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mRNA Profiling for Myocardial Injury In Experimental Cardiac Arrest Models

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Introduction: Cardiac arrest has a very poor prognosis and survival rate after restoration of spontaneous circulation (ROSC). Resuscitation techniques are one of the most significant contributors to the clinical outcome after cardiac arrest. Resuscitation aims for avoiding and/or alleviating the brain damage, myocardial function decline, and multi-organ failure due to ischemia/hypoxia during the event of cardiac arrest. Despite clinical research efforts to answer questions related to resuscitation techniques and the value of different resuscitation strategies, the 30-day mortality after an event of cardiac arrest is still high. Some of the great advancement of resuscitation techniques are Extracorporeal Life Support (ECLS) and Extracorporeal Membrane Oxygenator (ECMO) which provide means for restoring tissue perfusion, oxygenation, drug delivery and temperature control until cardiac function is restored. Several protocols that used ECLS and ECMO for neuroprotection and cardioprotection have been tested, including hypothermia and supplementation of different drugs. However, the results of such studies are not conclusive which can be attributed to lack of understanding of the molecular events associated with these protocols. Therefore, in this study we will investigate the genetic and molecular remolding associated with two models of post cardiac arrest resuscitation.

Scope of the study: This study was conducted to assess the genetic and molecular changes associated with two different models of resuscitation in the sitting of cardiac arrest. First model is hypothermic arrest followed by different rate of rewarming. Second model is a cardiac arrest followed by cardiopulmonary bypass (CPB) resuscitation with NO supplementation using ECMO. This study is conducted to fulfill the requirements for doctoral degree in cardiovascular sciences at the university of Verona.

Materials and methods: Male Sprague Dawley rats weighing around 400±50 g were used. Two main models of resuscitation in the sitting of cardiac arrest were used; the model of hypothermic cardiac arrest followed by different rates of rewarming using ECLS, and the model of experimental cardiac arrest and resuscitation using NO supplied through the ECMO. Cardiac tissues were collected after the experiment for molecular analysis. RNA sequencing was used to detect transcriptomic changes. The apoptosis was assessed using Tunnel assay

Results: First our results highlight the effect of different rates of rewarming post cardiac arrest. Slow rewarming showed a significant lower level of myocardial apoptosis compared to rapid rewarming. Rapid rewarming was associated transcriptomic derangement involving pathways related to lipid metabolism, inflammatory and apoptotic pathways, and calcium handling. **Second our results showed that resuscitation using nitric oxide (NO) has a deleterious effect of on the myocardium** as indicated by a significant increase in the apoptosis. The known beneficial effects of NO include inhibition of the sympathetic signaling, and smooth muscle relaxation couldn't overcome the complex metabolic derangement, and profound activation of inflammatory and apoptotic pathways.

Conclusions: Our study explained the beneficial effect of the slow rewarming over the fast rewarming after hypothermic arrest at the molecular and genetic level. We also challenged the idea of using NO as a protective agent during **resuscitation. NO has a deteriorating effect on the myocardium.** This study highlights the genetic remodeling associated with resuscitation in the sitting of cardiac arrest which would guide clinical decisions. In addition, this study spots the light on the possible molecular pathways that could be targeted to improve resuscitation process post-cardiac arrest and would results in improving the survival rate post-cardiac arrest.

1. INTRODUCTION

All the experiments reported in this thesis are the result of years of research supervised by Prof. Giovanni Luciani and Dr. Riham Abouleisa and performed in the cardiovascular research laboratory of the Biological Institutes directed by Prof. Giuseppe Faggian at the University of Verona, Verona, Italy and the Institute of Molecular Cardiology directed by Prof. Tamer Mohamed at the University of Louisville, Kentucky, USA. This very successful collaboration under the INVITE project funded by Horizon 2020 paved the road for me to accomplish this project and overcome many difficulties.

During the three years of PhD study, we aimed to understand the transcriptomic changes that occur in association with two main protocols of resuscitation after experimental cardiac arrest; the hypothermia rewarming and the neuroprotection effect of nitric oxide. The neurological impact of these protocols during resuscitation has been extensively studied in a project performed by my colleague Dr. Daniele Linardi. Hereby,

We are characterizing the transcriptomic effects of the studied protocols. As well, we expect this to have translational impact, as this will influence decisions in situations faced in every day clinical practice. Despite successful restoration of spontaneous circulation in many of cardiac arrest patients, many patients suffers from very low cardiac functions (1) with very poor prognosis. Understanding the genetic and molecular remolding associated with resuscitation will be the first step towards personalized resuscitation protocols that will definitely improve the treatment outcome.

2. REVIEW OF LITERATURE

2.1 Cardiac Arrest

Cardiac arrest is the cessation of the mechanical pump activity of the heart, with immediate interruption of the circulation and blood flow, which leads to rapid oxygen depletion and depression of brain function (2). Cardiac arrest can be accidental or controlled. Accidental cardiac arrest can occur in the presence of pre-existing heart disease, or in the absence of underlying cardiac pathologies. It can

be a result of altered electrical activity or a mechanical obstacle. Controlled cardiac arrest is induced in the operating room with the help of cardioplegia and extracorporeal circulation (CPB) to allow cardiac surgery to be performed. Cardiac arrest can be a result of electrical or mechanical mechanisms. The most common electrical mechanism is the appearance of ventricular fibrillation (VF), followed by asystole, pulseless electrical activity (PEA) or ventricular tachycardia (TV) without pulse. Pulseless VT and VF, which are typically the rhythms of onset of cardiac arrest from myocardial ischemia, decay in a few minutes to low-voltage VF, and finally to asystole (2).

Mechanical causes include rupture of the ventricle, cardiac tamponade, acute mechanical obstruction of a coronary artery, and acute rupture of a large-caliber vessel. The immediate consequence is the absence of systemic perfusion (3).

Cardiac arrest is a reversible clinical death condition that, if not properly treated, is destined to evolve into irreversible biological death mainly due to cerebral hypoxia. The probability that resuscitation maneuvers will be successful is conditioned by the environment in which the arrest occurs, the mechanism responsible, the patient's basic clinical condition and the effectiveness and readiness of the treatment carried out.

The causes of cardiac arrest can also be schematically divided into (4):

- cardiac (among which, the most frequent is ischemic heart disease);
- non-cardiac, less frequent, in turn divided into mechanical (cardiac tamponade, pulmonary embolism, tension pneumothorax, and others) and anoxic (e.g. airway obstruction and neurological events).

The onset of cardiac arrest is often instantaneous, with no clinical signs or warning symptoms. The evolution of cardiac arrest towards irreversible biological death depends critically on the time that elapses between the primary event and the implementation of the resuscitation maneuvers. The brain is very sensitive to anoxia resulting from the arrest of the circulation: in a few seconds there is loss of consciousness, while after about 4 minutes there is irreversible damage. The heart is less sensitive, but cardiac functions will be also deteriorating if arrest continues for more than a few minutes.

Nowadays, coronary heart disease is one of the leading causes of mortality: it is responsible for 1/3 of the deaths in the world's population over the age of 35 (3).
In Europe, according to current ERC guidelines, cardiovascular disease alone is responsible for about 40% of all deaths in the under-75 age group and in particular the Sudden from ischemic heart disease is responsible for more than 60% of deaths. It is estimated that the annual incidence of cardiac arrest varies between 0.3 - 3/1,000 inhabitants/year for the age between 20 and 75 years with a survival rate at hospital discharge that stands at around 10-20%. Of these, 30% develop post-anoxic encephalopathy (5).
Many trials for early prediction and proper management of cardiac arrest have been conducted. With this objective, concepts such as the chain of survival or early defibrillation were born, and for this reason we are constantly trying to raise awareness among the population on this subject and instruct them on the importance of the early execution of cardiopulmonary resuscitation maneuvers.
Another distinct form of cardiac arrest is the cardioplegic cardiac arrest needed to perform most cardiac surgeries. In the cardiac surgical field, a total of over 800,000 coronary artery bypass surgeries or valvular surgeries are performed every year, with approximately 1000 cardiac surgeries of this type carried out every day only in the United States (6). At the University hospital of Verona, about 1000 operations are carried out every year with the aid of cardiopulmonary bypass (CPB) and cardioplegic arrest obtained with the administration of cardioplegic solution in coronary ostia and venous coronary sinus. The cardioplegic solution helps stopping the heart and lower its energy requirements. In this case, the damage from ischemia and reperfusion involves only the heart that during the intervention will not be perfused. However, all other organs, including the brain, are instead perfused thanks to the CPB thus not usually subjected to damage except in specific cases as carotid atheroma, embolisms or any complications. During aortic arch surgery, a circulation stop is necessary. The interruption of blood flow; that until that moment of the intervention is supported by the CPB; will result is widespread ischemic hypoxic damage to all organs.
Strategies for protection from hypoxic-ischemic damage have been mainly aimed

at brain protection. In particular, in recent decades, 3 main brain protection strategies have been developed and spread for patients undergoing surgery on the aortic arch: circulation arrest in deep hypothermia (DHCA), retrograde cerebral perfusion (RCP), and anterograde cerebral perfusion (ACP). Therefore, the possible superiority of one of these 3 techniques is a constant topic of debate; moreover, the search for new perfusion techniques and an ever better and more correct management of body temperature during and after the arrest of the circulation is always underway and in continuous evaluation (7).

In fact, the recent use of cerebral perfusion has led to the use of higher temperatures during circulatory arrest at many centers that deal with aortic surgery, then applying moderate or even mild hypothermia instead of deep. Although the protective role played by hypothermia is now certain, and despite an increasing tendency to use hypothermia for example during recovery after cardiac arrest, it also brings with it some adverse effects. In addition, during aortic surgery, the achievement of deep hypothermia and subsequently the heating for the recovery of normal body temperature prolongs the time of the intervention and the time of assistance with CPB, for this reason it is of great interest to determine which is the most appropriate temperature to maintain a protective effect and at the same time to reduce the adverse effects and the operating times to the minimum possible (8).

At the beginning of the 70s, dr. Vladimir Negovsky called the condition that emerges as a result of cardiac arrest as post-resuscitation disease, today called post-cardiac arrest syndrome. He sought to recognize its etiology and the stages of the pathological processes following cardiac arrest (9, 10).

Post-cardiac arrest syndrome involves multiple organs, causing damage that occurs both during and after reperfusion. The four key components of post-cardiac arrest syndrome are:

- post-arrest brain damage,
- post-arrest myocardial dysfunction,
- systemic damage from ischemia-reperfusion,
- underlying chronic pathologies that flare up.

The severity of these problems after the resumption of spontaneous circulation are

not uniform and may vary from patient to patient. The outcome after resuscitation also is not uniform and is based on the severity of the ischemic insult, the causes of cardiac arrest and pre-arrest health status. If the resumption of spontaneous circulation (ROSC) is early, post-arrest syndrome may not occur.

2.2. Hypothermia

THERAPEUTIC HYPOTHERMIA: HISTORICAL NOTES

Hypothermia as a medical practice has ancient origins, although with different purposes and potentialities from the current ones. Illustrious examples, come to us already from ancient Greece: Hippocrates advised bandaging the limbs of wounded soldiers with ice and snow for analgesic and hemostatic purposes. Similarly, in the early nineteenth century Napoleon's surgeon, Baron Larrey, described how soldiers who were warmed up near fire after being operated on had a lower survival than those who were kept in a cold environment. In fact, even the concept of "total body therapeutic hypothermia" has ancient origins: from the study and simple observation of the phenomenon of animal hibernation, the ancient Egyptians used the practice of mummification in cold chambers in the hope of one-day restoring life to their dead (11).

