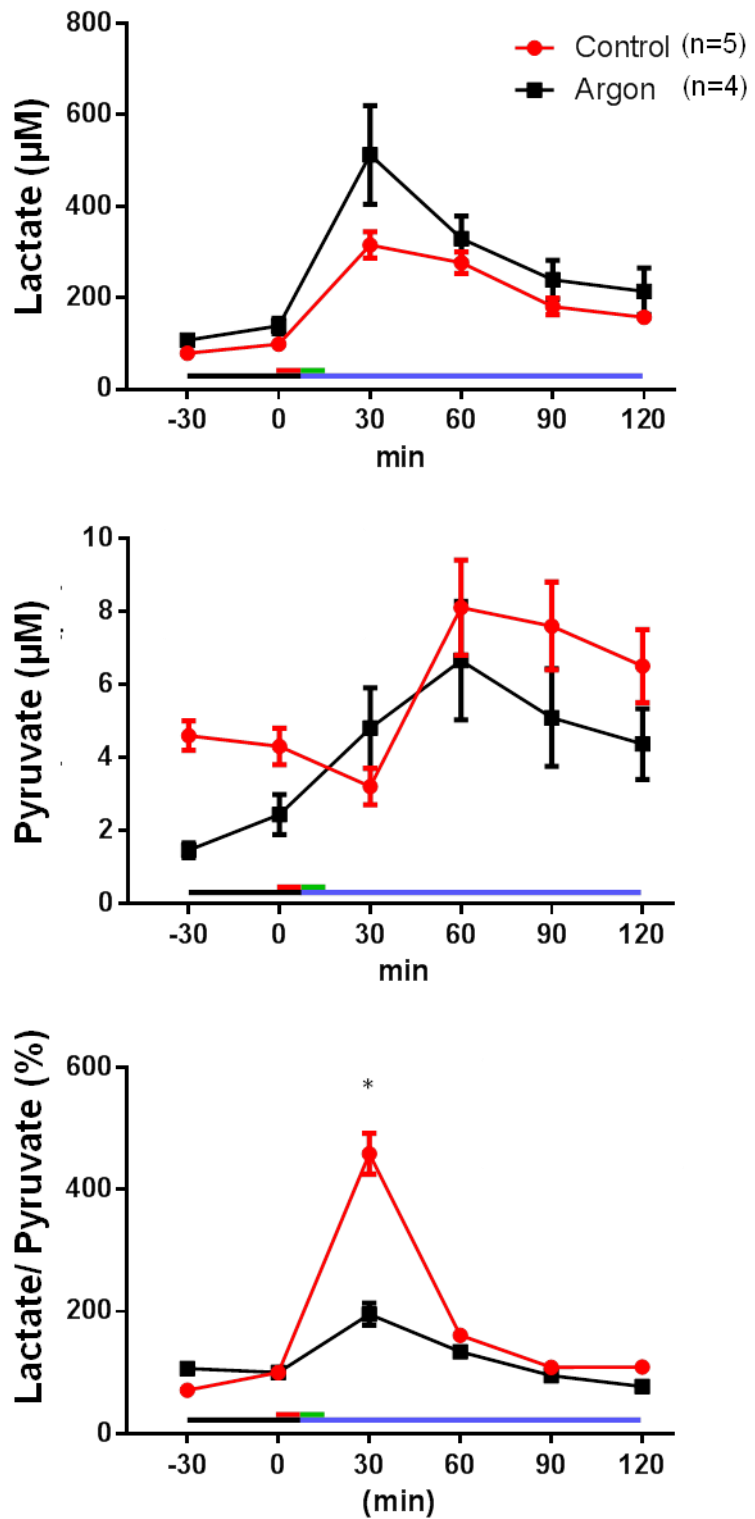


Figure 19. Extracellular levels of lactate and pyruvate in the dorsal hippocampus, and ratios between the lactic acid and pyruvic acid concentration, at baseline, during CA (red line) and CPR (green line), and after cardiac arrest. The black and the blue line over the X axis indicates the duration of ventilation with room air (at baseline) and the Argon or control ventilation (post cardiac arrest). Data shown as mean and standard error. Repeated measurement ANOVA with Tuckey's multiple comparisons test, * $p < 0.05$.

Finally, to assess the protective effect of Argon ventilation on the myocardium, plasma levels of hs-cTnT were measured. Reduction in hs-c TnT were detected in animals ventilated with Argon compared to controls (Figure 20).

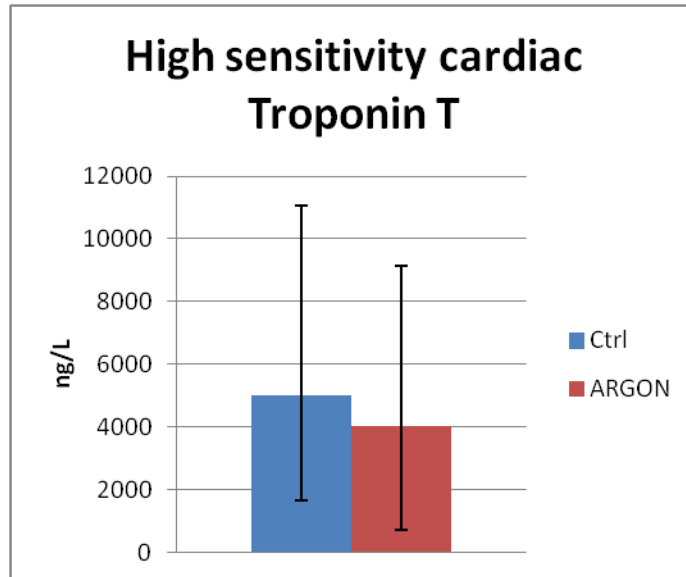


Figura 20. Plasma hs-c TnT in Argon treated animals (n= 13) and controls (n= 18), 120 mins after ROSC. Data shown as median [Q1-Q3].

5 Rat model of myocardial ischemia-reperfusion injury- Experiment 5

Six hours after reperfusion, ten rats survived in both groups. Ventilation with Argon did not affect neither infarct size nor heart weight (Table 14, Student T-test, p not significant). However, rats treated with Argon showed a lower plasma level of hs-cTnT compared to controls (Figure 21. 3128 ng/L in Ar vs. 7432 ng/L; Mann-Whitney test, p=0.046) together with a reduced neutrophil infiltration (Figure 21. 72 cells/mm² in Argon animals vs. 92 cells/mm² in controls, p=not significant).

	Argon	CTRL	p
Survival, n/tot	10/12	10/13	1.00
Infarct Size, %	33.89 ± 5,1 (n=11)	30.75 ± 3.7 (n=10)	0.63
HW, g	0.813 ± 0.04 (n=11)	0.884 ± 0.05 (n=11)	0.30

Table 14. Survival, Infarct size, heart weight (HW) in controls and Argon ventilated animals 6 hours after reperfusion. Infarct size is expressed as infarct area/ area at risk. Data shown as mean ± SEM.

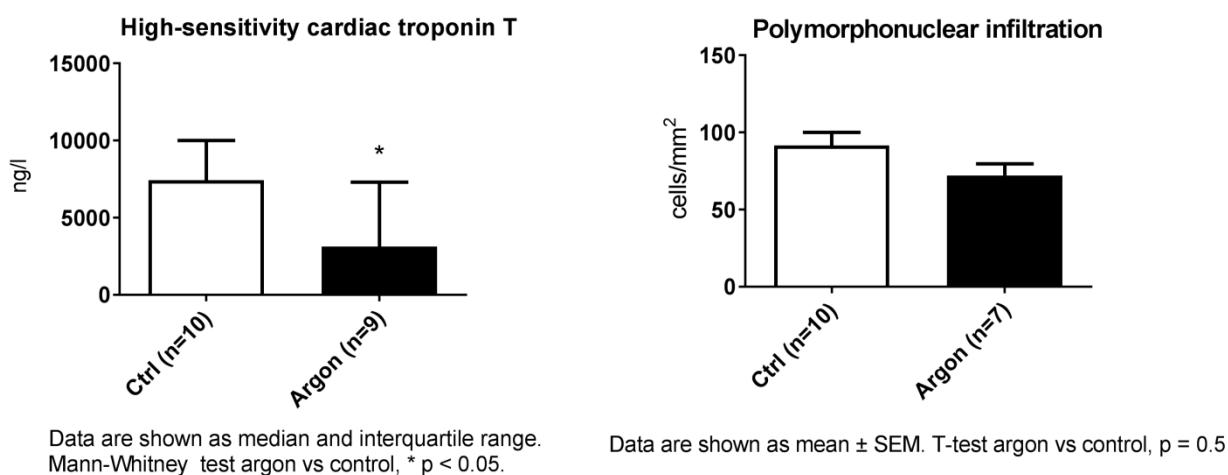


Figure 21. Plasma hs-c TnT and polymorphonuclear infiltration 6 hours after reperfusion.

Twenty-four hours after reperfusion, thirteen rats survived in both groups. A trend towards a reduction of infarct size and heart weight was observed in Argon treated animals compared to controls (Table 15, Student T-test, p not significant). Ventilation with Argon did not affect neither hs-cTnT nor cellular infiltration (Fig 22).

	Argon	CTRL	p
Survival, n/tot	13/16	13/17	1.0
Infarct size, %	36.22 ± 5.1 (n=13)	43.08 ± 5.7 (n=12)	0.38
HW (g)	0.89 ± 0.03 (n=13)	0.95 ± 0.03 (n=13)	0.12

Table 15. Survival, Infarct size, heart weight (HW) in controls and Argon ventilated animals 24 hours after reperfusion. Infarct size is expressed as infarct area/ area at risk. Data shown as mean ± SEM.

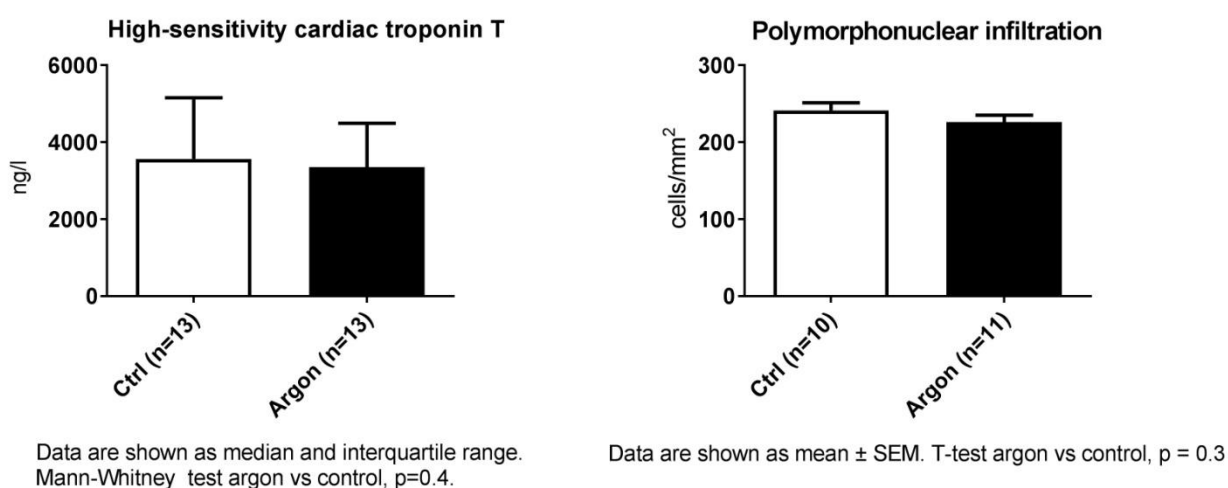


Figure 22. Plasma hs-c TnT and polymorphonuclear infiltration 24 h after reperfusion.

Discussion

1 Main findings

The main finding of my PhD studies is that a 4-h post-resuscitation treatment with Argon significantly improved neurologic recovery after CA. In details, animals that received Argon achieved a faster and complete neurological recovery, in contrast to controls. Different neurological tests together with histopathology confirmed the better recovery induced by Argon in two different swine models of CA and CPR. Our results consistently demonstrated the efficacy of Argon treatment in a preclinical model of CA. Argon efficacy has been tested in the large animal for the first time, corroborating the positive results previously obtained *in vitro* and in rats and providing, providing sufficient informations on the efficacy and safety aspect needed to proceed to clinical application.