The first modern attempts to exploit the beneficial properties of hypothermia date back to the first decades of the 20th century. Lawrence Smith and Temple Fay attempted to stop the growth of prostate cancer and cervix, even with a hypothermic local application, which was actually initially successful but did not last (12). Another failed attempt was that of Talbott in 1941 who used total body hypothermia as shock therapy in psychotic patients (13).

The following decades witnessed a considerable increase in interest in the subject that gradually led to accumulating knowledge about the protective mechanisms of hypothermia especially in terms of neuroprotection and which laid the foundations for current clinical applications. Early in the fifties, the neuroprotective effects of hypothermia were tested in patients with head trauma, and good results were obtained. For example, Botterell showed beneficial effects of hypothermia in the treatment of brain aneurysms by preserving brain tissue from surgery-induced anoxia and vasospasm (14). Rosomoff's experiments on dogs were numerous

studying the effects of hypothermia, which gave increasingly solid scientific evidence on the benefits of hypothermia after cerebral ischemic events and traumatic brain damage (15). Bigelow's laboratory experiments allowed to introduce temporary and reversible cardio-circulatory arrest first in dogs and then in cardiac surgical practice with allowing interventions with a bloodless operating field (16).

In these years, the first studies on therapeutic hypothermia after cardiac arrest were also reported. Benson et al. in a 1959 described the outcome of a dozen cardiac arrest patients treated with therapeutic hypothermia with a 50% survival of the treated group versus only 14% of non-treated. Meanwhile, Williams reported a survival rate of 83% of treated compared to 25% of untreated (17, 18).

However, together with the positive clinical evidence, the important side effects began to emerge, especially that associated with the use of deep hypothermia (20-30°C), which made this treatment impractical in many situations. Further experimental studies on animal guinea pigs, however, intensified since the 80s, brought to light the actual benefits at moderate hypothermia temperatures (32-35°C), with minor side effects and better clinical outcomes (19, 20).

These studies, therefore, allowed to acquire knowledge on the safety and feasibility of hypothermic treatment, such as to be able to carry out increasingly wide-ranging clinical studies. Then, two large randomized controlled clinical trials were published by the New England Journal in February 2002 that laid the foundations of the knowledge of therapeutic hypothermia as a means to significantly improve the outcome of patients who survived cardiac arrest.

The first study conducted by the Hypothermia After Cardiac Arrest Study Group included 274 patients suffering from cardiac arrest from ventricular fibrillation (21). These patients were randomized into two groups: 137 in the hypothermia group and 138 in the normothermia group. After a rapid cooling obtained with external devices and ice packs and a maintenance of the temperature between 32° and 34°C for 24 hours, followed by an 8-hour warm-up, patients treated with hypothermia showed a better neurological outcome than the group treated with the standard normothermic manner. Moreover, a good neurological outcome was

demonstrated at six months in 55% of hypothermic patients compared to 39% of normothermic patients, and mortality at six months was also reduced in the hypothermic group (35%) compared to the normothermic group (55%).

The second study was conducted by Bernard et al. who had already had good results with previous studies (22, 23). In the 2002 study, they included 77 patients of which 43 were treated with hypothermia and 34 with normothermia. To avoid the chills, patients were previously sedated and treated and the hypothermic state was induced starting from 2 hours after the resumption of circulation with the use of ice packs to the head, neck, torso and limbs until reaching the 33 ° C required by the protocol, a temperature that was maintained for 12 hours. The rewarming instead was active using hot air and blankets within 6 hours. The result of the study showed that 49% of patients treated with hypothermia had a good neurological outcome, evaluated at home discharge, against 26% of patients treated with normothermia. The difference in mortality between the two groups, however, did not prove statistically significant. The study also aimed to evaluate the hematological, biochemical and hemodynamic effects. The authors did not document clinically significant adverse effects in terms of arrhythmias, extent of myocardial damage (CK-MB levels were similar in both groups), alterations of the hemocoagulation structure or increased incidence of infections.

These two studies have therefore represented two milestones for the clinical practice of therapeutic hypothermia, and the interest on this topic has grown enormously since then. In 2003, on the basis of the two clinical studies mentioned above, the Advanced Life Support Task Force of the International Liaison Committee on Resuscitation (ILCOR) published an Advisory Statement placing hypothermia as a therapeutic indication for unconscious patients with restored spontaneous circulation after cardiac arrest (24) (Figure 1). In particular, they recommended the maintenance of a temperature between 32° to 34°C for a time between 12 and 24 hours after ROSC. However, the indication has been placed as Level of Evidence 1 only for patients with initial rhythm of ventricular fibrillation or ventricular tachycardia, while Level 4 for patients with other types of rhythms.

These same indications were then also included in the resuscitation guidelines of the ERC in the 2005, and then were modified in the 2010 guidelines where its use has also been expanded in patients who have gone from a non-defibrillable rhythm to a defibrillable one (25-27). However, large-scale prospective studies are still lacking. Moreover, these guidelines recognized that many of the previously accepted predictors of unfavorable outcome in comatose survivors of cardiac arrest are not reliable, especially if the patient has been treated with therapeutic hypothermia.

Figure 1: Role of hypothermia in the Chain of Survival.

EFFECTS OF HYPOTHERMIA:

In the specific context of hypothermia, the whole organism reacts with various reflex mechanisms in order to generate heat: shivering, the inhibition of sweating, a strong sympathetic response, and other mechanisms for increasing heat production. Shivering is triggered by the primary motor center of shivering located in the posterior hypothalamus which is activated by cold signals from the skin or spinal cord when a critical level is reached. The shivering at maximum intensity can result in an increase in oxygen consumption of 40% up to 100% from baseline and this increases heat production to 4-5 times of baseline (28). The sympathetic response triggers numerous phenomena such as peripheral vasoconstriction. Moreover, the activation of sympathetic fibers together with the release of hormones such as adrenaline, norepinephrine and in the long run also thyroid hormones constitute the effect called "chemical thermogenesis". However,

chemical thermogenesis is directly proportional to the amount of brown fat present which is rich in mitochondria. Unfortunately, the adult man has only a very small amount of brown fat, but it is much more relevant in the newborn, where it actually plays an important thermogenic role (29). This will instantly result in a series of physiological variations such as increased oxygen consumption, increased respiratory rate, increased heart rate, ejection volume and contractility, and an increase in the blood pressure (30).

The hypothalamic regulatory system, however, has a threshold limit value. At body temperatures below 29°C, the hypothalamus completely loses all regulatory capacity that already at 34°C is severely compromised. The factors that explain this are the reduced production of heat due to progressive drops in temperature, drowsiness and coma that depress the activity of the CNS preventing the development of shivering. Finally, vascular smooth muscles relaxation leads to a sharp vasodilation that is manifested by a reddening of the skin (31).

The physiological response of human body to a possible therapeutic hypothermia is contradicting to the therapeutic objective. For this reason, an adequate administration of sedatives, anesthetics, opiates and / or muscle relaxants is essential. It is worth mentioning that the greatest evidence on the protective effects of hypothermia comes from studies in the field of neuroprotection. These effects include:

a. Decreased metabolism:

Hypothermia is a significant modifier of blood flow and metabolism (32). Studies have confirmed that there is a 6% to 7% drop in metabolism for every 1°C below 37°C. At 36°C oxygen consumption rate is 2.9 ml/g/min, and at 25°C the rate is reduced to 0.90 ml/g/min and at 20°C it has been reduced to one fifth of normothermic values, with little effect below this temperature (33).

b. Apoptosis, Caspase and mitochondrial proteolysis:

Apoptosis involves several cellular processes such as mitochondrial dysfunction and the release of particular enzymatic proteins called Caspases (34). Numerous studies have shown that hypothermia disrupts the apoptotic pathway and the associated damage especially in the early stages of the process, inhibiting in

particular the activation of caspases (35). The effects of hypothermia also include the prevention of mitochondrial dysfunction, the reduction of the overload of excitatory neurotransmitters, and the modification of intracellular concentrations of ions (36).

c. Immune response and inflammation:

After events of tissue ischemia, a huge inflammatory response begins. Pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin-1 (IL-1), are released in large quantities (37). As well, there will be an activation of all the pathophysiological events that characterize the inflammatory response including chemotaxis of activated leukocytes, expression of endothelial adhesion molecules, activation of the complement system, and migration of neutrophils and macrophages. The latter in particular with their phagocytic action are responsible for further tissue damage.

Numerous animal experiments and some clinical studies have shown that hypothermia suppresses inflammatory reactions and the release of pro-inflammatory cytokines following ischemia (38, 39). Hypothermia also prevents or reduces DNA damage mediated by reperfusion, lipid peroxidation, and leukotriene production. It then decreases the production of nitric oxide by inducible nitric oxide synthase (iNOS), which is a key agent in the development of post-ischemic brain damage.

d. Production of free radicals:

Reactive oxygen species (ROS) including superoxide anion ($O2^-$), hydrogen peroxide (H2O2) and hydroxyl radicals (OH^-), play an important role in cell damage and death, being able to damage and oxidize numerous components. Cells normally have several antioxidant mechanisms, enzymatic and non-enzymatic, that prevent this type of injury, but the production of ROS after ischemia and reperfusion is so large that it cannot be neutralized.

Hypothermia significantly reduces the production of free radicals, and thus, allows endogenous antioxidant systems to prevent or at least mitigate oxidative damage, and allows the cell to repair any damage (40, 41).

e. Vascular permeability, and edema formation:

Damage from ischemia-reperfusion can lead to significant dysregulation of vascular permeability and the subsequent development of tissue edema. Ischemia-reperfusion damage compromises the endothelial cells functions and affects production of vascular endothelial growth factor (VEGF) and nitric oxide release (42). Moderate hypothermia significantly reduces these disruptions and decreases vascular permeability resulting from ischemia-reperfusion (43, 44). Hypothermia functions as a membrane stabilizer (45).

f. Activation of thrombosis and coagulation:

In case of cardiac arrest there is a marked activation of coagulation, which can lead to the formation of microthrombi with blockage of microcirculation in the brain and heart (46). Administration of anticoagulants such as heparin and recombinant plasminogen tissue improves reperfusion and survival in animal experiments (47). Hypothermia, on the other hand, has some anticoagulant effects such as mild platelet dysfunction at temperatures below 35°C, and an inhibition of the coagulation cascade at temperatures below 33°C (48).

g. Vasoactive mediators:

Several studies have shown that hypothermia affects the local secretion of vasoactive substances such as endothelin, thromboxane A2, and prostaglandin I2 in the brain and heart. These factors play an important role in the local regulation of blood flow, and should be balanced to maintain tissue homeostasis. This local homeostasis can be stopped after an ischemic or traumatic event, with a relative increase in the production of thromboxane A2 leading to vasoconstriction, hypoperfusion and thrombogenesis. This can be controlled, at least partially, by introduction of hypothermia (49).

Complications of hypothermia

In dealing with hypothermia-induced side effects, it should be noted that the distinction between real side effects and "physiological" consequences of hypothermia is often poorly delimited with certainty. It happens, in fact, that in some particularly critical patients the so-called physiological changes require adequate prevention and active treatment. While, on the contrary, in other patients

there may also be clear adverse effects that do not require any treatment without entailing a real risk for the patient.

a. Drug clearance:

Knowing that hypothermia depresses organs' function, we understand how this property that is exploited therapeutically turns out to be at the same time a disadvantage from the pharmacological point of view. In a state of hypothermia, not only the serum levels and clearance, but also the effects of various drugs can change. Recently Tortorici et al. in a work published in Critical Care Medicine have documented a significant reduction in the activity of enzyme systems based on cytochrome P450 with hypothermia, and analyzed the effects on various drugs widely used in Intensive Care (50). Mild-to-moderate therapeutic hypothermia decreases by 7-22% the systemic clearance of drugs metabolized by cytochrome P450 for each degree Celsius below 37°C. The potency and effectiveness of some drugs is also decreased. In most cases the effect of hypothermia is to increase drug levels or to emphasize its effect. The mechanism underlying this is a reduction in the activity of many liver enzymes combined with reduced liver perfusion and reduced bile production that will lead to a decrease in the excretion of certain drugs.

b. Electrolyte disturbances:

Electrolyte disturbances can develop especially during the induction phase of hypothermia. The reason for this is a combination of increased renal excretion of electrolytes and intracellular shift. Such electrolyte disturbances can increase the risk of arrhythmias and other adverse effects. Magnesium (Mg) may play an important role in reducing brain injury, myocardial damage and arrhythmias. In various animal models, it has been shown that significant depletion of Mg increases brain damage in models of head trauma and stroke. Clinical studies have similarly demonstrated a better neurological outcome with Mg supplementation in case of sub arachnoid hemorrhage (51, 52). However, the heating phase is also of concern as the potassium levels can increase due to release of intracellular stores of the ion. This is one of the reasons why rewarming should be done very slowly, giving the kidneys time to filtrate excess ion (53).

c. Hyperglycaemia:

The onset of hyperglycemia during hypothermia is a fairly frequent event linked to the simultaneous onset of insulin resistance and reduction of insulin secretion by the pancreatic islets. This requires active management as hyperglycemia can adversely affect outcome in critically ill patients while also increasing tissue injury during episodes of ischemia (54).

d. Metabolic effects and blood gases:

Hypothermia leads to increased synthesis of glycerol, free fatty acids, ketone acids, and lactate, causing mild metabolic acidosis. In contrast to pH levels measured at the extracellular level, the intracellular pH value increases slightly during cooling. Since induced hypothermia reduces metabolism, it will also reduce oxygen consumption and CO2 production. In fact, in blood samples of patients with hypothermia, pO2 and pCO2 will be overestimated, while the pH underestimated (55, 56).

e. Coagulation parameters:

Hypothermia induces mild hemorrhagic diathesis, with increased bleeding time due to effects on platelet count, platelet function, the kinetics of coagulation enzymes and tissue plasminogen activator inhibitor, and other enzymes in the coagulation cascade (57, 58). Hypothermia does not begin to affect platelet function until the temperature drops below 35°C, and clotting factors are only affected when the temperature drops below 33°C.

f. Infection:

Hypothermia is known to impair immune functions and inhibit several inflammatory responses. In fact, this side effect is exploited as a therapeutic advantage as the shutdown of inflammatory reactions could be one of the mechanisms by which hypothermia could exert its protective effects (59). This immunosuppression is due to inhibition of the secretion of pro-inflammatory cytokines, and chemotactic migration of leukocytes and phagocytosis (38). In fact, the reported incidence of infections with hypothermia from any cause is modest, except for the strongly increased risk of pneumonia when hypothermia is used for more than 24 hours. Hypothermia has also been reported to increase the risk of wound infections (60).

g. Chills:

They cause increased oxygen consumption and it is therefore necessary to suppress chills. Sedatives, analgesics and muscle relaxants or alternatively clonidine and neostigmine have been proved beneficial (61).

h. Other side effects:

Hypothermia is also associated with impaired bowel function and can aggravate gastric emptying problems. In addition, alterations in laboratory values may occur: in addition to hyperglycemia and electrolyte imbalances, the most frequent changes are an increase in amylase and transaminases, slight increase in serum lactate levels and ketone bodies and glycerol (leading to mild metabolic acidosis), and a decrease in platelet counts and sometimes even white blood cells (62).

Techniques and timing of induction of hypothermia

Numerous methods have been tested to reduce both the body temperature in total and locally the temperature of the heart or brain. The simplest and least invasive technique is definitely external cooling through ice packs, metal plates or cooling circuits. However, these techniques have the disadvantage of slowly lowering the temperature and being difficult to control, even inadvertently leading to excessive cooling. The methods used in clinical practice are divided into two large groups: invasive techniques and non-invasive techniques (63).

Among the non-invasive techniques we find blankets and helmets cooling through air or water circuits, ice pack applied to the head, neck, trunk and roots of the limbs, immersion in cold water and self-adhesive cooling plates coated with hydrogel through which water circulates at a controlled temperature. The latter device has also proved to be more effective than the others, especially in the treatment of refractory high fevers (64). Invasive techniques, on the other hand, include: nasal, rectal and naso-gastric cold washes, extracorporeal circulation systems (CPB or ECMO), jugular retrograde flow, peritoneal lavage with cold exchanges, intraventricular cerebral hypothermia and cold intravenous infusions.

In particular, the infusion of fluids through the intravenous route has proved to be particularly tolerable and feasible even in the pre-hospital setting. Bernard et al.

have long documented how rapidly infused 4°C Ringer solutions for about 30 ml/kg give a rapid drop in body temperature of 35.5°C to 33.8°C within 30 minutes with no side effects, particularly no signs of pulmonary edema (65).

However, according to Kliegel, this method is certainly effective in induction but not in maintaining the temperature, additional supports are often required for this purpose (66). That is why in clinical practice the association of several devices would be useful: for rapid induction invasive but more effective routes would be preferred, while if a longer maintenance is required, the non-invasive ways is to be considered especially for their given lower rates of side effects.

It is no coincidence that the latest indications on the overall duration of hypothermia recommend a minimum time of 12-24 hours (24). Many studies on animal guinea pigs have been done over the years and have shown how earlier therapeutic hypothermia is induced, the faster the temperature target is reached, the greater the chance of obtaining a positive outcome. Even a delay of only 15 minutes proved crucial in significantly reducing the chances of an improvement in the outcome, although there would still be a positive effect in slowing down brain damage (67). Many authors, however, have subsequently denied this dogma of "as soon as possible" demonstrating with their studies how even a delay in cooling can still lead to benefits (68-70).

Clinical applications of therapeutic hypothermia

a. Cardiopulmonary resuscitation:

Hypothermia for at least 12-24h has proven to be effective and protective, especially if started early, against both myocardial and cerebral damage. It still remains a topic of discussion whether its use can be extended not only to patients resuscitated after cardiac arrest by defibrillable rhythms but also to patients with PEA or asystole that seems of a poor prognosis. It is therefore unclear whether the less striking results in these patients are linked to the worst underlying clinical situation or whether hypothermia is actually not efficient in counteracting this (71).

b. Cerebral Trauma:

Brain trauma is the most common cause of death and disability in young people in

western countries. While the primary damage that occurred at the time of the trauma is likely to be irreversible, secondary damage, which also develops over days, is potentially preventable and treatable. This damage is linked to cerebral edema, the resulting intracranial hypertension, reduced blood flow of the injured area with further ischemia and increased risk of cerebral hernias which greatly increase the risk of death. Hypothermia treatment have been associated a reduction in intracranial pressure, while the net effect on survival and neurological outcome still presents conflicting results (71).

c. Stroke:

Animal studies indicate a clear benefit of hypothermia in case of stroke, but have also shown that the time window available for hypothermic treatment may be more limited than that of post-arrest damage. In particular, the therapeutic intervention is aimed at limiting damage in the ischemic penumbra. So far, there are only animal experiments and preliminary clinical trials suggesting that hypothermia may help limit neurological damage in stroke patients but there are no controlled clinical trials available in humans (71).

d. Fever in patients with neurological damage

Fever is very common in neurological patients in intensive care, affected by ischemic stroke, head trauma, intracerebral hemorrhage and subarachnoid hemorrhage but also after cardiac arrest. It is known that fever can adversely affect neurological outcomes, and several studies have reported worse neurological outcomes and higher mortality in patients who develop fever from causes. There are, therefore, indications for now to prevent or immediately treat fever with hypothermia in all ventilated patients with neurological lesions in intensive care if it occurs within the first 24-48 hours (71).

e. Intraoperative hypothermia

Intraoperative hypothermia has been used clinically since the 1950s by Bigelow et al. to allow intracardiac operations in a bloodless field. Usually, the goal of intraoperative hypothermia is to increase the time available for specific surgical procedures, reduce metabolism and confer protection to the brain and/or spinal cord during local vascular occlusion or complete circulatory arrest. An important

difference between intraoperative applications of hypothermia and other therapeutic applications is that treatment can be started before and during the insult resulting in a certainly greater protection. Currently, hypothermia is widely used in neurosurgery, vascular surgery and cardiac surgery (72, 73).

Accidental hypothermia

A complementary aspect to induced hypothermia is that which arises due to accidental causes. Accidental hypothermia is present at every latitude and in every season, however, it is more common in urban settings, where homelessness, alcohol and substance abuse, and psychiatric disorders often coexist (74). Primary or acute accidental hypothermia can be recognized, resulting from exposure to cold or immersion in cold water of a previously healthy individual, and secondary or chronic accidental hypothermia, a complication of a serious systemic disease, the mortality of which is higher than the previous one (75). There are numerous variables that make individuals vulnerable to heat loss, first of all extreme ages. Thermal perception decreases in the elderly, who are more prone to immobility, malnutrition, systemic diseases, as well as dementia and psychiatric diseases, that interfere with the production and storage of heat. On the other hand, newborns have high loss of body heat due to the high ratio of surface area to body mass, as well as the lack of an effective thrill reflex and adaptive behavioral responses (76). Endocrinological variables may also be responsible for the appearance of hypothermia. Hypothyroidism, especially in severe cases where myxedematous coma is reached, it causes a reduction in basal metabolic rate and hinders thermogenesis. Other conditions with similar effects are adrenal insufficiency and hypopituitarism. In people with diabetes, hypothermia may occur as a result of hypoglycemia, diabetic ketoacidosis or lactic acidosis. At the neurological level they can act as predisposing factors both brain injuries (trauma, cerebrovascular accidents, subarachnoid hemorrhages or hypothalamic lesions), and spinal cord injuries (blockage of the sympathetic nerve impulse responsible for the induction of chills and systemic vasoconstriction). As for the role played by exogenous factors, ethanol causes vasodilation, reduces thermogenesis and gluconeogenesis,

as well as limits judgment; phenothiazines, barbiturates, benzodiazepines, tricyclic antidepressants and many others induce vasoconstriction mediated by the central nervous system; anesthetics block the reaction of the thrill. Whatever the etiology behind the development of hypothermia, the presence of sepsis is an important negative prognostic sign (76).

To understand the role of these organ dysfunctions in the development of hypothermia, it is necessary to refer to the role played by them in thermoregulation and in the adaptive response to alterations of thermostasis. The anterior hypothalamus (preoptic region) is the main actor of the mechanisms underlying thermoregulation. At the initial activation of these systems, follows a progressive depression of all organs and systems, which occurs with variable kinetics.

Unlike induced hypothermia, in accidental hypothermia has a number of factors that can affect the rate at which cooling occurs, and these are represented by body mass, age, insulation (clothing and subcutaneous fat), shiver, movements, temperature gradient, percentage of surface in contact with a medium cold and local conditions, such as wind and turbulence of water in case of drowning. The crucial factor in any case is whether critical cerebral hypoxia occurred before protective cooling was established (77).

Early hypothermia is the main reason why it is possible to survive without developing neurological damage. In children, survival in cases of diving hypothermia is even better, due to the faster speed with which cooling occurs compared to adults, especially in infants. It is even likely that the aspiration of cold water in children can lead to an immediate cooling of the heart and carotid arteries, and consequently of the brain (78). In general, the duration of submersion (head under water) is a direct measure of anoxic damage: a duration of 2.5-5 minutes predicts positive outcomes, >10 minutes is associated with negative outcomes, while with a duration of more than 25-30 minutes the chances of survival are practically nil. In the case of avalanche victims, the cooling rate is extremely variable, but even in this case survival decreases dramatically after 35 minutes. If these subjects are found in cardiac arrest, outcomes are extremely poor, even if rewarmed with ECLS (79).