Pigs have emerged as established and widely accepted preclinical animal model for CPR research. This species allows for state-of-the-art measurements of myocardial and cerebral functions, ischemic injury, together with inflammatory, immune and oxidative stress reactions. Furthermore, observations on this model are comparable to those made in humans (Babini 2017). The efficacy of Argon in terms of neuroprotection was replicated in both short-duration CA and in a more severe CA model. Data obtained from the experiments on animals subjected to the CA of longer duration added clinical relevance to the experiment with shorter interval of untreated CA, which was purely descriptive. Indeed, clinical studies report an average emergency medical service arrival time to the CA scene of 6-8 min; however, considering the intervals for emergency call, ambulance dispatch, arrival to the patient and onset of CPR, it is reasonable that the no-flow duration accounts for even longer periods. Based on that, the duration of untreated CA in experimental models should reasonably last more than 8 min to adequately reflect the clinical OHCA environment (Nielsen 2013, Rea 2010, Nichol 2015, Hasselqvist-Ax 2015, Dankiewicz

2016). Experimental no-flow duration may be associated with changes in severity of post CA syndrome. Therefore, the studied interventions may either overestimate or fail to show any effect on outcome.

The use of Ar ventilation for neuroprotection after CA was explored in studies performed in rats (*Brücken 2013, 2014, 2015 and 2017, Zuercher 2016*). All these studies reported both neurological recovery, evaluated with the aid of various neurobehavioral tests, and tissue damage, assessed by histopathology and biochemistry. Most of these studies were from the same research group (*Brücken 2014*) that investigated the effect of 1 h of ventilation with a mixture of 70% Ar in O₂ initiated 1 hr after resuscitation. After a no-flow of 7 min, post resuscitation ventilation with Argon led to a better and faster neurological recovery during the following 7 days, assessed by a battery of tests, including the rodent neurological deficit score, the open field and the Morris water-maze tests, compared to the control ventilation (*Brücken 2013*). This good functional recovery was paralleled by a significantly lower brain damage in the neocortex and the hippocampal CA 3 and 4 regions. An additional study introduced further data showing that the 70% Argon-induced neuroprotection persisted even when the onset of the treatment was delayed up to 3 hrs after resuscitation (*Brücken 2014 and 2015*). More recently, neuroprotection of Argon was investigated in conjunction with mild therapeutic hypothermia, based on the hypothesis that the combination of these two interventions could yield to a boosted protection, further improving neurological outcome after CA (*Brücken 2017*). Thus, in the same model, rats were treated with mild therapeutic hypothermia at 33°C (6 hrs) and 1 hr ventilation with 70% Argon. Surprisingly, the results showed that the combined treatment, similarly to a control ventilation with air/O₂, generated worse neurological recovery together with greater neuronal degeneration in hippocampal CA1 region, when compared to mild therapeutic hypothermia alone. Unfortunately, in this study the treatment with Argon

alone, as reference, was absent and thus no further considerations on the effect of Argon can be made.

A dose dependency of Argon treatment emerged from our study. Indeed, in experiment 2 Argon treatment after CA was confirmed to improve both neurological outcome and histopathological brain damage compared to controls, regardless of Argon concentration in the inhalation mixture. However, the neuroprotective effects were more pronounced when the inhalation mixture contained 70% Argon in place of 50%. Interestingly, the hemodynamic parameters were improved in animals treated with Argon 70% compared to controls and to those treated with Ar 50%, suggesting that only Argon at high concentration affects hemodynamic.

The dose-dependent effect observed in our study was supported by a previous study employing a rat model of CA and CPR, ventilated with Argon at a concentration of either 40% or 70% after resuscitation (*Brücken 2014*). In addition, in mouse hippocampal slices subjected to a focal mechanical trauma mimicking traumatic brain injury (TBI), exposure to Argon also prevented neuronal injury in a dose dependent manner with a maximum at 70% concentration (*Brücken 2015*). Our results corroborate these findings and suggest that Argon at least *in vivo* exerts a maximum effect at the highest concentration.

The choice of testing the specific concentration of 50% Argon came from a previous publication, where Loetscher et al. investigated the effects of Argon in two distinct *in vitro* models of neuronal trauma induced by oxygen glucose deprivation (OGD) and traumatic brain injury (TBI) using either 25%, 50% or 75% Argon (*Loetscher 2009*). While there was no significant difference in neuroprotective efficacy between the different Argon concentrations in the OGD setting, a peak effect at 50% Argon was observed in the TBI model. A trend towards lesser neuronal damage in animals subjected to 24 hrs ventilation with Argon 50% after resuscitation compared to controls was reported also in a model of cardiac arrest in rodents induced with KCl and esmolol. As primary outcome, neuronal

damage was assessed by histopathology and for secondary outcome neurologic tests were performed (Zuercher 2016).

Numerous signalling pathways and molecular targets appeared to be involved in the biological effects provided by Argon. However, the specific contribution of each pathway or molecule is still unclear. Potential mechanisms of action involved in the Argon protection are introduced and described in many *in vitro* studies. Thus, Argon appeared to possess oxygen-like properties, which could explain its neuroprotective effects against hypoxia and ischemia, by partially restoring mitochondrial respiratory enzyme activity and reducing NMDA-induced neuronal death (David 2012). Moreover, Ar plays anti-apoptotic effects modulating the molecular pathways involved in cell survival in the following manners: increasing extracellular signal-regulated kinase (ERK) 1/2 phosphorylation, already after 30 min of exposure, which leads to a reduced ERK 1/2-caspase-3 cleavage, blocking the apoptosis cascade (Fahlenkamp 2012, Zhao 2016, Ulbrich 2015); upregulating the expression of the anti-apoptotic protein B-cell lymphoma-2 (BCL-2) (Zhuang 2012, Zhao 2016); activating the toll like receptor (TLR) 2 and 4, which reduce caspase-3 activity (Zhao 2016, Ulbrich 2015 and 2016); and mediate the intracellular signaling involved in the production of pro-inflammatory cytokines, growth factors, and cell survival (Ulbrich 2016). The above mechanisms of action have been confirmed in the *in vivo* studies, by either functional tests or histological and biochemical essays in animals subjected to a variety of injuries and treated with inhaled Argon as potential therapeutic agent.

Our experiment 4 explored the effect of Argon treatment on the brain extracellular levels of glutamate and GABA. Indeed, brain extracellular levels of the 2 neurotransmitters was reported to increase rapidly following the onset of ischemia, remain elevated during ischemia, and then decline over 20–30 min following reperfusion (Phillis 2003). The elevated levels of glutamate can contribute to the demise of surrounding neurons (Rothman 1986, Choi 1997), whereas the inhibitory amino acid GABA is considered to be

neuroprotective (Lyden 1997). Regarding GLU extracellular brain level, despite what is described in literature, we did not observe neither increase in GLU level during CA nor statistically significant differences between groups in GLU level consequent to the Ar or control ventilation. In contrast, GABA extracellular levels increased during the ischemic insult, even if we did not observe any difference between groups according to treatment. It should be noted that this data is to be considered preliminary because of the low number of animals per experimental group. Moreover, we observed lower brain extracellular lactate/piruvate ratios in animals treated with Argon, suggesting Argon treatment can alleviate the deterioration of the energy supply in the brain during the early phases of reperfusion (Madl 2004).

2 Hemodynamic effects and safety

Importantly, our studies demonstrated that the improved neurological recovery provided by the Argon treatment is associated with no effects on hemodynamics, respiratory function and gas exchange in both preclinical models. The absence of detrimental effects after Argon inhalation was also confirmed by data obtained in anesthetized domestic healthy pigs subjected to 4-h ventilation with Argon (Experiment 3). Several publications support our observations. In fact, no study has ever reported safety concerns related to exposure to inhalation of Argon. Hemodynamic parameters and blood gases were monitored during Argon ventilation in several *in vivo* studies (Brücken 2013, 2014, 2015 and 2017, Zuercher 2016, David 2012, Alderliesten 2014, Broad 2016, Ryang 2011, Pagel 2007, Martens 2017). In all these studies, Ar affected neither hemodynamics nor ventilation, in terms of respiratory gas exchange. In addition, in three studies body weight was also reported as an indicator of animal's wellbeing during the days after Ar ventilation and none of them reported significant changes (Zuercher 2016, Höllig 2016, Zhuang 2012). Furthermore, two studies aimed to specifically evaluate the safety of Argon ventilation in pigs. In one study, neonatal piglets were ventilated with Argon at different concentrations, ranging from 30% to 80% in

O₂, for a total of 3 h. Again, effects on hemodynamics and blood gases were evaluated, together with those on electrical brain activity (*Alderliesten 2014*). Prolonged ventilation with Ar was confirmed to be safe, at each concentration, in this setting. In the other study a 6-h ventilation with Argon 79% in pigs, showed no toxic effects, as demonstrated by analysis of serum biomarkers and histological assessment of liver and kidney function and structure (*Martens 2017*). There are 3 additional studies which evaluated the safety of Argon referring to its use as substitute of CO₂ in inducing pneumoperitoneum during laparoscopic surgery (*Roberts 1997, Eisenhauer 1994, Mann 1997*). Argon insufflations into the abdominal cavity produced hemodynamic modifications, mainly related to increases in abdominal pressure and perhaps to a potential effect on systemic vascular resistances. No significant changes in respiratory functions were observed. Since Argon is lesser soluble than CO₂, an increased risk of hemodynamic instability exists in the instance of accidental gas embolism (*Roberts 1997*). Finally, because Argon is denser than air, it could be argued that ventilation with such a gas could increase respiratory resistances (*Behnke 1939*). However, none of the preclinical studies have reported such a condition after prolonged exposure to Argon. Moreover, a study with a model of vigorous ventilation in a respiratory resistance machine, showed no changes in respiratory resistance at atmospheric pressure, and up to 4 atm, when Argon 80% was compared to air (*Behnke 1939*). To conclude, Argon administration could be considered safe and its potential use in humans should now be addressed.

3 Cardioprotection by Argon

Our data indicated that ventilation with Argon was associated with a slightly smaller left ventricular infarct size and lesser hs-cTnT release in pigs with myocardial infarction and CA. The presence of cardioprotective effect of Argon after myocardial ischemia/reperfusion was also demonstrated previously, although the number of studies was limited. Overall, these studies provided evidence for a reduced infarct size and an improved left ventricle systolic function, evaluated either by echocardiography or magnetic

resonance. Moreover, beside increasing cell viability (*Mayer 2016*), Argon also diminished early after depolarizations in the ventricle tissue, limiting the onset of ischemia-triggered arrhythmias. When Argon was applied on the human right atrial appendages after ischemia reperfusion injury, the recovery of contractile activity was increased compared to the control treatment (*Lemoine 2017*). To address specifically the effect of Argon inhalation on myocardial protection and its mechanism of action, we employed a rat model of coronary occlusion and reperfusion. The mechanism of cardioprotection by Argon described in two different paper are based on the inhibition of the opening of the mitochondrial permeability transition pore. Indeed, apart from ischemia, reperfusion can also contribute to cell death, and, particularly on cardiomyocytes, it can induce ventricular arrhythmias and contractile dysfunction subsequent to the opening of the mitochondrial permeability transition pore. Mitochondrial permeability transition pore is a non- selective channel of the inner mitochondrial membrane that opens at reperfusion, causing mitochondrial calcium - phosphate overload and alteration of mitochondrial membrane potential. For such pathophysiological effects, this channel could be a target in cardioprotection. Phosphatidylinositol-3-kinase (PI3K), ERK 1-2 and 70 kDa ribosomal protein s6 kinase (p70s6K) inhibit mitochondrial permeability transition pore opening by their actions on several downstream signaling molecules that modulate the transition state of the pore either directly (e.g. endothelial nitric oxide synthase, p53, glycogen synthase kinase) or indirectly by affecting the pro- and anti-apoptotic B cell lymphoma protein. Thus, Ar by acting on the same pathways, directly or indirectly, via reperfusion injury salvage kinase (RISK), prevents the mitochondrial permeability transition pore opening (*Lemoine 2017, Pagel 2007*). Instead, we explored the hypothesis that Argon inhalation may reduce cardiac ischemia/reperfusion injury and this could be due to a reduction in inflammatory response. Nevertheless, although ventilation with Argon reduced myocardial damage in terms of hs-c TnT release, we were not able to attribute this beneficial effect to the reduction of inflammatory response.