Management of accidental hypothermia

The diagnosis is relatively simple if the individual has a history of prolonged exposure to the external environment, at low temperatures, without adequate clothing; it is more complex if secondary accidental hypothermia occurs, in a clinical context that is not well known. Hypothermia is confirmed by central temperature measurement at least two locations. The possible sites that can be used, in descending order of invasiveness are the pulmonary artery, esophagus, bladder, rectum, tympanic membrane, oral cavity and skin. Generally, a rectal probe, placed at least 15 cm deep, and an esophageal probe, inserted up to 24 cm below the larynx, are used (76).

Accidental hypothermia is staged using the measured core temperature and clinical findings as parameters (Table 1) (80).

Table 1: Stages of accidental hypothermia

Stage	*Clinical presentation*	*Core Temperature*
HT I (mild)	Conscience preserved, thrill	35-32° C
HT II (moderata)	Altered Conscience, with/without thrill	28-32° C
HT III (severe)	Unconscious subject, preserved vital signs	<28° C
HT IV (severe)	Apparent death, absent vital signs	variabile

In hypothermic patients who have developed cardiac arrest, there are numerous factors that can affect outcomes: hypoxia (the single most important factor); characteristics of the patient (age, comorbidities, trauma, etc.); cooling speed; environment (wind, water, snow); features of the arrest itself (body temperature, whether hypoxia preceded arrest, delay in the establishment of cardiopulmonary resuscitation, speed of transfer to the hospital); proximity of adequate hospital structure; appropriate preparation of hospital staff (81). Almost, these subjects always require prolonged CPR (cardiopulmunary resuscitation). NIRS (near-infrared spectroscopy) is increasingly used to monitor brain oxygen saturation

regionally, and to predict ROSC and possible neurological outcomes, although the evidence is still scarce (82). The use of vasopressors would seem to be associated with improvements in ROSC, but not with better neurological outcomes, which would be even worse in association with the use of higher doses of adrenaline (83). The 2015 ERC guidelines recommend avoiding the administration of adrenaline in hypothermic cardiac arrest and limiting defibrillation to three attempts until 30°C is reached (84). In contrast, the American Heart Association guidelines allow further attempts in association with warming attempts and consider the administration of adrenaline according to the ALS algorithm standard (85). In any case, patients with hemodynamic instability or cardiac arrest should be transported as soon as possible to centers provided with ECLS support, which must be contacted in time in order to ensure the correct organization of the clinical sitting before the patient is admitted. In addition, during the transfer, the continuity of the CPR must be ensured.

Rewarming in hospital setting

After the first resuscitation maneuvers, once arrived in the hospital environment, it is possible to implement a series of strategies aimed at obtaining heating. These can be classified into passive (which consist of protection from further heat loss), external active (in which heat is supplied through the body surface), and internal active (heat is fed directly into the body). Passive heating simply consists of covering and insulating the patient in a warm environment: covering also the head reaches a heating rate of 0.5-2 ° C per hour. This is the best technique in previously healthy patients who have experienced acute, mild, primary and accidental hypothermia. Instead, the application of heat directly to the extremities should be avoided, to avoid the establishment of a peripheral vasodilation and therefore to precipitate the "post-fallen" effect of the central temperature, in which a continuous drop in temperature occurs even after the cessation of exposure to cold. By applying heat also to the trunk this phenomenon can be prevented or at least reduced. Heating central, however, is necessary when the temperature is below 32 ° C, when there is cardiovascular instability, in extreme ages, in the presence of functional alterations of the CNS or endocrine insufficiency, as well as in

suspicions of secondary hypothermia. Active external heating is achieved by means of forced aeration thermal blankets, or alternatively with tools that allow the circulation of hot water through external surfaces that exchange heat, the use of radiant heat sources or hot compresses. Active central heating is represented by the heating of the respiratory tract with humidified and heated oxygen (at 40-45 ° C) by means of a mask or endotracheal tube; crystalloid solutions heated to 40-42 ° C, the effectiveness of which is however guaranteed only by the administration of large volumes of liquids; gastrointestinal or bladder irrigation with heated fluids, or closed chest washing, far more effective in severely hypothermic patients, and peritoneal lavage with dialysate at 40-45°C (86, 87).

Finally, ECLS using arteriovenous extracorporeal membrane oxygenation (VA-ECMO) or CPB is the treatment of choice to be used in case of hypothermic cardiac arrest or severe circulatory instability not responsive to ALS techniques. ECLS is a safe method, and with survival rates higher than all other methods. In fact, it immediately restores circulation, provides tissue oxygenation and CO2 removal and ensures rapid and controllable heating (81). Most patients in primary hypothermia maintain a rhythm that guarantees perfusion up to 28 ° C, consequently, the indications for the use of ECLS in patients arrested but with a central temperature between 28 ° and 32 ° C are more controversial, since it is very likely that cardiac arrest is linked to causes other than hypothermia, and consequently the benefits on neurological outcomes would be less marked. The use of ECLS in HT III patients (therefore not yet in cardiac arrest) can be considered in certain situations: I) in case of failure of heating with other less invasive method; II) in the presence of life-threatening arrhythmia; III) hypotension (systolic pressure <90 mmHg); IV) respiratory failure; V) refractory acidosis. Even elderly patients, with a limited tolerance to the low flow state typical of severe hypothermia, they may benefit from treatment with ECLS (88).

Currently, among the various methods available, the privileged one is ECMO, and in particular its veno-arterial variant. ECMO is a system born as a bridge therapy in subjects with respiratory and/or cardiovascular failure. During the procedure the blood is drained from the patient's vascular system, circulated outside the body via

a mechanical pump, then reintroduced into the circulation. While outside, blood passes through an oxygenator and a heat exchanger. Inside the oxygenator hemoglobin is completely saturated with oxygen, while carbon dioxide is removed. The two main types of ECMO are venous-venous (VV) and venous-arterial (VA). The reasons that lead to prefer this system over other extracorporeal heating techniques are the speed with which it can be positioned, the lower need for heparinization compared to CPB and the possibility of prolonging cardiorespiratory support if necessary, considered in fact the most significant advantage. A series of retrospective studies also showed a better survival rate with this technique (89). The fastest route to establish VA-ECMO is through cannulation of the femoral artery and vein. It is necessary to provide general anesthesia, designed to prevent the patient from waking up, and to start warming up with a circuit temperature approximately equal to that of the patient at admission, to avoid excessive gradients (90). Flows should also be increased gradually, to avoid the formation of air bubbles and cell damage induced by ischemia-reperfusion. Normally used heating rates vary between 1° C every 5 minutes and 1° C every hour depending on the situation, however the optimal rate is not yet known. Ventilation should be started as soon as the ECLS begins. Circulatory support must be guaranteed until a stable rhythm is recovered, adequate perfusion and spontaneous oxygenation and a central temperature >32 ° C. It is essential to try to avoid post-resuscitation hyperthermia. Inotropics and vasopressors may be used at the time of weaning, while ECMO discontinuation may be considered if ROSC does not occur at 32-25°C, as well as on the basis of clinical evaluations, such as the presence of uncontrollable hemorrhage, different etiology underlying the CA or severe hypoxic brain damage.

2.3 NITRIC OXIDE (NO)

The substance initially recognized as responsible for vascular relaxation was called endothelium-derived relaxing factor (EDRF), it was later shown that this factor includes nitric oxide (NO). NO is now well known as an important mediator of many physiological functions, and its role in cardiac pathophysiology is now recognized. NO is a lipophilic molecule, extremely reactive due to the presence of

an unpaired electron that gives it radical properties. Lipophilia and the absence of electric charge allow it to spread freely through cell membranes. Nitric oxide is one of the smallest bioactive molecules in the human body, being composed only by the union of a single nitrogen atom (N) with one of oxygen (O). At atmospheric pressure it is a gas, and at the cellular level it reacts with various compounds (oxyhemoglobin, molecular oxygen, superoxide anion, amines, thiols, etc.) reducing its half-life to a few seconds (≈ 5 seconds) (91).

Nitric oxide synthesis

Endothelial nitric oxide modulates vascular tone and blood pressure through the action of cyclic guanosine monophosphate (cGMP) stimulating the relaxation of smooth muscle, inhibition of platelet aggregation and their adhesion to the endothelium, and preventing the proliferation of smooth muscle (prevents thickening of the vascular wall). There are two categories of fundamental mechanisms of nitric oxide production in the human body, one enzyme dependent, the other independent enzyme. Enzyme-dependent mechanisms include the three isoforms of nitric oxide synthase (NOS) and the xanthine oxidase pathway in case of tissue acidosis. The mechanism independent of an enzyme pathway concerns the transformation of nitrites. NOS transforms L-arginine into NO and L-citrulline in the presence of NADPH, FMN, FAD, heme and tetrahydrobiopterin as cofactors; this reaction occurs in the presence of oxygen (92, 93).

Neuronal NOS (nNOS) and endothelial NOS (eNOS) are produced in a constitutive manner and are therefore also referred to as constitutive NOS (cNOS). The amount of NO released by them is in fractions of nM; the inducible NOS (iNOS) instead produces μM amounts of NO, that is an amount 1000 times higher than that released by the cells under basal conditions. A key difference between the three isoforms of nitric oxide synthase is the stimulus required for the production of NO. While two neurotransmitters, glutamate and acetylcholine, are responsible for the activation of nNOS and eNOS, the expression of iNOS is induced by pro-inflammatory cytokines (INFα, TNFα, IL-10). NF-kB and STAT1 are two important transcriptional factors for iNOS mRNA that are activated the first by TNFα and IL-1β, the second by INFγ (94, 95).

NO performs multiple functions in the human organism and affects almost all biological systems. The molecular targets with which NO interacts are in fact numerous. NO production has also been demonstrated in cardiomyocytes. Production during myocardial ischemia in humans was measured by Node et al. measuring the difference in arteriovenous coronary concentration of nitrates plus nitrites. In adult rat ventricular cardiomyocytes, cNOS and iNOS have been demonstrated (96, 97).

Decoupling of cNOS

A damaged endothelium produces a large amount of superoxide anion ($O2^{\bullet}$) compared to the endothelium under normal conditions. Low concentrations of L-arginine promote the decoupling of cNOS and the formation of $O2^{\bullet}$ while the addition of L-arginine restores the production of NO and abolishes that of $O2^{\bullet}$. The simultaneous presence of NO and $O2^{\bullet}$ determines the triggering of a reaction that leads to the formation of peroxynitrite ($ONOO^{\bullet}$), a highly cytotoxic component. $ONOO^{\bullet}$ can be protonated and form peroxynitric acid (ONOOH) which, at low concentrations, can undergo rapid isomerization and form nitrate ($NO3^{-}$) and a proton (H^{+}). However, at high concentrations, the ONOOH gradient between the cytoplasmic membrane of the endothelial cell and the blood facilitates its spread. During the diffusion process the ONOOH molecule is subjected to hemolytic or heterolytic cleavage. Hemolytic cleavage leads to the formation of hydroxyl radical ($OH\bullet$) and nitric dioxide radical ($NO2\bullet$), both powerful oxidants. Heterolytic cleavage leads to the formation of another powerful oxidant; the nitronium ion. These oxidants are the major cause of oxidative stress generated by the dysfunctional endothelium. Oxidative stress reduces the bioavailability of NO in the cardiovascular system, causes vasoconstriction of capillaries and increases blood pressure. It also reduces some cNOS cofactors by promoting their decoupling and leading to further formation of radicals. These act on numerous cellular components leading to enzymatic inactivation, DNA damage, lipid peroxidation and membrane damage; which can contribute to the pathogenesis of atherosclerosis, cardiovascular, neurological, pulmonary diseases and cancer (98).