The significance and innovation of this thesis work is the characterization of a promising and inexpensive therapeutic approach after CA and CPR: inhalation of Argon. Although noble gases are known as "inert" gases, these monatomic colorless, odorless agents can produce biological effects. Compared to other noble gases, and especially to the most widely investigated xenon, Argon has many advantages. Used at normobaric pressures, Argon has no hemodynamic properties and lacks hypnotic and anesthetic effects, thus making open-circuit administration feasible. In addition, Argon is ubiquitous in the atmosphere, being even more common than carbon dioxide, and it is available via extraction from liquefied air. Thus, Argon administration might be substantially inexpensive. Application of this promising gas may expand the neuro- and cardiac care.

ArgonArgonArgonArgonArgonArgonArgonArgonArgonArgonArgonArgonArgonArgonA
rgonArgonArgonArgonArgonArgonArgonArgonArgonArgonArgonArgonArgonConsidering the
promising results of Argon inhalation after CA experimentally obtained and the apparently
safe profile of its administration, the protocol of the “CardioPulmonary Resuscitation with
Argon” – CPAr study, an upcoming phase I clinical trial, was submitted and approved to
the Ethical Committee and is presented in the Appendix as preliminary draft. The aim of
the CPAr study is to evaluate safety and feasibility of Argon ventilation in patients
resuscitated from cardiac arrest, prior to its use as therapeutic agent.

4 Limitations

We recognize limitations in this thesis work. First, while the principal results obtained in the pig model of CA and CPR are very robust, the mechanistic explanation of the positive actions were limited to small rodents and are still preliminary. Thus, further work to elucidate the mechanism of Argon’s neuroprotection is needed. Second, the effects of Argon were not compared with those of other noble gases. However, earlier investigations have reported similar neuroprotective effects of Argon and the widely studied xenon.

ArgonArgonFinally, different onsets and durations of treatment have to be tested. Accordingly, further studies will be performed to address these issues.

5 Conclusions

The substantial knowledge on management of Argon gained from these studies should facilitate its clinical testing and possible its entrance in the clinical practice. Indeed, I believe this project has great translational relevance. Once the therapeutic approach with inhaled Argon in the preclinical setting is optimized, its subsequent clinical application with an optimized therapeutic strategy would be the next step.

Compared to the fragmented informations emerging from the available literature on the clinical use of Argon, the approach adopted in the PhD studies reported within this thesis represents a structured investigation of different aspects of the Argon-based treatment of CA, and represents a valid background in order to move into the clinical setting.

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Appendices

Appendix 1

CLINICAL STUDY PROTOCOL

Rationale

Ar, as shown in the above discussed preclinical studies, might diminish the neurological and myocardial damage after any hypoxic-ischemic insult. Indeed, Ar has been tested in different models of ischemic insult, at concentrations ranging from 20% up to 80%. Overall, Ar emerged as a protective agent on cells, tissue and organs, showing less cell death, reduced infarct size and faster functional recovery. More specifically, encouraging data has been reported in animal studies on cardiac arrest in which a better and faster neurological recovery was achieved when Ar was used in the post-resuscitation ventilation. More importantly, these beneficial effects played by Ar have been replicated in different studies, enrolling both small and large animals (12–16,48). Finally, ventilation with Ar in O₂ has been demonstrated to be safe both in animals and humans (35,60,63). Thus, based on this evidence, a clinical translation is advocated.

Thus, the CardioPulmonary resuscitation with Argon – CPAr study has been conceived. The aim of the CPAr study is to evaluate safety and feasibility of Ar ventilation in patients resuscitated from cardiac arrest, prior to its use as therapeutic agent.

Methods

Study design

CPAr study is phase I, open label randomized controlled intervention study performed in patients resuscitated from out-of-hospital (OH) CA and admitted to ICU of two tertiary hospitals in Italy. The study will be submitted for approval by the Institutional Ethical Committees and will be conducted in compliance with the World Medical Association Declaration of Helsinki. Only patients meeting the inclusion criteria and from whom informed consent for the use of the data are obtained, either directly from the patient or from her/his legal representative, are included in the study.

Inclusion and exclusion criteria

Inclusion criteria are: ICU admission after resuscitation from witnessed non-traumatic OHCA of presumably cardiac etiology with a presenting shockable rhythm; age ≥ 18 years; unconsciousness after ROSC; duration of CPR ≤ 30 min; CPR performed according to European Resuscitation Council 2015 guideline (64); initiation of study intervention ≤ 2 hrs from ROSC. Exclusion criteria are: age < 18 years; non-witnessed CA; CA of traumatic origin or from a non-presumably cardiac cause; CA with a non-shockable presenting rhythm (pulseless electrical activity and asystole); pregnancy; presumable irreversible death or known terminal illness; pre-CA CPC ≥ 3 ; participation in another clinical trial within the previous 30 days; initiation of the study intervention > 2 hrs from ROSC; refused informed consent to the use of data.

Study procedures

Upon ICU admission, the ventilation of the eligible patients is performed with the ventilator Bellavista 1000, IMT, Switzerland, a CE-approved device for clinical use and compatible with the ventilation with Ar 70% / O₂ 30%. A 30-min ventilation with air 70% and oxygen 30% is begun at ICU admission to evaluate the capability to maintain adequate arterial saturation with such a FiO₂. Ventilator is set with pressure control mode with a tidal volume of 6-8 ml/kg, a PEEP of 5-10 cmH₂O and a respiratory ratio (RR) adjusted to obtain an arterial carbon dioxide partial pressure (paCO₂) of 35-40 mmHg. Normoxia with an oxygen arterial saturation (SaO₂) of 95%-98% are the target for ventilation. After this 30-min ventilation trial: 1) if the ventilation targets are not reached the study is discontinued and patient care continued according to 2015 ERC guidelines; 2) if the ventilation targets are reached, the patient is randomized to: a 4 hrs ventilation with Ar 70%/oxygen 30%; or a 4 hrs ventilation with oxygen 30% in room air. After completion of the ventilation period, ventilation is switched to regular oxygen/air ventilation and subsequent management is performed according to 2015 ERC guidelines and local post-resuscitation protocols. In both groups, blood gases are measured at hourly intervals to determine pH, PaO₂, PaCO₂, HCO₃⁻ during ventilation and till one hour after completion of the experimental treatment. Subsequent blood gas analyses will be performed according to the local post-resuscitation care protocols.

The following data are collected: age, gender, body mass index, Sequential Organ Failure Assessment (SOFA) score, cause of CA, characteristics of the OHCA according to Ustein style, post-CA interventions (i.e. coronary angiography; percutaneous coronary intervention, hemodynamic support), pre-existing pathological conditions.

Blood samples at 0, 6, 24, 48, and 96 hrs post-ROSC are withdrawn for analysis of: hs-cTnT; creatinine; transaminases; electrolytes; and lactates. NSE is measured at 48, 72 and 96 hrs after hospital admission. Survival to ICU discharge, hospital discharge and 1-month is evaluated together with the cerebral performance category (CPC) score. The responsible physician will call on the phone the patient to check for his vital status and neurological recovery at 1 month since the inclusion.

Study endpoints

The *primary endpoint* of the study is to identify any early adverse events related to Ar inhalatory treatment: inability to adequately ventilate using Ar/O₂ within the predetermined setting (FiO₂ 30% and PEEP 5–10 cmH₂O), i.e. an arterial O₂ saturation < 95%; and/or appearance of detrimental Ar-related hemodynamic adverse effects, i.e. arterial hypotension not responsive to fluids and/or vasopressor/inotropic drugs and malignant arrhythmias.

In addition, data on potential delayed effects of Ar on survival and CPC at ICU discharge and at 1 month later, are collected.

Statistics

Since there is no data on the occurrence of adverse events and/or clinical safety of Ar treatment after OHCA, a formal sample size calculation cannot be performed. The sample size has been selected in order to establish sufficiently precise measures of safety and feasibility, while it is not powered to address any clinical efficacy. A number of 50 patients (25 per group) has been chosen to be included, as considered reasonable to detect an increase in adverse events related to Ar, in comparison to the control treatment. For this safety study, a concurrent control group was also considered, in order to better discriminate any adverse event related to Ar, but also to acquire data to be used for a correct sample size calculation for a subsequent phase II study.

CONCLUSIONS

Cardiac arrest is a public health issue with a severe impact on mortality and morbidity. Annually about 275.000 people in Europe experience OHCA, with an incidence rate of 38/100000 people/year . Approximately 70-90% of individuals with OHCA die before reaching the hospital and those who survive cardiac arrest are likely to suffer post-cardiac arrest syndrome. This syndrome has a high mortality rate due to its unique pathophysiological process involving multiple organs. The main four components of this syndrome are: 1) post-CA brain injury, 2) post-CA myocardial dysfunction, 3) systemic ischemia and reperfusion response, 4) persistent precipitating pathology. Moreover, as many as 30% of survivors of CA manifest permanent brain damage and in some instances only 2–12% of resuscitated patients have been discharged from the hospital without neurological impairments.

Despite the efforts to reduce brain injury and cardiovascular impairment after CA, outcome results are controversial and further approaches need to be established. So far, many supportive measures have been proposed and applied in clinical practice, but only temperature management appears as a possible neuroprotective treatment. In order to overcome this lack of treatment option, novel therapeutic approaches have been conceived and tested in the last decade, including volatile

anesthetics and noble gases, such as Ar, to allow for cerebral and myocardial preservation after CA. This study, will confirm that administration of Ar is safe and feasible and would provide preliminary data on clinical outcome, essential to design a subsequent phase II clinical study on efficacy.

Appendix 2

Supplemental Table 1. From *Scolletta 2012*.

Table 1. Most commonly used biomarkers of organ injury after cardiac arrest.								
Biomarker	Use	Cutoff	Timing	Sensitivity (%)	Specificity (%)	FPR	TH	Limitations
<i>Heart injury</i>								
Troponin	AMI Diagnosis	0.6–14.5 ng/ml	< 12 h	72–88	75–95	NA	No effects	Need to be combined with ECG Specific only if ST-elevation Elevated after coronary angiography
CK-MB	AMI Diagnosis	60 ng/ml	< 12 h	88	88	NA	NA	Not specific of AMI Elevated because of CPR Elevated because of defibrillation
BNP	Outcome	80–230 pg/ml	Admission	83–87	87–96	NA	NA	Limited studies Elevation in other conditions with HF
<i>Brain injury</i>								
NSE	Outcome	> 33 mg/l	< 72 h	72–80	84–100	0–23%	Reduced levels	Elevated if hemolysis Elevated if use of LVAD/IABP Elevated in some cancers
S100B	Outcome	0.2–1.5 mg/l	< 72 h	70–80	85–100	0–16%	NA	Elevated if heart and aorta injury Elevated in some cancers
<i>Inflammation</i>								
Procalcitonin	Outcome	0.5–1.0 ng/ml	< 24 h	70–85	80	NA	NA	Elevated in inflammatory conditions Elevated if underlying infection

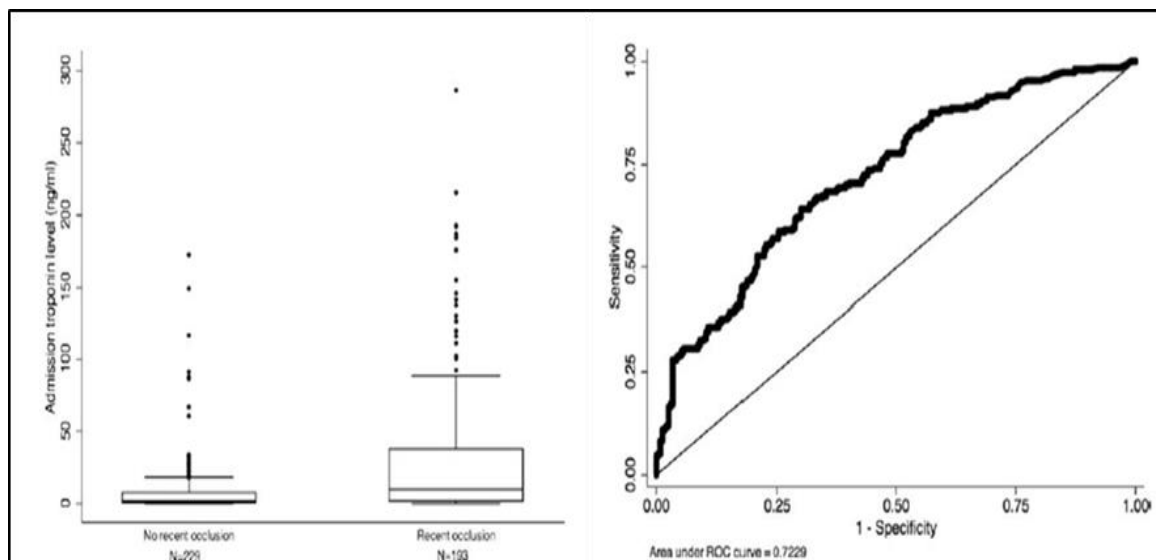
AMI: Acute myocardial infarction; BNP: Brain natriuretic peptide; CK-MB: Creatine kinase-MB; CPR: Cardiopulmonary resuscitation; FPR: False-positive rate; HF: Heart failure; IABP: Intra-aortic balloon pump; LVAD: Left ventricular assist device; NA: Not available; NSE: Neuron-specific enolase; TH: Therapeutic hypothermia.

Supplemental Table 2. From *Hochholzer 2010*.

Table II. Comparison of selected cardiovascular biomarkers			
	Prognostic impact	Diagnostic impact	Therapeutic impact
Markers of necrosis			
Creatine phosphokinase MB	+++	+++	++
Myoglobin	++	++	++
Troponin	++++	++++	++++
Markers of myocardial dysfunction or stress			
Atrial natriuretic peptides	+++	+++*	?
Brain natriuretic peptides	++++	++++*	+++*
Copeptin	++	+	?
Proadrenomedullin	++	+	?
Markers of inflammation			
Adiponectin	++	?	?
C-reactive protein	++++	?	++
Growth differentiation factor 15	+++	?	+
Interleukin 6	+++	?	?
Soluble ST2			
Tumor necrosis factor α	++	?	?
Myeloid-related protein 8/14	+	?	?
Markers of ischemia			
Choline	++	?	?
Heart-type fatty acid-binding protein	++	++	?
Ischemia modified albumin	+	+	?
Markers of plaque destabilization/rupture			
Lipoprotein-associated phospholipase A2	+++	?	?
Matrix metalloproteinase-9	++	?	?
Myeloperoxidase	+++	++	?
Placental growth factor	++	?	?
Pregnancy-associated plasma protein A	+++	+	?
Secretory phospholipase A2	+	?	?
Soluble fms-like tyrosine kinase 1	+	+	?
Soluble intercellular adhesion molecule 1	+++	?	?
Markers of platelet activation			
Soluble CD40 ligand	++/?	?	?
Soluble P-selectin	++	?	?

+, Some evidence by small studies; ++, intermediate evidence from several studies or one large study or trial; +++, good evidence from several large studies or trials; +++++, excellent evidence; ?, conflicting results or no results available or not applicable.
 This table only gives an overview of the evidence published for the various markers. It does not indicate the clinical utility of different markers (eg, a marker might be very useful for risk stratification, but not feasible for the clinical setting due to limitations in detection or because it is also elevated at important differential diagnoses).
 * For stratification of patients with heart failure.

Supplemental Figure 1. Troponin levels dispatched according to the presence of a recent coronary lesion. Troponin I receiving operating characteristic (ROC) curve predicting a recent coronary lesion. From *Dumas 2012*.



Appendix 3

Duration of untreated cardiac arrest and clinical relevance of animal experiments: the relationship between the “no-flow” duration and the severity of post-cardiac arrest syndrome in a porcine model

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Abstract

Introduction. Duration of untreated cardiac arrest (CA), also known as “no-flow” time, is one of the main determinants of the severity of post-cardiac arrest syndrome (PCAS). However, CA models present a large variation in the duration of untreated CA, with often limited clinical relevance. The present study investigated the effect of different no-flow durations on post-resuscitation myocardial and neurological injury and survival in a pig model with the aim to identify an optimal duration that adequately reflects the clinical scenario.

Methods. An established model of myocardial infarction followed by CA and cardiopulmonary resuscitation (CPR) was used. The left anterior coronary artery was occluded and ventricular fibrillation induced in 22 male pigs. Animals were divided in 3 groups of different no-flow durations, namely short (8-10 min), intermediate (12-13 min), and long (14-15 min), prior to mechanical CPR and defibrillation. Arterial and central venous pressures were invasively monitored. Left ventricular (LV) systolic function, expressed as ejection fraction (EF), was echocardiographically assessed together with thermodilution cardiac output (CO) and high sensitivity cardiac troponin T (hs-cTnT) assay. Neurological impairment was evaluated by neurological scores, serum neuron specific enolase (NSE), and histological neuronal degeneration.

Results. All pigs were successfully resuscitated except for one in the longest no-flow group. More than 60% of animals survived when the duration of CA was ≤ 13 min, compared to only 20% for a duration ≥ 14 min. Moreover, neuronal degeneration and neurological scores showed a trend towards a worse recovery for longer durations of no-flow; No animals achieved a good neurological recovery for a no-flow ≥ 14 min, in comparison to a 56% for a duration ≤ 13 min ($p=0.043$). Serum NSE levels were significantly correlated with the no-flow duration level ($r=0.892$, $p<0.001$). Longer durations of CA were characterized by significantly lower LV EF and CO compared to short durations ($p<0.05$). The longer was the duration of no-flow, the higher was the total number of defibrillation delivered ($p=0.033$) and this number of defibrillations correlated with LV EF impairment ($r=-0.523$, $p=0.038$) and with plasma hs-cTnT release ($r=0.679$, $p=0.002$).

Conclusion. This porcine model showed a linear relation between the duration of untreated CA and the severity of PCAS. Indeed, longer no-flow durations caused greater post-resuscitation myocardial and neurological dysfunction and reduced the survival rate, in comparison to shorter durations. A duration of untreated CA of 12-13 min may be an optimal choice for clinically relevant CA models.

Key words: No-flow; cardiac arrest; outcome; post-cardiac arrest syndrome; animal model.

Introduction

Cardiac arrest (CA) is burdened by high mortality and severe neurological impairment among survivors.¹ Up to 64% of out-of-hospital (OH) CA victims die on the scene or on the way to the hospital during ambulance transport.² Moreover, more than half of the successfully resuscitated patients die within 72 hr after hospital admission,^{3,4} such that final survival is approximately 8% in Europe, with great differences among countries.² Patients who eventually survive to hospital discharge, frequently suffer of a persistent neurocognitive impairment that deeply impacts the quality of life.^{1,4,5} Post-cardiac arrest syndrome (PCAS) is the responsible for these subsequent in-hospital mortality and poor neurological outcome.³

Novel therapeutic approaches have been conceived and tested in order to improve outcome of CA. Indeed, experimental models represent the starting point for evaluating the effect of new interventions prior to their implementation into the clinical practice. However, CA models are often not consistent in methods and experimental designs, such that their findings might be hardly reproduced in human trials. An example is represented by the duration of untreated cardiac arrest, known as “no-flow” time, that although recognized as one of the main determinants of the PCAS severity,⁶ remains highly variable in the experimental studies.^{7,8,9} This potentially raises concerns on the adequacy of available experimental models in reflecting the real clinical scenario.¹⁰

Clinical studies report an average emergency medical service (EMS) arrival time to the CA scene of 6–8 min;^{4,11–14} however, considering the intervals for emergency call, ambulance dispatch, arrival to the patient and onset of cardiopulmonary resuscitation (CPR), it is reasonable that the no-flow duration accounts for even longer periods. Based on that, the duration of untreated CA in experimental models should reasonably last more than 8 min to adequately reflect the clinical OHCA environment. Indeed, a shorter or a longer experimental no-flow duration may be associated with a lesser or a more severe PCAS in the animal, respectively,⁶ such that the studied interventions may overestimate or fail to show any effect on outcome of CA.

The present study investigates the effect of different durations of untreated CA on post-resuscitation myocardial and neurological injury and survival in a pig model of CPR with the aim to identify an optimal no-flow interval to be used for interventional experimental studies.

Materials and method

All procedures involving animals and their care were in conformity with national and international laws and policies. Approval of the study was obtained by the institutional review board committee and governmental institution.

Animal Preparation

Twenty-two male domestic pigs (40 ± 1 kg) were fasted the night before experiment except for free water access. Anesthesia was induced by intramuscular injection of ketamine (20 mg/kg) followed by intravenous administration of propofol (2 mg/kg) and sufentanyl (0.3 μ g/kg) through an ear vein access. Anesthesia was then maintained by continuous intravenous infusion of propofol (4–8 mg/kg/hr) and sufentanyl (0.3 μ g/kg/hr). A cuffed tracheal tube was placed, and animals were mechanically ventilated with a tidal volume of 15 mL/kg and FIO₂ of 0.21. Respiratory frequency was adjusted to maintain the end-tidal PCO₂ (EtCO₂) between 35 and 40 mmHg, monitored with an infrared capnometer.⁸ For measurement of aortic pressure, a fluid-

filled 7F catheter was advanced from the right femoral artery into the thoracic aorta. For measurements of right atrial pressure, core temperature, and cardiac output, a 7F pentalumens thermodilution catheter was advanced from the right femoral vein into the pulmonary artery. Conventional pressure transducers were used (MedexTransStar, Monsey, NY). Myocardial infarction was induced in a closed-chest preparation by intraluminal occlusion of the left anterior descending (LAD) coronary artery with the aid of a 6F balloon-tipped catheter inserted from the right common carotid artery.⁸ For inducing ventricular fibrillation (VF), a 5F pacing catheter was advanced from the right jugular vein into the right ventricle. The position of all catheters was confirmed by characteristic pressure morphology and/or fluoroscopy. Frontal plane electrocardiogram was recorded. Heart rate and rhythms were monitored by electrocardiograms (EKG).

Experimental procedure

The balloon of the LAD coronary artery catheter was then inflated with 0.7 mL of air to occlude the flow. If VF did not occur spontaneously, after 10 min it was induced with 1 to 2 mA AC current delivered to the right ventricle endocardium.⁸ Ventilation was discontinued after onset of VF. After 8 to 15 min of untreated VF, CPR, including chest compressions with the LUCAS 2 (PhysioControl Inc, Lund, Sweden) and ventilation with oxygen (tidal volume of 500 mL, 10 breaths/min), was initiated. More specifically, six pigs had a no-flow time of 8 min, two of 10 min, five of 12 min, three of 13 min, one of 14 min and five of 15 min. For homogeneous comparison, animals were assigned to three groups of no-flow duration: "short" 8-10 min (n=8); "intermediate" 12-13 min (n=8); and "long" 14-15 (n=6). After 5 min of CPR, defibrillation was attempted with a single biphasic 150-J shock, using an MRx defibrillator (Philips Medical Systems, Andover, MA). If resuscitation was not achieved, CPR was resumed and continued for 1 min before a subsequent defibrillation. Adrenaline (30 µg/kg) was administered via the right atrium after 2 and 7 min of CPR. Successful resuscitation was defined as restoration of an organized cardiac rhythm with a mean arterial pressure (MAP) of more than 60 mmHg. After that, if VF reoccurred, it was treated by immediate defibrillation. After successful resuscitation, anesthesia was maintained, and animals were monitored during the 4-h treatment. Forty-five minutes after resuscitation, the LAD coronary artery catheter was withdrawn. Temperature of the animals was maintained at 38°C ± 0.5°C during the whole experiment. After 4 h of treatment, catheters were removed, wounds were repaired, and the animals were extubated and returned to their cages. Analgesia with butorphanol (0.1 mg/kg) was administered by intramuscular injection. At the end of the post-resuscitation observation period, up to 96 h, animals were reanesthetized for echocardiographic examination and blood sample withdrawn. Animals were then sacrificed painlessly with an intravenous injection of 150 mg/kg sodium tiopental, and heart and brain were harvested. Autopsy was performed routinely for potential injuries due to CPR or obfuscating disease.