Physiology of nitric oxide release from the endothelium

The pressure and friction force determined by the flow of blood on the endothelial surface are the cause of flow-induced NO release. This force that is expressed on the endothelium is the cause of an immediate response (in milliseconds or seconds) that is expressed with an increase in ion permeability and intracellular calcium levels; and a delayed response (minutes to hours) triggered by chronic stress that includes the expression of certain genes (99).

The amount of NO released directly depends on the wall stress and the speed of the flow. Wall stress results in the formation of a thin layer of NO below the endothelial cell membrane since cNOS is placed at the level of the membrane. Therefore, the highest concentration of NO is recorded in the endocardium which is exposed to a highly turbulent flow. A reduction in the presence of NO determines an increase in platelet and leukocyte adhesion, promotes platelet aggregation and the formation of clots on the endothelial surface (100).

Nitric oxide in cardiac activity

Recent studies have shown that endogenous NO agonists, exogenous NO donors or increased cGMP promote myocardial relaxation and directly depress cardiac contractility (negative inotropic effect) (101). With the increase in preload there is a gradual, not sudden, but significant increase in both the peak and the baseline level of NO, while as a result of a reduction in ventricular filling the concentration of NO decreases (102). NO is released by the beating heart with a pulsatile pattern and its synthesis is directly related to ventricular load conditions, contributing to the regulation of cardiac performance beat to beat.

Although many cell types express cNOS, the predominant action in the regulation and synthesis of load-dependent NO seems to depend on the activity of microcirculation endothelial cells; in fact, most of the NO produced by the endocardium is quickly dissipated into the bloodstream where it inhibits the local aggregation of platelets. Myocytes also express cNOS but do not represent a significant source of NO, at least in response to a change in load.

The importance of endothelial NO in regulating vascular tone at the coronary and systemic level has been experimentally demonstrated by regional inhibition of its synthesis with NG-monomethyl-L-arginine (L-NMMA) competing with L-

arginine as a substrate of NOS (103). Regional blockade of NO synthesis significantly reduces blood flow demonstrating the importance of regional NO synthesis, supporting the bioactive role of nitrites since its consumption increases greatly with exercise and with the inhibition of NO (104).

Several studies have shown that NO circulates in plasma also in the form of S-nitrosothiols (SNOs); IN FACT, NO reacts with protein thiols, forming nitrosylated compounds that can take on new physio-chemical properties. Nitrosothiols such as S-nitro-cysteine (SNOC), S-nitro-glutathione (GSNO) and S-nitro-albumin (SNOALB), are capable of releasing NO exhibiting properties similar to those of NO itself; these are divided into high molecular weight (HMW) and low molecular weight (LMW) SNOs. SNOALB is definitely the most abundant HMWSNOs, probably transnitrosilation from GSNO and SNOC is the main mechanism for the formation of SNOALB and appears to be more effective than the mechanism of transnitrosilation from free NO or NO donors drugs (105).

The abundance of S-nitrosothiols in plasma suggests that these could constitute an important reservoir of NO due to their relatively long half-life under physiological conditions compared with the short half-life of free NO or LMWSNOs. These low- and high molecular weight S- nitrosothiols likely play a role in the stabilization and spread of NO in the vascular bed, where NO can change vascular tone (106).

NO donors

Due to the instability and short half-life of NO there is considerable interest in the role of more stable NO adducts for the regulation of vascular tone in vivo. Therefore, the possibility of using artificially created peptides to allow an increase in the presence of NO in the circulation has been explored. Many groups of researchers have synthesized NO-donors that should be able to release NO selectively at the target site. For example, V-PYRRO/NO and 2-(acetyloxy) benzoic acid 3-(nitroxymethyl) phenyl ester may release NO into the liver (107, 108). However, these NO-donors have not yet been applied in clinical situations because the mechanisms of action are not yet fully understood. Therefore, the search for a safer NO-donor is feverish; a NO transporting protein that has high efficiency of S-nitrosylation, high stability of the nitrosylated form in circulation

and a high efficiency of S-transnitrosilation within cells in need of NO. As a candidate for this role, human serum albumin (HSA) has become increasingly important, because it is the most abundant plasma protein and because endogenous S-nitrosothiols in human plasma are widely associated with HSA. In addition, S-nitrosylated-HSA (S-NO-HSA), such as the one developed by Seth Hallström, is much more stable than low molecular weight S-nitrosothiols (109). Restoring blood flow in ischemic tissues is a stage of many surgical procedures. Especially after prolonged ischemia, reperfusion can lead to changes in vascular motility and lead to an increase in the concentration of many reactive species and an increase in permeability leading to edema.

In a study involving treatment with S-NO-HSA in a rabbit that had been induced an ischemia of the hind leg lasting 2 hours followed by reperfusion data have been obtained that detect that S-NO-HSA preserves the function of eNOS, prevents its decoupling, stabilizes the basal production of NO, decreases the production of oxidized species and therefore has beneficial effects in reducing the damage from ischemia/reperfusion. It is evident a deficiency of NO at the end of ischemia and at the beginning of reperfusion indicating the effectiveness and necessity of the use of a NO-donor drug. It is also shown that pre-treatment before induction of ischemia is more effective in reducing damage. The measurement of high-energy phosphates also demonstrates greater preservation of mitochondrial function when the drug is used.

In a second study, a model was recreated that simulated the cold removal and preservation of organs in an isolated rabbit heart that underwent a 6-hour ischemia. Semsroth et al. thus demonstrates how with the use of S-NO-HSA substantially increased cardiac output, diastolic function, myocardial perfusion compared to controls. These data may suggest the concrete possibility of the use of NO-donor drugs in cardiac preservation in cold ischemia during cardio-pulmonary by-pass and in the preservation of the organs to be transplanted (110).

In another experiment, Dworschak et al. recreated severe myocardial ischemia with cardio-pulmonary by-pass in pig's heart (111). In animals treated with S-NO-HSA, an increase in oxygen extraction at the myocardial tissue level was thus evident,

presumably because the number of perfused capillaries remains higher and fewer shunts are formed. After severe myocardial ischemia increased coronary flow and higher O2 consumption were associated with better heart function. In fact, the systolic pressure of the left ventricle (LVSP) and the mean arterial blood pressure (MAP) were remarkably higher in treated animals after weaning from cardio-pulmonary by-pass.

Also in a study of hot, unprotected, pig heart ischemia, it was noted that the infusion of S-NO-HSA preserved the function of eNOS, stabilized NO production, decreased production of $O2^{\bullet}$ and $ONOO^{\bullet}$ and had beneficial effects on hemodynamics dependent on the reduction of damage from ischemia/reperfusion (112). After weaning from cardio-pulmonary by-pass, MAP decreased in both groups (treated and untreated), however, the decrease in the S-NO-HSA group was significantly less pronounced after 15 minutes and 75 minutes after weaning. In addition, in the treated group MAP stabilized after 120 min, while in the untreated group it continued to decline. Moreover, MAP in the treated group was significantly lower. LVSP increased initially in both groups, however only in those treated it remained at high levels, while returned to baseline levels in controls.

Clinical applications of NO

The use of nitric oxide by inhalation (iNO) in full-term or near-term infants is now well established in clinical practice, and was approved by FDA in 1999 (113). This type of treatment is indicated in infants older than 34 weeks with hypoxemic respiratory failure that is not responsive to standard treatments.

Acute hypoxemic respiratory failure can be caused by various pathologies, such as infections, meconium aspiration syndrome, respiratory distress syndrome (RDS), and persistence of fetal circulation (114-116). The pathologies that receive the most benefits from this type of treatment are those associated with pulmonary hypertension and left right extra pulmonary shunt, while in other forms, such as those characterized by alterations of the parenchyma or with predominantly intrapulmonary shunts (sepsis, pneumonia, RDS) should be associated with treatment with iNO other therapies aimed at resolving the underlying pathology (117, 118). It is important to exclude by echocardiography congenital cyanotic

heart diseases that can give pulmonary hypertension but do not respond to iNO therapy: even iNO therapy was found to be harmful in these patients, as pulmonary vasodilation given by the drug could increase the left preload. For the same reason it is important to detect a situation of left ventricular failure by echocardiography (119).

Other contraindications to iNO therapy in the newborn are: severe malformations, lethal chromosomal abnormalities and severe coagulopathy (114). Since the response to iNO therapy improves by optimizing alveolar recruitment, it is advisable to treat these patients with exogenous surfactant and ventilator support before initiating the treatment (120, 121).

Several studies in full-term or near-term infants have shown improved oxygenation and reduced need for ECMO. However, the use of iNO in premature infants with gestational age <34 weeks is not recommended (122, 123).

The dosages considered effective in the treatment of newborns are 20 ppm as the initial concentration. In some cases, such as if there is severe pulmonary hypertension with poor response, a dose between 30-40 ppm may be considered. Dosages in higher concentrations do not increase efficacy and indeed pose a greater risk of toxicity by increasing met-hemoglobin levels. The parameter by which the response to therapy is evaluated is the rapid improvement of oxygenation: the lack of an improvement of 20-25% in the first 4-6 hours indicates the lack of response to the treatment. If the patient does not respond to therapy, alternative therapies such as ECMO should be considered. The average duration of a treatment varies from 3 to 7 days, then a gradual and progressive reduction of doses to final concentrations is carried out which should ideally be 1 ppm or less. It is important in some cases to increase FiO2 preventively, to prevent possible rebound effects characterized by desaturation crises and pulmonary hypertension. With regard to long-term outcomes, such as the degree of neurological deficit and the frequency of re-hospitalization, no significant differences were demonstrated compared to the control groups.

Recently the medical gas nitric oxide has been used in cardiac surgery in an unconventional way by P. Checchia in 2013 and C. James in 2016 (124, 125).

Checchia et al. connected the NO dispenser directly to the CPB oxygenator in 8 children undergoing tetralogy surgery of Fallot detecting reduction of mechanical ventilation time, stay in intensive care and better cardiac performance. In 2016, however, at the Melbourne hospital as many as 101 children who underwent various congenital heart disease repair procedures received NO 20 ppm directly through the CPB oxygenator during surgery. James et al. reported an improvement in cardiac output and a reduction in low cardiac output syndrome in the treated children. These studies have been a source of inspiration for one of our experimental models.

NO effects on the post cardiac arrest syndrome

Nitric oxide has various physiological characteristics that can give a benefit in ischemia-reperfusion damage: is a powerful vasodilator that inhibits activation and adhesion of platelets and leukocytes, has the ability to inhibit enzymes responsible for the formation of reactive oxygen species (ROS) and acts directly as a scavenger for ROS. These effects seem to be mediated by various mechanisms but the prevailing one is the activation of molecular pathways through the increase in the amount of cGMP produced by guanylate cyclase. The benefits of inhaled nitric oxide on extra-pulmonary organs have been demonstrated by various experiments on animal models; first in mice from Hataishi et al., where administration of iNO for 24 hours post-reperfusion limited cardiac damage and improved systolic and diastolic functions, later on the porcini model, where the same results were reconfirmed (126, 127). Studies on the effects of iNO in extra-pulmonary organs have also been conducted with good results in humans, in the context of liver transplantation and in the context of cardiopulmonary bypass, where inhalation of 20 ppm of iNO during and after CPB demonstrated a decrease in myocardial damage and an improvement in left ventricular dysfunction (128, 129).