Measurements

Hemodynamics, ETCO₂, and electrocardiogram were recorded continuously on a personal computer-based acquisition system (WinDaq DATAQ Instruments Inc, Akron, OH). The coronary perfusion pressure was computed from the differences in time-coincident diastolic aortic pressure and right atrial pressure. Cardiac output (CO) was measured by thermodilution technique (COM-2; Baxter International Inc, Deerfield, IL). Echocardiography was performed using a phase-array multifrequency 2.5- to 5-MHz probe (CX50, Philips, The Netherlands), and left ventricular (LV) ejection fraction (EF) was calculated using the modified single-plane Simpson's rule.⁸ Arterial

blood gases were assessed with i-STAT System (Abbott Laboratories, Princeton, NJ). Plasma high-sensitivity cardiac troponin T (hs-cTnT) and serum neuron-specific enolase (NSE) were measured with electrochemiluminescence assays (Roche Diagnostics Italia, Monza, Italy).

As previously described,⁸ neurologic recovery was assessed with the neurologic alertness score (NAS), ranging from 100 (normal) to 0 (brain death), and with the swine neurologic deficit score (NDS), ranging from 0 (normal) and 400 (brain death). Finally, the functional recovery was evaluated prior to sacrifice according to overall performance categories (OPCs) as follows: 1 = normal, 2 = slight disability, 3 = severe disability, 4 = coma, and 5 = brain death or death.⁸ Outcome was defined poor when OPC was ≥ 3 . Scores were assessed by veterinarian doctors blinded to treatment.

At sacrifice, the brains were carefully removed from the skulls and fixed in 4% buffered formalin. Standardized 5-mm coronal slices were taken. The hippocampal CA1 sector and the cortex were chosen as regions of interest and were paraffin embedded. Five-micrometer-thick sections were then obtained and stained with hematoxylin-eosin. The proportion of neuronal loss and degeneration/necrosis (shrunken neurons with deeply acidophilic cytoplasm and pyknotic nucleus) was quantified as absent (0), rare (1), few (2), and numerous (3). An experienced pathologist, blinded to treatment, performed the assessments. For transmission electron microscopy (TEM) analysis, samples from hippocampus were reduced and fixed with 4% paraformaldehyde and 2% glutaraldehyde in phosphate buffer 0.12 mol/l pH 7.4 overnight at 4°C, followed by incubation at room temperature for 2 h in 2% OsO₄. After dehydration in a graded series of ethanol preparations, tissue samples were cleared in propylene oxide, embedded in epoxy medium (Epoxy Embedding Medium kit; Sigma-Aldrich) and polymerised at 60°C for 72 h. Ultra-thin (60 nm thick) sections of areas of interest were obtained with a Leica EM UC6 ultramicrotome (Leica Microsystems), counterstained with uranyl acetate and lead citrate and examined with Energy Filter Transmission Electron Microscope (EFTEM, ZEISS LIBRA® 120) equipped with YAG scintillator slow scan CCD camera.

Myocardial infarct was assessed by tetrazolium chloride (TTC) staining. The LV was sliced into 5-mm-thick transverse sections, which were incubated (20 min) in a solution of TTC and then transferred to 4% formalin overnight before image analysis. Infarct size was reported as percentage of TTC-negative area relative to LV area.⁸

Statistical Analysis

One sample Kolmogorov–Smirnov Z test was used to confirm normal distribution of the data. For comparisons of time-based variables, repeated measures analysis of variance (ANOVA) was used. For comparisons between groups at the given time points, one-way ANOVA with Tukey's multiple comparison was used for normally distributed variables, while Kruskal-Wallis test with Dunn's multiple comparison was used for not normally distributed variables. When the dependent variable was categorical, χ^2 test was performed. Linear correlations between parametric variables were calculated using the Pearson correlation coefficient. Spearman test was performed for the non-parametric variable correlation analyses. For survival analysis, Kaplan-Meier survival curves and log-rank (Mantel-Cox) test were used. Data are expressed as mean \pm SEM, except for CPR duration, number of defibrillations delivered, hs-cTnT and NSE, presented as median [Q1-Q3]. A $p \leq 0.05$ was regarded as statistically significant. Data analyses were performed using GraphPad Prism (version 6.05 for Windows, GraphPad Software, USA).

Results

No significant differences in hemodynamic parameters, EtCO₂, cardiac function and blood gases analysis were observed between groups at baseline (Table 1). Duration of CPR was similar in the 3 groups (*p* not significant) and all animals were successfully resuscitated except for 1 animal in the long duration group. More than 80% of animals in the short and 60% in the intermediate no-flow duration survived for more than 3 days, in contrast to only 20% in the long duration group (*p*=0.007 long vs. short, Table 1 and Figure 1). The longer was the duration of no-flow, the higher was the total number of defibrillations delivered (*p*=0.042), with a 5-fold difference between the long and the short duration groups (*p*<0.05, Table 1 and Figure 2).

Resuscitation outcomes, post-resuscitation hemodynamics, and blood gas analyses data are shown in Table 1. No significant differences in MAP were observed among the groups, except for a trend towards a higher MAP in the short no-flow group compared to the others (Table 1). Animals subjected to longer durations of no-flow presented significantly higher post-resuscitation HR and RAP and a lower LV CO, compared to those in the shorter durations (*p*=0.040, *p*<0.001, and *p*=0.032, respectively Table 1).

LV EF decreased in each group after resuscitation (Figure 3). Post-resuscitation LV EF was significantly lower in the long duration group compared to the short one (*p*<0.05). LV EF at 4 h post-resuscitation was inversely correlated with the duration of no-flow (*r*=-0.65, *p*=0.006, Figure 3). Moreover, an inverse relationship between LV EF and the number of delivered defibrillations was observed (*r*=-0.52, *p*=0.038, Figure 2).

LV infarct size was comparable after short or intermediate duration of no flow, while it was greater in the single animal that survived in the long duration group (Table 1). Hs-cTnT release significantly correlated with the LV infarct size (*r*=0.77, *p*=0.004). There was a trend towards higher plasma levels of hs-cTnT in the long duration group, but there was no correlation between hs-cTnT and the duration of no-flow (Figure 4). Nevertheless, levels of hs-cTnT were significantly related to the number of defibrillations received by the animals (*r*=0.679, *p*=0.002, Figure 2).

Sixty-two % of the animals resuscitated in the short and 50% in the intermediate no-flow duration group survived with a good neurological recovery (OPC ≤ 2), in contrast to no animal in the long duration group, in which only one of the 5 resuscitated animals survived and had a OPC of 3 (*p*=0.043, Figure 5). NAS and NDS showed a trend towards a worse recovery for longer durations of no-flow, with the worst scores observed in the group with the longest duration of untreated CA (Figure 5). NSE release paralleled the trend observed in the neurological recovery, with higher circulating levels in the instance of longer durations of no-flow (*p*=0.021 for short vs. intermediated group, Figure 4). Indeed, the duration of untreated CA strongly correlated with NSE release (*r*=0.892, *p*<0.001, Figure 4).

Neurological recovery and NSE data were confirmed by the histological findings, showing greater neuronal degeneration/necrosis in hippocampus and in cortex for increasing no-flow durations, with the single animal survived in the long duration group showing the most severe neuronal injury with associated gliosis and perivascular inflammation (Figures 5 and 6). Ultrastructural imaging showed mitochondria in neurons undergoing degenerative processes of increasing severity, i.e. disruption of cristae and reduction of matrix density, for longer no-flow durations;

neuropil, normally composed by a tightly packed myriad of axons, dendrites and glial processes, also presented a progressive vacuolization and loss of structure (Figure 7).

Discussion

The present study systematically investigated, for the first time, the effect of different no-flow intervals on PCAS severity in an experimental model, describing a linear association between the duration of untreated CA and the correspondent heart and brain injury. Indeed, longer no-flow durations were associated with lower percentage of survival and worse post-resuscitation myocardial dysfunction and neurological recovery compared to shorter durations. These findings can be applied experimentally in order to design more clinically relevant studies.

Duration of no-flow is one of the key features that deeply impact CA survival^{13,15,16} and neurological injury.^{14,17} Designing realistic models for preclinical research, with specific attention on the duration of untreated CA, is therefore crucial to correctly evaluate the potential benefits of new interventions for the treatment of CA and PCAS. For this reason, the no-flow duration should be wisely selected in order to obtain an experimental model that closely reflects the pathophysiology of CA and PCAS and whose observations might be easily translated clinically.¹⁰ In our model, only when the duration of no-flow exceeded 12 min, data on survival were similar to those observed clinically,¹⁸ while with shorter durations a survival above 80% was still present. A duration of untreated CA of 12-13 min showed the best compromise in terms of severity of myocardial and neurological injury and survival, with outcomes that resembled those observed in the clinical scenario.⁴ In our opinion the no-flow duration of 12-13 min may be an optimal choice for CA experimental models, while with longer no-flow intervals, i.e. ≥ 14 min, the mortality was unacceptably high. Moreover, in the view of using a preclinical model to investigate new neuroprotective interventions, a CA duration ≥ 14 min determined probably a too extensive neuronal and mitochondrial damage with important loss of ultrastructure, such that any potential benefit might be obfuscated.

Surprisingly, CA duration is extremely variable in CPR models.⁷⁻⁹ In a review including 42 experimental articles, significant differences in research methodology have been reported, highlighting a poor clinical representativeness in the majority of the studies. More specifically, the duration of untreated CA ranged from 0 to 15 min, with the majority of the experiments employing a duration as short as 3 min or even lesser.¹⁰ In other recent reviews on animal models of CA, longer no-flow intervals, close to 8 min, were reported.^{19,20} Nevertheless, in our model, an untreated CA with a duration up to 10 min caused a limited injury, considering that all the animals were easily resuscitated and almost 90% of them survived during the whole observational period.

After the initial success of CPR, the majority of patients die within 72 h from hospital admission, due to a variable severity of PCAS.^{3,6} Most prominent are post-resuscitation cardiac failure and ischemic brain damage. In our model, when the duration of untreated CA was < 14 min, a survival with a good neurological recovery was observed in approximately 60% of animals, while it occurred in 0% for a longer duration. This was concordant with the trend observed in the behavioral scores used to assess the neurocognitive status during the days after resuscitation and with the neuronal degeneration, which paralleled the duration of untreated CA, both in the hippocampus and in the frontal cortex. Thus, our results suggest that a clinically relevant model of CA should use longer durations of no-flow, i.e. $> 12-13$ min, to achieve a good balance between post-resuscitation neurocognitive injury and mortality, with a more clinical relevance.

The myocardial dysfunction observed after CA is a transient phenomenon due to cell stunning rather than permanent injury, that usually spontaneously reverses within the following 2-3 days.^{3,17,21} In our model, the post-resuscitation myocardial dysfunction, evaluated by LVEF, was deeply depressed in all groups of animals during the first 4 h post-resuscitation, with a greater impairment after a duration > 14 min. The reason for a worse myocardial dysfunction after a longer untreated CA may be explained as the result of a more severe ischemic insult, as supported by the 2-fold greater infarct size in the single animal that survived and by the higher plasma hs-cTnTs in this group of animals. Another explanation for the greater post-resuscitation myocardial dysfunction after longer durations may be represented by the greater number of defibrillations delivered to the animals.²² Indeed, repetitive defibrillations are known to increase the severity of post-resuscitation myocardial dysfunction²³ and in our study, the longer was the duration of no flow, the greater was the number of defibrillation attempts and such a number of electrical countershocks was significantly related with the systolic dysfunction, represented by the LVEF impairment, and with the myocardial injury, reported as hs-cTnT release.