The study conducted by Kida and Ichinose also shows that the administration of iNO in mice after cardiac arrest and subsequent cardiopulmonary resuscitation demonstrated a decrease in the development of abnormalities of water diffusion in the brain and consequently an improvement in neurological outcomes and survival rate (130). A decrease in the expression of genes encoding pro-inflammatory

proteins such as cytokines and NADPH oxidase was also observed in mice treated with iNO, suggesting that this may play a key role in the development of neurological dysfunction.

A 2013 review reports on all the mechanisms involved in ischemia and reperfusion damage in the brain and how these mechanisms are affected by the administration of nitric oxide or of NO-donor molecules. Nitric oxide reduces the activation of NF-KB, reduces the inflammatory response, reduces caspase activation and thus apoptosis eventually leading to a reduction in the ischemic area and an improvement in cerebral blood flow (131).

2.4. EXTRACORPOREAL MEMBRANE OXIGENATION (ECMO)

Definition and history

extracorporeal membrane oxygenation (ECMO) is a circulatory support, used to support respiratory function and possibly also cardiac function. It is based on the principles of cardiovascular bypass used in cardiac surgery, with the difference that ECMO is a closed circuit (the external reservoir for blood is absent) and is studied to be used on the patient for a prolonged period and not just for a few hours. The main indication for ECMO is cardiogenic shock with organ dysfunction, or rapidly reversible cardiac dysfunction (myocarditis, overdose, deep hypothermia). It is to be considered a "bridge" therapy: the patient is treated while waiting for him to recover vital functions or for an effective therapeutic strategy to be adopted (132). The first operation in which a human heart-lung machine was used was performed by Gibbon in 1953, in 1972 ECMO was used for the first time outside the operating room. 200 with encouraging results on children and infants suffering from respiratory failure. To date, ECMO is used in the clinical practice of intensive care as cardiorespiratory support in pediatric patients. As for adult patients, the first studies were not at all encouraging, but recently with the improvement of the components (such as the implementation of the centrifugal pump and the new oxygenator) new studies suggest excellent results also on this type of patients.

Components

The ECMO circuit consists of a pump, an oxygenator, a heat exchanger, arterial and venous cannulas and a set of tubes to connect the patient to the machine.

Cannulas can be of different diameter and length, have a shaped tip to facilitate the penetration of the arterial vessel, reinforcing metal coils and a rigid proximal portion that must be connected to the pipes. Venous cannulas are usually thicker and longer than arterial ones. The pump is centrifugal; it works by creating a pressure differential that allows blood to flow. A sensor calculates the flow in L/min. This type of pump has the advantage of giving less hemolysis than other types and stops in case of presence of air in the circuit. This type of pump is non-occlusive, so there may be a return flow of blood, especially if the ECMO works with low pressures and the pressure generated by the heart of the patient. It is important to always keep this retrograde flow monitored and be ready to clamp the arterial tube in case the pump stops working. The circuit is composed of sterile PVC pipes treated with anticoagulants to reduce the risk of thrombi (133).

The blood passes through an oxygenator, which allows the exchange of gases. It is composed of multiple fibers with a diameter <0.5mm, covered with a hydrophobic polymer (polymethylpentene) that allows the passage of gas through a pressure gradient but not liquid. The capacity of the membrane is ten times lower than that of a healthy lung (3000 vs.200-250 mL/min). The heat exchanger is used to heat, by convection, the patient's blood; hot water circulates around the oxygenator and indirectly heats the blood (133).

Technique:

The principle of ECMO is to collect the patient's venous blood and pass it through a pump and an oxygenator in order to reintroduce oxygenated blood into the patient. Venous blood is typically drained by a large-caliber vein such as the femoral vein, is oxygenated, decarboxylated through a membrane and finally reflux into the circulation via venous or arterial access, depending on the ECMO type. In fact, there are two types of ECMO: venoarterial ECMO (ECMO-VA), used to fully support cardiopulmonary function and venous ECMO (ECMO-VV), which instead only supports the respiratory function by oxygenating the blood (figure 3) (134-136).

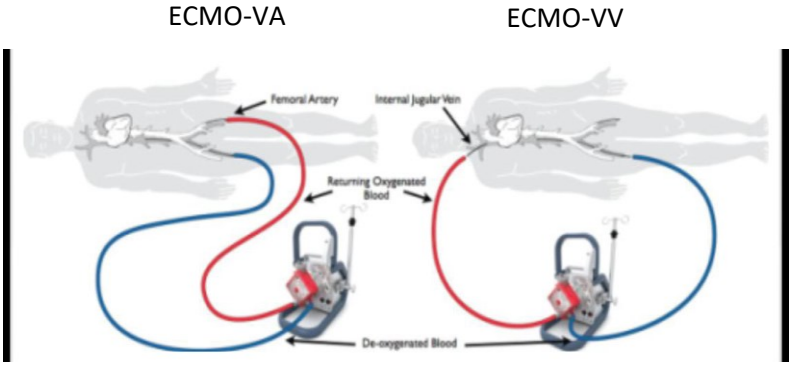

Figure 2: schematic presentation of ECMO-VA and ECMO-VV

ECMO-VA

The most frequent indications for the use of ECMO-VA are represented by all causes of cardiogenic shock refractory to medical treatments: myocardial infarction, myocarditis, refractory cardiac arrest, post-CPB cariogenic shock, transplant rejection, drug toxicity, accidental hypothermia, pulmonary embolism, acute and chronic heart failure.

In these cases, heart pump failure quickly leads to tissue hypoxia due to the inability to maintain adequate flow and therefore consequently to multiple organ deficiency. The most used technique for inserting this type of device is the femoro-femoral surgical approach: the femoral vessels are accessed at the level of the shoe triangle and after locating the artery and the femoral vein, the two vessels are catheterized using the Seldinger technique. The patient is decoagulated with a bolus of unfractionated heparin; the venous cannula is placed first in the vena cava and then in the right atrium under echocardiographic control, then we continue with the insertion of the arterial cannula. Subsequently, a reperfusion catheter is placed below the arterial catheter, to ensure perfusion at the level of the lower extremities. A percutaneous approach via ultrasound control is also possible, which still requires surgical removal.

It is also possible to directly cannulate the right atrium and the descending aorta (ECMO VA-C): this happens frequently in cases of post-CPB cardiogenic shock as the sternum is already open the result makes it easier to install (137).

ECMO-VV

This type of support is usually used in patients with normal heart function, it tends to be patients with an ARDS (acute respiratory distress syndrome) severe, unresponsive to conventional therapies. This type of ECMO ensures the exchange of gases and the maintenance of mechanical ventilation at low volumes (gentle ventilation) in order to put the lung "at rest" and allow its healing. The cannulation sites are mainly femoro-jugular: the outflow cannula is inserted into the femoral vein while the influent cannula is inserted at the level of the internal jugular vein. A percutaneous technique is used for cannulation (138).

ECMO use in cardiac arrest

In pediatric and neonatal patients The use of ECLS is predominantly indicated in refractory cardiac arrest, with the possibility of reasonable treatment options: the main strategies for which it is used are mainly (139):

1.As a bridge for recovery (in reversible pathologies).

2.As a bridge to another therapy (such as VAD or oxygenator).

3.As a bridge for organ transplantation.

4.As a bridge to the therapeutic decision (allows the recovery and temporary stabilization of the patient).

The indications for ECLS, according to ELSO guidelines, in the pediatric patient are divided into two categories:

- related to cardiac surgery and catheterization; for failure of weaning from cardiopulmonary bypass, as a support in election during high-risk catheterization procedures, for low post-operative cardiac output, to stabilize an unstable patient before an operation; cardiac arrest, different types of shock,
- in-hospital cardiac arrest not responsive to cardiopulmonary resuscitation, if the ECMO specialist is readily available.

As for the contraindications there are absolute and relative ones, in particular there are prematurity and low birth weight, lethal chromosomal abnormalities (trisomy 18, trisomy 13), uncontrollable bleeding and irreversible brain damage.

In adult patients, the main indication is cardiogenic shock refractory to therapy with inotropics and vasoconstrictors and possibly to the use of intra-aortic

counterpulsator. Typical causes leading to this condition are myocarditis, acute myocardial infarction, peripartum cardiomyopathy, chronic non-responsive heart failure, post-cardiotomy shock (140).

The use of ECMO in cardiac arrest is indicated when:

-the time elapsed between the start of the CPR and ALS maneuvers and the ECMO implantation time is less than 100 minutes

-patients under the age of 65 years,

-the rhythm causing cardiac arrest is not asystole

- hypothermic cardiac arrest

Absolute contraindications are represented by patients with irrecoverable heart and not a candidate for transplantation or VAD, advanced age, chronic organ dysfunctions such as emphysema, cirrhosis and renal failure, patient compliance, prolonged CPR without adequate tissue perfusion.

Despite advances in techniques, ECLS protocols and instrumentation, mortality of cardiac arrest remains high. The incidence of cardiac arrest in Europe is 2,500/100,000 inhabitants per year of these patients only 40% arrive at the hospital after adequate CPR and ROSC. Of these patients, only a third survive 30 days after the event and are discharged from the hospital. So only 12% of patients who have suffered cardiac arrest are alive after 30 days. The number of surviving patients may increase with the use of ECMO. According to data from the ELSO registry in the period between 2003 and 2014, 30-day survival in patients undergoing ECLS after cardiac arrest is 29%. Despite the advancement of knowledge and techniques of ECLS, mortality of cardiac arrest continues to be affected by the increase in patients' comorbidities, particularly in the elderly and the onset of irreversible neurological damage (141).

3. SCOPE OF THE STUDY

The aim of the studies conducted by our team at the University of Verona over the past 7 years was to find alternative methods to improve cardioprotection and neuroprotection in the presence of ischemia damage and reperfusion caused by a stop of spontaneous blood circulation. We hypothesized that identifying the molecular effects of the used protocols would provide an explanation for some clinical observations, an understanding of underlying mechanisms, answers to non-conclusive clinical results, and develop possible targets for future research.

Aims of the study: characterization of the molecular and genetic changes associated with two main models of resuscitation post-cardiac arrest:

1) **Model of hypothermic cardiac arrest followed by different degree of rewarming using ECLS**. It is known that slow rewarming results in better recovery so we hypothesized that fast rewarming may induce genetic and molecular changes that would deteriorate the myocardium. In this study we would identify transcriptomic variation in the myocardium exposed to both fast and slow rewarming protocols and investigate the effect of the fast and slow rewarming on the myocardial apoptosis.

2) **Model of cardiac arrest followed by CPB resuscitation with NO supplementation using ECMO**. NO supplementation was shown to have neuro-protective effects but little was known about its effect on the myocardium. We hypothesized that NO might have cardio-protective effects during resuscitation especially if supplied through ECMO. In this study we would investigate the transcriptomic effects associated with NO supplementation on the myocardium and the test the effect effects of NO supplementation on myocardium apoptosis.

The models will be described in full details in the materials and methods section, then the protocols used with the different experimental models will be discussed. Afterwards the protocols of the various studies will be exposed, and one by one presented results, discussion and conclusion.

4. Materials and methods:

4.1. General surgical protocols

All experimental animal surgeries were performed at C.I.R.S.A.L. (Interdepartmental Research Centre on Laboratory Animals) of the Biological Institutes, University of Verona. The housing, handling and sacrifice of the animals have been carried out according to current regulations (Declaration of Helsinki and "Guide for the Care and Use of Laboratory Animals – Institute of Laboratory Animal Resources – National Institutes of Health").

Male rats of the Sprague Dawley strain weighing 400±50 g were used, kept in temperature and humidity controlled rooms with typical light-dark cycle, and fed with standard diet and water.