We recognize several limitations in the interpretation of our findings. First, the study did not assess the effect of different CPR or “low-flow” durations, which are known to potentially play a role in outcome.²⁴ Second, the effect of body temperature on neurological outcomes was not evaluated, although target temperature management (TTM) is currently a standard of care after OHCA for its beneficial effects on survival and neuroprotection.^{4,25,26} Nevertheless, the quality of CPR was standardized and the duration of CPR was similar in all the groups. Moreover, all the animals received the same post-resuscitation management, with the core temperature maintained at normothermia during the 4 h of intensive observation. Indeed, the main focus of the study was to assess the impact of the sole no-flow duration on outcome of CA, excluding any other potential bias, i.e. TTM or duration of CPR. Therefore, our results are robust in order to show the direct impact of no-flow duration on the severity of PCAS and outcome of CA.

Conclusions

In this porcine model, a linear relation between the duration of untreated CA and the severity of PCAS was observed. Indeed, longer no-flow durations caused greater post-resuscitation myocardial and neurological dysfunction and reduced the survival rate, in comparison to shorter durations. A duration of untreated CA of 12-13 min may be an optimal choice for clinically relevant CA animal models, because it yields survival rates and neurological outcomes similar to those observed in the clinical scenario.

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Table 1. Resuscitation outcomes, hemodynamics and blood gas analysis

	Short	Intermediate	Long	p
	8-10 min	12-13 min	14-15 min	value
	n=8	n=8	n=6	ANOVA
Total defibrillations, n	3 [1;10]	10 [2;11]	15 [11;26] *	0.042
Duration of CPR, min	315 [300;509]	361 [325;455]	480 [334;757]	0.307
Successful resuscitation, n/n (%)	8/8 (100)	8/8 (100)	5/6 (83)	0.247
Long-term survival, n/n (%)	7/8 (88)	5/8 (63)	1/5 (20) *	0.078
Heart Rate, beat/min				
BL	99 ± 10	93 ± 8	86 ± 8	0.602
PR2h	127 ± 6	156 ± 13	164 ± 11 *	0.040
PR4h	130 ± 8	129 ± 12	143 ± 5	0.595
Mean Arterial Pressure, mmHg				
BL	99 ± 4	101 ± 3	96 ± 5	0.702
PR2h	82 ± 6	85 ± 6	92 ± 2	0.571
PR4h	92 ± 7	76 ± 2	78 ± 6	0.107
Right Atrial Pressure, mmHg				
BL	5 ± 1	6 ± 1	8 ± 1	0.081
PR2h	7 ± 1	8 ± 1	9 ± 1	0.346
PR4h	7 ± 1	7 ± 0	12 ± 0 ***##	<0.001
End-Tidal CO₂, mmHg				
BL	36 ± 0	37 ± 1	36 ± 0	0.406
PR2h	36 ± 0	36 ± 0	37 ± 1	0.302
PR4h	36 ± 1	36 ± 0	37 ± 0	0.633

LV CO, L/min				
BL	4.5 ± 0.5	4.4 ± 0.3	4.0 ± 0.3	0.735
PR2h	3.3 ± 0.3	3.3 ± 0.3	2.8 ± 0.6	0.604
PR4h	3.2 ± 0.3	3.2 ± 0.4	1.9 ± 0.1 *	0.032
LV infarct size area, %	9.7 ± 2.0	6.5 ± 0.7	13.1	§
pH				
BL	7.495 ± 0.017	7.519 ± 0.016	7.513 ± 0.027	0.636
PR2h	7.434 ± 0.026	7.394 ± 0.042	7.395 ± 0.017	0.583
PR4h	7.462 ± 0.017	7.429 ± 0.027	7.410 ± 0.011	0.251
PaO ₂ , mmHg				
BL	79 ± 11	83 ± 5	83 ± 2	0.931
PR2h	89 ± 7	109 ± 8	84 ± 10	0.111
PR4h	88 ± 3	100 ± 11	70 ± 9	0.074
PaCO ₂ , mmHg				
BL	38 ± 2	37 ± 1	37 ± 1	0.789
PR2h	40 ± 1	39 ± 1	41 ± 1	0.615
PR4h	42 ± 1	40 ± 1	42 ± 1	0.302
HCO ₃ , mmol/L				
BL	29.3 ± 1.0	30.3 ± 1.2	29.8 ± 1.2	0.828
PR2h	27.2 ± 0.9	24.5 ± 2.0	25.4 ± 0.7	0.353
PR4h	29.9 ± 1.1	26.8 ± 1.3	26.7 ± 0.4	0.088
BE, mmol/L				
BL	6.4 ± 1.1	7.4 ± 1.5	6.7 ± 1.6	0.865
PR2h	3.1 ± 1.4	-0.2 ± 2.6	0.5 ± 1.0	0.402
PR4h	6.1 ± 1.3	2.5 ± 1.5	2.0 ± 0.4	0.089

Data are expressed as mean±SEM. BL, baseline; PR2h 2 h post-resuscitation; PR4h 4 h post-resuscitation; CO, Cardiac Output; EF, Ejection Fraction; PR, post-resuscitation; LV, left ventricle.

* $p < 0.05$ and ** $p < 0.01$ vs. “short”; # $p < 0.05$ and ## $p < 0.01$ vs. “intermediate”.
§ ANOVA was not performed due to a single animal survived in the “long” group.

Legend to Figures

Figure 1. Kaplan–Meier of post-resuscitation survival after an untreated cardiac arrest of short, intermediate, and long duration.

Figure 2. A. Total number of defibrillations prior to successful resuscitation after an untreated cardiac arrest of short, intermediate, and long duration between groups. B. Correlation between total number of defibrillations and duration of untreated cardiac arrest. C. Correlation between total number of defibrillation and left ventricular ejection fraction (LV EF) at 4 h post-resuscitation. D. Correlation between total number of defibrillations and plasma high sensitive cardiac troponin T (hs-cTnT) at 4 h post-resuscitation.

Figure 3. A. Left ventricular ejection fraction (LV EF) at baseline (BL), and at 2 and 4 h post-resuscitation (PR), and at sacrifice, after an untreated cardiac arrest of short, intermediate, and long duration. B. Correlation between the no-flow duration and LV EF at PR 4 h.

* $p < 0.05$ vs. short duration group.

Figure 4. A. Plasma high sensitive cardiac troponin T (hs-cTnT) levels at baseline (BL), and at 2 and 4 h post-resuscitation (PR), and at sacrifice, after an untreated cardiac arrest of short, intermediate, and long duration. B. Correlation between the duration of untreated cardiac arrest (CA) and the release of hs-cTnT at PR 4 h. C. Serum neuronal specific enolase (NSE) levels at baseline and at sacrifice. D. Correlation between the duration of untreated CA and the release of NSE at sacrifice.

* $p < 0.05$ vs. short duration group (long duration group was not included in the analysis due to sample size of survived animals, $n=1$).

Figure 5. A. Overall Performance Category (OPC, upper figure), B. Neurological Alertness Score (NAS), and C. Neuronal Deficit Score (NDS), after an untreated cardiac arrest of short, intermediate, and long duration. D. Neuronal degeneration in the hippocampal CA1 sector and in cortex after an untreated cardiac arrest of short, intermediate, and long duration.

* $p < 0.05$ vs. long duration group.

Figure 6. A. Hematoxylin-eosin staining 200x magnification of hippocampal CA1 sector (A) and cortex (B) after an untreated cardiac arrest (CA) of short, intermediate, and long duration.

Short duration: hippocampus - some degenerated/necrotic neurons characterized by shrinkage, deeply acidophilic cytoplasm and pyknotic nucleus are intermixed with numerous normal neurons; cortex - normal histological appearance of the nervous tissue.

Intermediate duration: hippocampus - numerous degenerated/necrotic neurons with some normal neurons still detectable (on the left side of the picture); cortex - presence of some degenerated/necrotic neurons.

Long duration: hippocampus - almost all neurons are degenerated/necrotic; cortex - severe damage characterized by the presence of numerous degenerated/necrotic neurons, gliosis and perivascular inflammatory cells cuffing.

Figure 7. Ultrastructural analysis of hippocampus. Representative images by transmission electron microscopy of neurons (mitochondria indicated by white arrows) and neuropil (single

vacuoles indicated by asterisks and agglomerated vacuoles encircled), after an untreated cardiac arrest (CA) of short, intermediate, and long duration.

Short duration: mitochondria show only irregular shapes and moderate damage of inner membranes; neuropil normally composed by a tightly packed myriad of axons, dendrites and glial processes presents some vacuolated and enlarged processes.

Intermediate duration: mitochondria show disruption of cristae and remarkable reduction of matrix density; neuropil shows a more evident vacuolization.

Long duration: mitochondria show a complete loss of internal components; the confluence of vacuoles determines an edematous-like massive loss of the neuropil.