For all experiments, regardless of the cardiac arrest model used, the phase of anesthesia, intubation, preparation and monitoring were always the same.

First, the rats were anesthetized with diethyl ether vapors, and were intubated by the oro-tracheal route with an atraumatic tube consisting of a 14G. venous cannula. Then The rats were mechanically ventilated with a mechanical rodent respirator (Harvard Model 687, Harvard Apparatus, Holliston MA) with a mixture of oxygen and sevofloran anesthetic 2% (Forene; Abbott, Baar, Switzerland). Respiratory support was continued for the duration of the operation with a fraction of inhaled oxygen (FiO2) of 90%, a tidal volume of 10 ml/kg and a frequency of 80 breathing acts per minute. Ketoprofen 2mg/kg was injected subcutaneously to maintain analgesia and repeated every 2 hours. During the surgical procedure, the temperature of the room was maintained between 23° and 25°C and a layer of insulating material (cork) was placed between the body of the animal and the operation table. The rats were placed in a supine position, the thoracic and the ventral surfaces of were shaved, and the skin was disinfected with chlorhexidine., Monitoring of the ECG and the body temperature is maintained during the entire course of the experiments. The right femoral artery is isolated and a miniaturized catheter with a diameter of 2-Fr (model SPR 838, Millar Instruments, Houston, TX) is inserted for monitoring systemic arterial pressure. Finally, the right femoral vein is cannulated with 24 G cannula for the infusion of 2 mg/kg of pancuronium-

bromide to obtain complete muscle relaxation and washed with Normal Saline to avoid thrombosis of the cannula.

4.2. Tissue collection

At the end of the reperfusion period all rats were sacrificed and the heart was quickly taken through median sternotomy. The samples taken were immediately placed in liquid nitrogen and then stored at -80 °C.

4.3. RNA extraction and RT-PCR

The heart was homogenized with QlAzol lysis reagent (Qiagen Cat# 79306). RNA was extracted following the miRNeasy micro kit protocol (Qiagen Cat# 217084). 100 µl Qiazole was added to mashed cardiac tissue. then 20 µl chloroform was added and vortex. the mix was incubated on ice for 3 minutes. Afterwards, samples were centrifuged (17000xg) at 4ºC for 15 minutes. Supernatant was collected and 1.5 times volume of ethanol 100% added and passed through the provided spin columns followed by wash with the kit wash buffer. DNAase was added to each column to lysis any DNA and columns were incubated in 37 ºC for 1 h. Later, the columns were washed twice with wash buffers then 1 time with ethanol 70%. Finally, RNA was collected by adding nuclease free water to columns and centrifuging at 17000xg for 2 minutes. RNA quality and concentration was assessed through nanodrop using the Cytation 1 reader.

One microgram of each RNA sample was reverse transcribed to cDNA using a mixture of oligo-dT and random hexamer primers (SuperScript IV VILO Master Mix, ThermoFisher Scientific Cat # 11756050). the cDNA was diluted 5 times then 1ul was used in Real-time PCR. Real-time PCR was conducted with Taqman fast advanced master mix (Thermo Fisher 4444557), and primers specific to targeted genes (table 2) using the Quant studio 5 real-time PCR detection system (Applied Biosystems). The RT-PCR cycle included 40 cycles of DNA denaturation at 95 ºC, primer annealing at 54 ºC, and primer extension at 72 ºC. The delta delta CT analysis method was used to determine the fold change of the gene expression.

Table 2: TaqMan Primers used for RT-PCR assay

Target	*Primer*	*Catalogue #*
Atp5a1	Rn01527025_m1	4331182
Cox-2 (Ptgs2)	Rn01483828_m1	4331182
Camk2a	Rn01258147_m1	4331182
TNF	Rn99999017_m1	4331182
Fas	Rn00685720_m1	4331182
DHPR (Qdpr)	Rn02025866_u1	4331182
ATG-9a	Rn01400691_m1	4331182
IL-1a	Rn00566700_m1	4331182
NFKb1	Rn01399572_m1	4331182
ADRA1a	Rn00567876_m1	4331182
B1AR	Rn00824536_s1	4331182
B2AR	Rn00560650_s1	4331182

4.4. Bulk RNA sequencing and analysis

RNA isolated from rat heart as previously described (section 4.3) Using the Ovation RNA-seq System v2 Kit (NuGEN), total RNA (20–50 ng) was reverse transcribed to synthesize the first-strand cDNA using a combination of random hexamers and a poly-T chimeric primer. The RNA template was then partially degraded by heating, and the second-strand cDNA was synthesized using DNA polymerase. Double-stranded DNA was then amplified using single primer isothermal amplification (SPIA). Random hexamers were then used to amplify the second strand cDNA linearly. cDNA samples were fragmented to an average size of 200 bp using the Covaris S2 sonicator. Libraries were made from the fragmented cDNA using the Ovation Ultralow V2 kit (NuGen). Following end-repair and ligation, the libraries were PCR amplified for 9 cycles. A Bioanalyzer assessed library quality on High-Sensitivity DNA chips (Agilent), and concentration was quantified by qPCR (KAPA). The libraries were sequenced on a Novaseq sequencer with a single-read, 50- cycle sequencing run (Illumina). Sample QC was

assessed using FastQC http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Reads were aligned to the GRCh38 genome using Tophat 2.0.13. Gene expression was tallied by Subread feature Counts using Ensemble's gene annotation rat genomes. Finally, we calculated differential expression P-values using edgeR 8. Here, we first filtered out any genes without at least two samples with a FPKM (Fragments per kilo base of transcript per million mapped fragments) between 0.5 and 5000. FPKMs below 0.5 indicate non detectable gene expression, and FPKMs above 5000 are typically only seen in mitochondrial genes. If these high-expression genes were not excluded, their counts would disproportionately affect the normalization. After excluding these genes, we renormalized the remaining ones using "calcNormFactor" in edgeR, then calculated P-values for each gene with differential expression between samples using edgeR's assumed negative–binomial distribution of gene expression. Next, we calculated the false discovery rates (FDRs) for each P-value with the Benjamini-Hochberg method based on the built-in R function "p.adjust.". The genes that showed a fold change of 2 or more with significance of padj <0.05 are identified as the differentially expression genes between the groups. The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.8 was used for the GO term enrichment analysis (https://david.ncifcrf.gov/) which assigns genes to a set of predefined bins depending on their functional characteristics. Pathview (https://pathview.uncc.edu/analysis) open source online software was used for pathway analysis and generation of Kyoto Encyclopedia of Genes and Genomes (KEGG) graphs (142, 143). In brief, this software presents the log2 fold change of gene expression in one group compared to the other group using the KEGG graphs for known pathways. Positive log2 fold change which means higher expression of genes is presented with red color, while negative log2 fold change which means lower expression of genes is presented with green color.

4.5. Immunofluorescence

Frozen tissues were defrosted and washed with PBS and kept in 4% paraformaldehyde for 48 h. The hearts were then washed with PBS, then placed in

10% sucrose solution for 1 h followed by 20% sucrose solution for 1 h at room temperature, then placed in 30% sucrose solution overnight at 4ºC. The hearts were then processed into frozen Optimal Cutting Temperature compound (OCT) blocks and kept at -80ºC for 24 h. Next, the heart was sectioned using a cryostat (Leica Inc.) in 8µm thick sections, placed on slides, and kept at -20ºC until staining. To start staining, the OCT was removed from the section by 3 cycles of heating at 95ºC for 5 min then washing in PBS for 30 min. The sections were permeabilized with 0.1% Triton X-100 for 15 min (Millipore Cat# 55163804) and then blocked with 3% bovine serum albumin (BSA) in PBS for 60 min at room temperature (VWR Cat# 0332). The sections were then probed with primary antibody (1:200 in 1% BSA) for 1.5 h. Then washed three times with PBS. They were then labeled with secondary fluorescent antibody (1:200 in 1% BSA). Table 3 shows a list of primary and secondary antibodies used in this study. Tissue sections were then washed three times with PBS and stained with DAPI 1µg/ml (Biotium Cat# 40043) to stain the nucleolus blue.

Table 3: Antibodies used for Immuno-fluorescent staining

Target	Antibody	Cat#
Cardiac Troponin	Mouse monoclonal Anti-Cardiac Troponin T antibody	Thermos Fisher (MA5-12960)
Anti-mouse IgG	Goat anti-Mouse IgG (H+L), F.I.T.C.	Thermos fisher (A16079)

4.6. Apoptosis assay:

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was conducted using Click-iT™ Plus TUNEL Assay kit (Invitrogen Cat# C10619). Slides of 8µm thick heart sections were prepared as described in section 4.5. To start the assay, OCT was removed by heating at 95ºC for 5 min then washing in PBS for 30 min. Slides were immersed in 4% paraformaldehyde (Electron Microscopy Sciences Cat# 15713-S) for 15 minutes at 37ºC, followed by twice washing in PBS for 5 minutes each. Tissues were then incubated for 15 minutes with Proteinase K solution, a permeabilization reagent, followed by a 2 times washing in PBS for 5 minutes each. The tissues were then immersed in 4%

paraformaldehyde for 15 minutes at 37ºC followed by twice washing in PBS for 5 minutes each, then rinsed in deionized water. Afterwards, tissues were incubated for 10 min at 37ºC with 100 µl of terminal deoxynucleotidyl transferase (TdT) reaction buffer, then 60 min at 37ºC with 100 µl TdT preformed reaction mixture. Finally, the visualization reaction was done by incubating tissues for 30 minutes at 37ºC with 50 µl of the Click-iT Plus TUNEL reaction cocktail. The samples were blocked with 3% BSA, immunostaining protocol for troponin staining followed as described in section 4.5, and nuclei stained blue with DAPI. For positive control, DNA strand damage was induced by incubating permeabilized section with 1 unit of DNase I (DNase Max® kit - Qiagen Cat#15200-50) for 30 minutes at 37ºC. Imaging was conducted for the heart section using the high content imaging instrument, Cytation 1. Gen5 software was used for counting the TUNEL positive signal localized with cardiomyocyte nuclei.

5. Model of hypothermic cardiac arrest followed by rewarming using ECLS

Hypothermia, either accidental or therapeutic, is a common scenario in the setting of cardiac arrest. Rewarming is a key element in resuscitation after hypothermic cardiac arrest however, the rate of rewarming is a matter of controversy. As most clinical reports point to the superiority of slower rewarming rates, we hypothesized that slower rewarming carry an advantage of tissue protection at molecular levels. Accordingly, we aimed to investigate the transcriptomic differences in heart tissues from experimental hypothermic arrest models exposed to two different rates of rewarming during resuscitation; fast and slow rewarming.

5.1. Surgical Methods:

The general surgical procedures as described in section 4.1 were followed. To induce hypothermic cardiac arrest, the rats were placed in a supine position on the operating table and wrapped with a previously frozen pad of gel until a rectal temperature is 15°C. At this point, the electrocardiographic trace detects a variable rhythm between a rate of ventricular fibrillation, marked bradycardia (20 bpm) or asystole. The average blood pressure detected in these phases varied between 40mmHg and 20 mmHg. At this point the ventilator was turned off, and the rats were left in a state of hypothermic cardiac arrest for 60 minutes.

During the cardiac arrest phase, the rats were connected to the Cardio-pulmonary bypass (CPB) circuit. The thoracic and ventral surfaces were shaved and the skin was disinfected with chlorhexidine. The right external jugular vein was then isolated and a 24 G cannula with is inserted and advanced along its entire length sufficient to reach the level of the right atrium of the rat. (the position of the cannula is modified to obtain the best possible venous drainage during CPB). The right common carotid artery was cannulated with a 24 G catheter, advanced to the aortic arch and then connected to the arterial perfusion line of the circuit. At the time of insertion of the venous catheter, rocuronium bromide 1-2 mg/kg for muscle relaxation and 500 IU/kg of heparin for anticoagulation were administered.