Figure 1

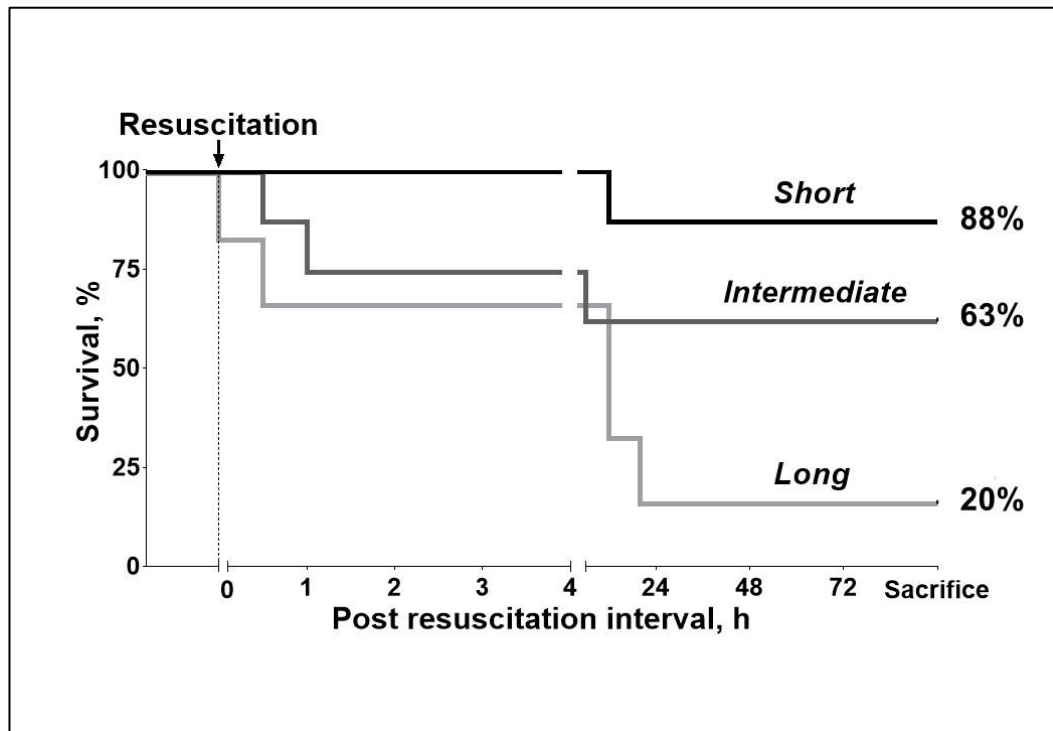


Figure 2

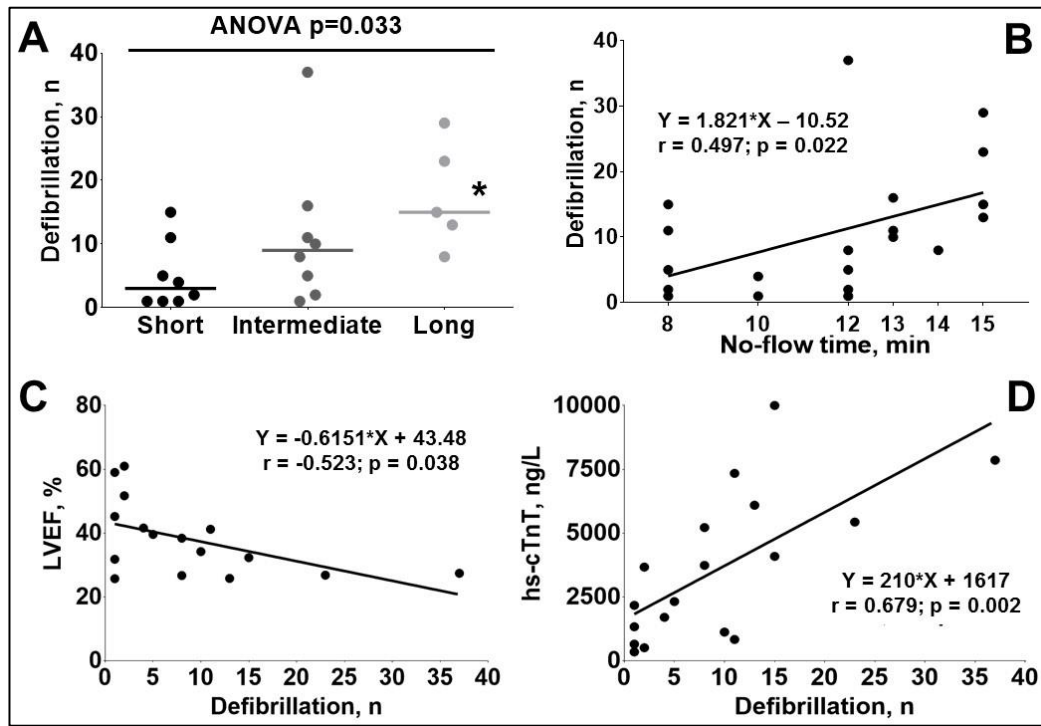


Figure 3

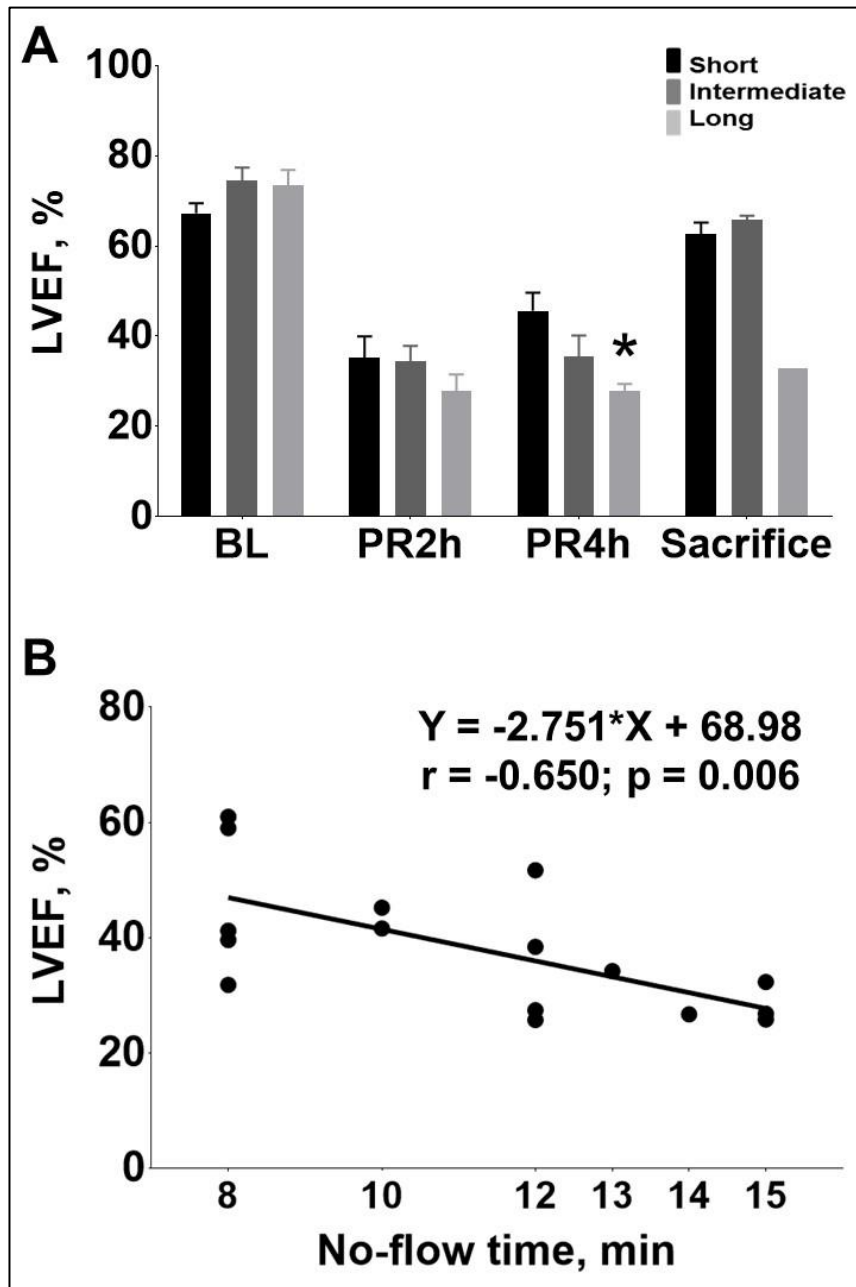


Figure 4

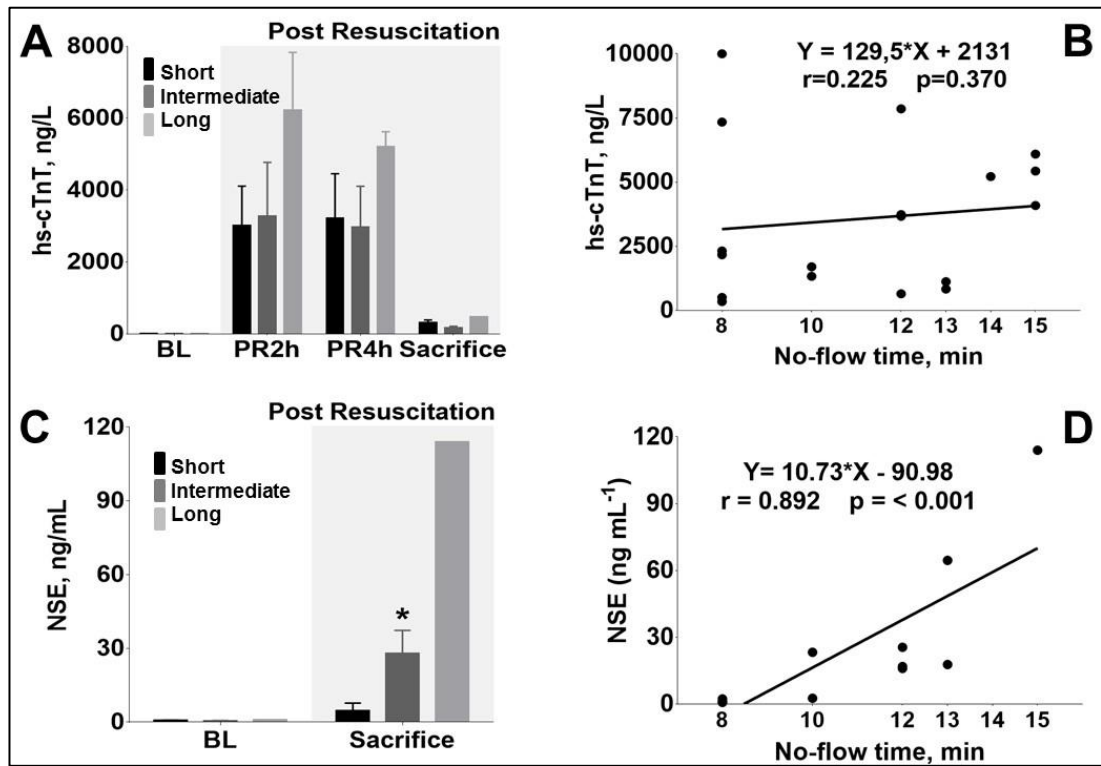


Figure 5

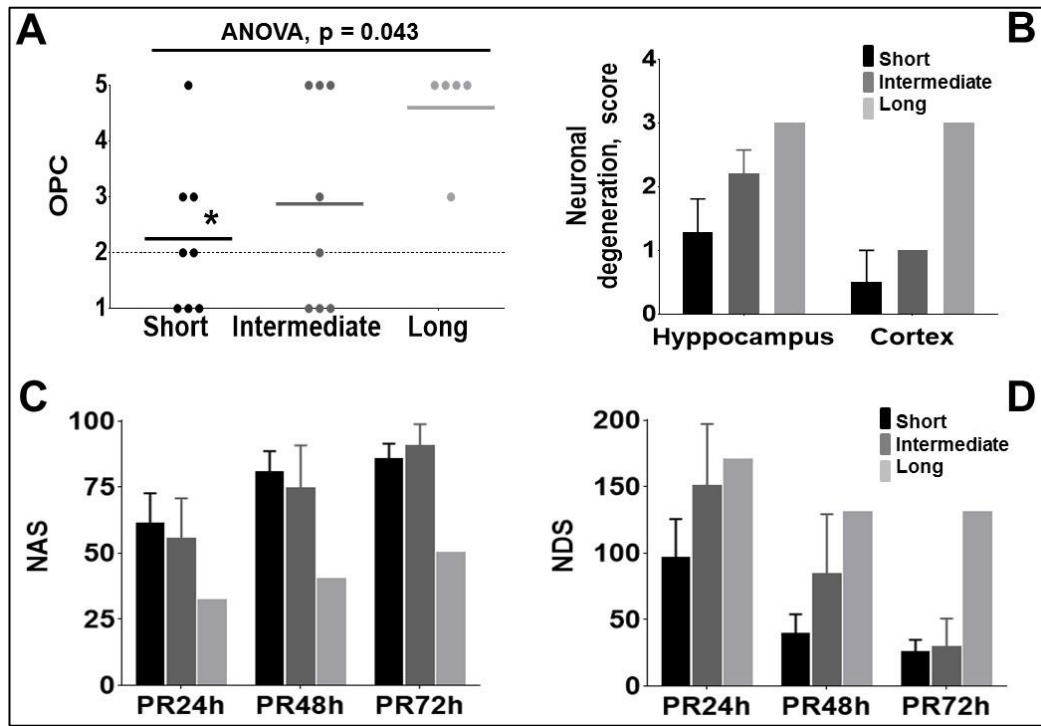


Figure 6

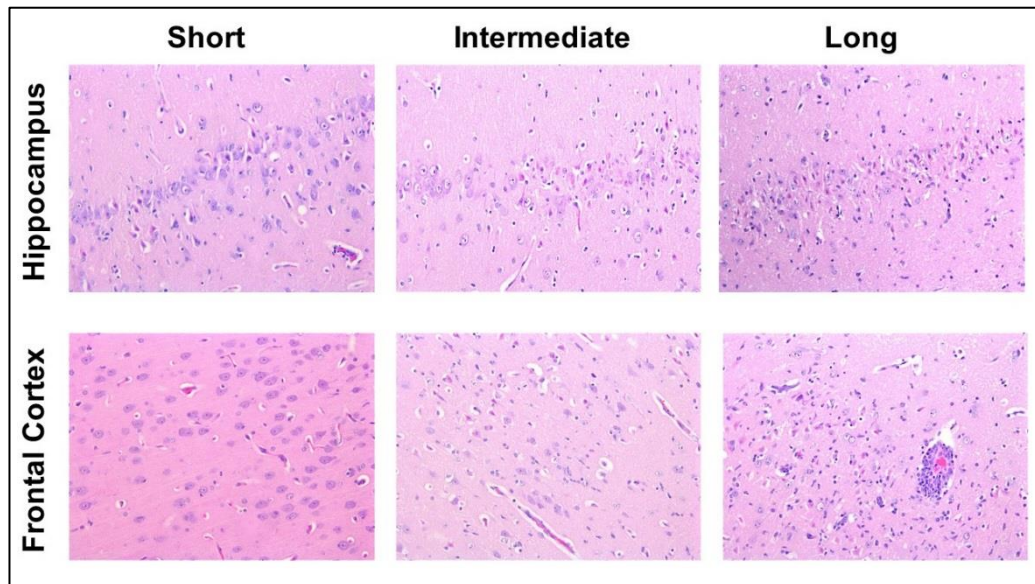
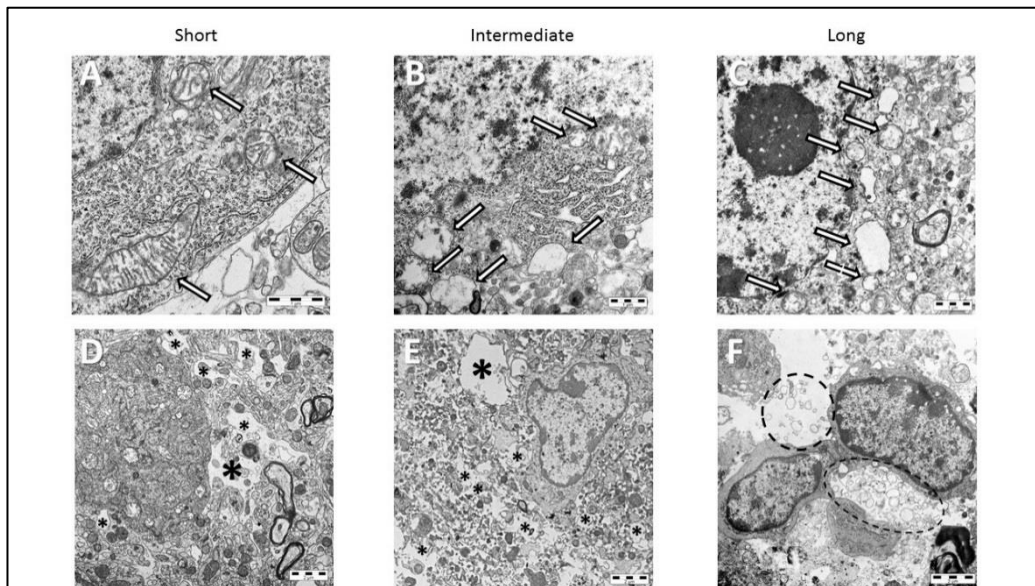


Figure 7



Student's background

Publications derived from the thesis work

Full articles:

1. Ristagno G, Fumagalli F, Russo I, Tantillo S, Zani DD, Locatelli V, De Maglie M, Novelli D, Staszewsky L, Vago T, Belloli A, Di Giancamillo M, Fries M, Masson S, Scanziani E, Latini R. Post-resuscitation treatment with Argon improves early neurological recovery in a porcine model of cardiac arrest. *Shock* 2014 Jan;41(1):72-8.
2. Babini G, Grassi L, Russo I, Novelli D, Boccardo A, Luciani A, Fumagalli F, Staszewsky L, Fiordaliso F, De Maglie M, Salio M, Zani DD, Letizia T, Masson S, Luini MV, Pravettoni D, Scanziani E, Latini R, Ristagno G. Duration of Untreated Cardiac Arrest and Clinical Relevance of Animal Experiments: The relationship Between the "No-Flow" Duration and the Severity of Post-Cardiac Arrest Syndrome in a Porcine Model. *Shock*. 2017 May 30.
3. Nespoli F, Redaelli S, Fumagalli F, Magnoli M, Olivari D, Roberto Latini R, Giuseppe Ristagno G. Preclinical and clinical uses of the noble gas Argon. Evidence of protection after ischemia and study protocol for the CPAr (CardioPulmonary resuscitation with Argon) trial in cardiac arrest patients. Submitted at *Anesthesiology*.

Abstracts:

1. Giuseppe Ristagno, Francesca Fumagalli, Ilaria Russo, Simona Tantillo, Davide D Zani, Valentina Locatelli, Marcella De Maglie, Deborah Novelli, Lidia Staszewsky, Angelo Belloli, Mauro Di Giancamillo, Michael Fries, Serge Masson, Eugenio Scanziani, Roberto Latini. Post-Resuscitation Treatment With Argon Improves Neurological Outcome in a Porcine Model of Myocardial Infarction and Cardiac Arrest. *Circulation*. 2013;128:A12276.

2. F. Fumagalli, S. Scala, R. Affatato, M. De Maglie, D. Zani, D. Novelli, C. Marra, A. Luciani, D. De Zani, M. Luini, T. Letizia, D. Pravettoni, L. Staszewsky, S. Masson, A. Belloli, M. Di Giancamillo, E. Scanziani, R. Latini, G. Ristagno. Post-resuscitation treatment with inhaled Argon improves outcome even after a prolonged untreated cardiac arrest in a porcine model. *Intensive Care Medicine Experimental* 2016, 4(Suppl 1):28.

Congress presentations related to the thesis work

- F. Fumagalli, S. Scala, R. Affatato, M. De Maglie, D. Zani, D. Novelli, C. Marra, A. Luciani, D. De Zani, M. Luini, T. Letizia, D. Pravettoni, L. Staszewsky, S. Masson, A. Belloli, M. Di Giancamillo, E. Scanziani, R. Latini, G. Ristagno. Post-resuscitation treatment with inhaled Argon improves outcome even after a prolonged untreated cardiac arrest in a porcine model. Abstract at ESICM congress, Milan, Oct 1-5, 2016. *Intensive Care Medicine Experimental* 2016, 4(Suppl 1):28. Poster session.
- F. Fumagalli, S. Scala, R. Affatato, M. De Maglie, D. Zani, D. Novelli, S. Ceriani, A. Luciani, M. Luini, T. Letizia, D. Pravettoni, L. Staszewsky, S. Masson, A. Belloli, E. Scanziani, R. Latini, M. Di Giancamillo, G. Ristagno. Effetti protettivi del trattamento inalatorio con Argon in un modello di arresto cardiaco e rianimazione cardiopolmonare nel maiale. Abstract at Italian Resuscitation Council congress, Milan, Oct 20-21, 2016. Poster session.

Other publications

- Vicinanza C, Aquila I, Cianflone E, Scalise M, Marino F, Mancuso T, Fumagalli F, Giovannone ED, Cristiano F, Marotta P, Torella A, Iaccino E, Latini R, Agosti V, Veltri P, Urbanek K, Isidori AM, Saur D, Indolfi C, Nadal-Ginard B, Torella D. c-kit^{Cre} Knock-ins Fail to Identify, Effectively Recombine and Fate-Map Cardiac Stem Cells. Accepted at Nature.
- Fumagalli F, Silver AE, Tan Q, Zaidi N, Ristagno G. Cardiac rhythm analysis during ongoing cardiopulmonary resuscitation using the Analysis During Compressions with Fast Reconfirmation technology. Heart Rhythm. 2017 Sep 14.
- Lavanga M, Baselli G, Fumagalli F, Ristagno G, Ferrario M. The possible role of the vagal nervous system in the recovery of the blood pressure control after cardiac arrest: a porcine model study. Physiol Meas. 2017 Jan;38(1):63-76.
- He M, Gong Y, Li Y, Mauri T, Fumagalli F, Bozzola M, Cesana G, Latini R, Pesenti A, Ristagno G. Combining multiple ECG features does not improve prediction of defibrillation outcome compared to single features in a large population of out-of-hospital cardiac arrests. Crit Care. 2015 Dec 10;19:425.
- Ristagno G, Mauri T, Cesana G, Li Y, Finzi A, Fumagalli F, Rossi G, Grieco N, Migliori M, Andreassi A, Latini R, Fornari C, Pesenti A; Azienda Regionale Emergenza Urgenza Research Group. Amplitude spectrum area to guide defibrillation: a validation on 1617 patients with ventricular fibrillation. Circulation. 2015 Feb 3;131(5):478-87.
- Hackenhaar FS, Fumagalli F, Li Volti G, Sorrenti V, Russo I, Staszewsky L, Masson S, Latini R, Ristagno G. Relationship between post-cardiac arrest myocardial oxidative stress and myocardial dysfunction in the rat. J Biomed Sci. 2014 Aug 19;21:70.

- Fumagalli F, Russo I, Staszewsky L, Li Y, Letizia T, Masson S, Novelli D, Rocchetti M, Canovi M, Veglianese P, Gobbi M, Latini R, Zaza A, Ristagno G. Ranolazine ameliorates postresuscitation electrical instability and myocardial dysfunction and improves survival with good neurologic recovery in a rat model of cardiac arrest. *Heart Rhythm*. 2014 Sep;11(9):1641-7.
- Ristagno G, Li Y, Fumagalli F, Finzi A, Quan W. Amplitude spectrum area to guide resuscitation-a retrospective analysis during out-of-hospital cardiopulmonary resuscitation in 609 patients with ventricular fibrillation cardiac arrest. *Resuscitation*. 2013 Dec;84(12):1697-703.
- Ristagno G, Fries M, Brunelli L, Fumagalli F, Bagnati R, Russo I, Staszewsky L, Masson S, Li Volti G, Zappalà A, Derwall M, Brücken A, Pastorelli R, Latini R. Early kynurenine pathway activation following cardiac arrest in rats, pigs, and humans. *Resuscitation*. 2013 Nov; 84(11):1604-10.
- Hydroxytyrosol attenuates peripheral neuropathy in streptozotocin-induced diabetes in rats. Ristagno G, Fumagalli F, Porretta-Serapiglia C, Orrù A, Cassina C, Pesaresi M, Masson S, Villanova L, Merendino A, Villanova A, Cervo L, Lauria G, Latini R, Bianchi R. *J Agric Food Chem*. 2012 Jun 13;60(23):5859-65.
- Efficacy of aminaftone in a rat model of monocrotaline-induced pulmonary hypertension. Zambelli V, Santaniello A, Fumagalli F, Masson S, Scorza R, Beretta L, Latini R. *Eur J Pharmacol*. 2011 Sep 30;667(1-3):287-91.

Honors and awards

- ‘Young Investigator Award’, American Heart Association (AHA) 2013, Resuscitation Science Symposium, Dallas (TX, USA), 15 November 2013, for the abstract “Ranolazine Increases Amplitude Spectrum Area during Untreated Ventricular Fibrillation and Cardiopulmonary Resuscitation”.
- ‘Lucien Dreyfus Foundation Award’, European Cardiac Arrhythmia Society (ECAS) 2015, Paris (France), April 19-21, for the abstract "Ranolazine Ameliorates Post-Resuscitation Electrical Instability and Myocardial Dysfunction and Improves Outcome in a Rat Model of Ventricular Fibrillation".
- ‘Best Abstract IRC 2016’ award, Italian Resuscitation Council (IRC) National Congress 2016, Milan, Italy, 20-21 October 2016, for the abstract “Effetti protettivi del trattamento inalatorio con Argon in un modello di arresto cardiaco e rianimazione cardiopolmonare nel maiale”.
- April 2017, Member of the Steering Committee of the AMSA trial: AMplitude Spectrum Area to guide defibrillation during cardiopulmonary resuscitation in out-of-hospital cardiac arrest patients.

Grants

1. The Laerdal Foundation for Acute Medicine – Project Grant Support. Period: 2015-2018. Principal Investigator for the study “Effects of ranolazine during CPR or after ROSC on post-resuscitation myocardial dysfunction and survival in a rat model of cardiac arrest and CPR”.
2. Italian Ministry of Health “Ricerca Finalizzata - Giovani Ricercatori”. Period: 2014-2017. Co-investigator, Responsible of Unit of Work for the project “Preclinical optimization of treatment with inhaled Argon to improve neurological outcome and survival after cardiac arrest”.