The CPB circuit consists of a roller pump (Stöckert SIII, Sorin, Germany), a hollow

fiber oxygenator with standard industrial characteristics (Sorin, Mirandola, MO, Italy), a regulator vacuum with a pressure of -30/-40 cmH2O to facilitate venous drainage. All these parts were connected by plastic pipe of 1.6 mm internal diameter. The total filling volume of the CPB circuit including the oxygenator, was 6 ml and consisted of 50 % colloid solution and 50 % Lactate Ringer. The gas exchange surface was 450 cm^2. The heat exchange surface 15.8 cm2 (Figure 3). Once the venous and arterial accesses were prepared, the rats were connected to the CPB circuit and maintained with a flow rate of 80-100ml/kg/min and an average blood pressure range of 70-90 mmHg. To apply different rates of rewarming a heat exchanger, which is capable of reaching a maximum temperature of 45 °C was used. The thermal gradient is adjusted according to the predetermined rate of heating until 35°C reached. If necessary, topical heating is also provided with a warm infrared lamp.

Finally, rats were randomized to receive either fast (45 minutes), or slow (90 minutes) assisted rewarming to a target core temperature of 35ºC. A control group consists of normal hearts that not exposed to cardiac arrest and isolated from rats at the same age and weight.

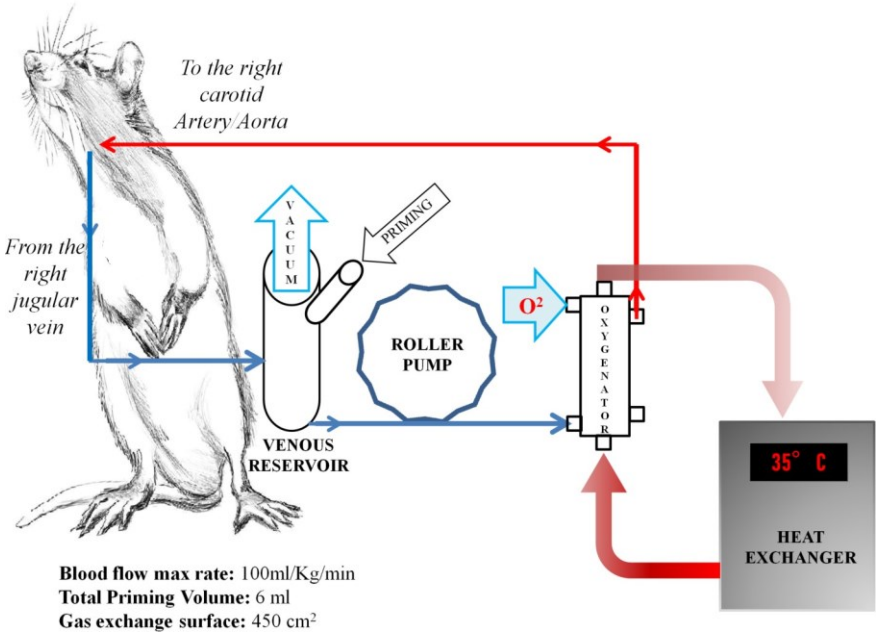

Figure 3: Schematic presentation of the CPB circuit used for rewarming after

hypothermic cardiac arrest.

5.2. Results:

5.2.1 Bulk RNA sequencing to characterize the general transcriptional changes accompanied different rates of rewarming:

Rats were subjected to hypothermic cardiac arrest then randomly subjected to either fast (45 minutes) or slow (90 minutes) rewarming. RNA from the hearts was isolated and sequenced to investigate the transcriptional changes associated with different rate of rewarming. Our data reviled that around 100 genes was differentially expressed between the fast and slow rewarming groups (Figure 4). The data was presented as fragments per kilo base of exon per million mapped fragments (FPKM) which is a gene expression unit used for normalizing counts.

The gene ontology (GO) analysis of the differentially expressed genes between fast rewarming and slow rewarming models show that fast rewarming is associated with a significant upregulation of genes involved in the signal transduction, response to stress, ubiquitination, and viral response (figure 5). Fast rewarming was associated with repression of pathways related mainly to metabolism (figure 6). This data indicates that the rate of rewarming results in many transcriptomic changes that may impact the final outcome of resuscitation.

5.2.2 Pathway analysis using Pathview software to highlight the main molecular pathways that been affected by the profound transcriptional changes

To further illustrate the key transcriptomic changes and to obtain a better visualization of the difference between the slow rewarming and fast rewarming post hypothermic cardiac arrest, Pathview analysis software was used and KEGG graphs were generated. Metabolic, signaling, inflammatory, apoptotic, phagocytic, autonomic pathways, ETC were analyzed. The fold change of the genes changed in the fast rewarming compared to slow rewarming was presented on the KEGG graph. The Pathview software analysis shows that fast rewarming was associated with metabolic dysregulation. A reduction in the fatty acids oxidation pathway was observed as indicated by downregulation of the long chain fatty acids ligase enzyme (figure 7). An increase in fatty acids elongation was evidenced by

upregulation of the key enzyme very-long-chain 3-oxoacyle-COA synthase (figure 8). Enhanced catabolism of branched chain amino acids was evidenced by upregulation of methylmalonyl-COA mutase and methylmalonate-semialdhyde dehydrogenase (figure 9). Finally, inhibition of oxidative phosphorylation was evidenced by downregulation of complex I and complex IV subunits (figure 10).

Fast rewarming was also found to be associated with activation of pro-inflammatory and pro-apoptotic signaling pathways compared to slow rewarming. Fast rewarming is associated with derangement of MAPK signaling pathway that leads to inhibition of differentiation signaling and activation of inflammation/apoptosis signaling (figure 11). Fast rewarming is associated with potentiation of cytokine-cytokine receptor interactions (figure 12), activation of NF-kappa B pathway (figure 13), activation of autophagy and phagosome formation (figures 14,15), apoptotic signaling pathways (figure 16).

Finally, Pathview software analysis shows that fast rewarming is associated with downregulation of Na^+/K^+ pump, dihydropyridine receptors (DHPR), and the protein phosphatase 2 (PP2A) (figures 17,18).

This data reflects the unfavorable transcriptomic effects of fast rewarming on the cellular response to stress which may impact the final outcome of resuscitation.

5.2.3 RT-PCR confirms the effects of fast rewarming on metabolic, inflammatory, and apoptotic gene expression

To confirm our findings from the bulk-RNA data, we conducted RT-PCR analysis of the key differentially expressed targets genes for samples from negative control (hearts that did not subjected to hypothermic cardiac arrest), fast rewarming and slow rewarming post hypothermic cardiac arrest groups. ATP synthase is a part of complex V of the electron transport chain involved in oxidative production of ATP. ATP synthase gene expression was significantly decreased (0.4-fold change) in fast rewarming compared to negative control. While there is no significant difference in the slow rewarming compared to negative control. Similarly, fast rewarming was associated with a decreased expression of the calcium handling proteins including DHPR (0.5-fold change), and CAMKII (0.6-fold change) compared to negative controls. Fast rewarming showed an increased expression of the genes involved in

autophagosome protein ATG-9 (4-folds change), apoptotic signaling protein (Fas-L) (4-folds change) and necrotic factor TNF-α (5-folds change) compared to negative controls (figure 16). This data confirms our sequencing findings and provides a stronger evidence on affection of the metabolic, calcium handling, autophagic and apoptotic pathways with fast rewarming.

5.2.4 TUNEL assay shows increased apoptotic markers in tissues exposed to fast vs slow rewarming:

Finally, to identify the effect of these transcriptomic changes on tissue viability, we conducted TUNEL assay for assessment of the apoptotic nuclei. Tissues exposed to fast rewarming protocol has showed significantly higher percentage of TUNEL positive nuclei compared to slow rewarming (50%±7 vs 18%±3) (figure 20). This clearly indicates that the transcriptomic changes associated with fast rewarming lead to more tissue injury and apoptosis compared to slow rewarming.

gene	Fast rewarming			Slow rewarming	
H3f3b	405.8555	413.4695	406.0195	79.45659	192.1508
RGD156564	30.60941	38.77405	31.2138	7.159305	11.66254
Tspan12	25.33226	22.63816	22.15874	10.79175	12.30587
OC1003602	21.26971	21.43707	21.3928	11.34289	9.20857
Chmp2b	18.03205	15.82754	14.61566	7.622708	8.370844
Tob1	18.12133	14.89813	18.19091	4.726977	4.586748
Ccl6	13.7957	11.9074	10.89625	3.143454	2.376211
Fosl2	10.399	7.779583	9.493885	2.123872	0.331194
Mapk6	8.13918	6.805616	8.076909	2.675885	3.188609
B3gnt2	7.515351	7.588461	9.080263	2.656939	1.049798
Lnx1	8.211464	7.451836	7.139404	3.282385	4.138801
Lima1	5.881717	6.03527	6.207022	2.032361	2.362784
Arhgap29	6.354652	6.4991	6.858145	1.89842	1.537118
Spcs3	5.932269	6.030236	6.52725	1.294835	2.596052
OC1009118	9.834249	9.505618	6.606763	0	0.623644
Kazald1	5.342342	6.185296	5.301469	1.94841	2.696519
Rai14	3.117054	2.765546	2.914126	1.474281	1.018865
RGD131186	4.152871	3.745183	4.488046	0.619671	1.404167
Cyth3	4.01478	4.182202	4.513964	0.053857	1.228264
Fbxo30	4.536896	4.212525	3.628091	0.985752	0.710942
Sulf1	2.87031	3.43137	2.93423	1.590766	1.263281
Erlin1	2.942429	2.762361	2.686814	1.34668	1.259999
Fam204a	1.792463	1.751455	1.887652	0	0.5534
Slc31a1	2.677302	2.507048	2.575487	0.446373	0.435043
Pdcl	2.620374	2.223048	2.359059	0	0.279619
Rps15al2	1.771799	1.570961	1.465203	0.536102	0.313497
Sh3rf1	2.442587	2.047795	2.014959	0.734531	0.417601
Slc25a24	1.79013	2.02515	1.819762	0.653942	0.365024
Nmnat3	2.08	2.111804	2.137803	0.960892	1.035082
Pdgfrl	2.26779	1.867524	2.302776	0	0.671871
Endod1	1.565728	2.073397	1.928792	0.441078	0.171953
Ddx10	1.314067	1.057801	0.97229	0.219728	0.091779
Ift22	0.931561	1.296006	1.459632	0	0.078076
Tlr3	1.436276	1.648416	1.330841	0.353424	0.647574
Kif16b	1.640833	1.577774	1.478478	0.375335	0.606197
Mertk	1.232036	1.062858	1.016088	0.022669	0.344665
Kit	1.102326	1.136197	1.247037	0.380231	0.14043
Hspa1a	1.691518	2.017367	2.06173	0.100137	0.175672
Mipol1	1.848711	1.820999	1.330061	0	0.183997
Dclre1b	1.126632	1.191606	0.992718	0	0.355724
Trim59	0.641947	0.528967	0.623187	0.114009	0
Ipo11	1.003072	0.947883	0.785841	0.143765	0.252209
Klrb1b	1.388595	0.972363	1.13439	0	0.145183
Ube3d	0.794596	0.792592	1.037522	0	0.166492
Lrat	0.529619	0.675029	0.64966	0	0.117137
Ace2	0.617998	0.616439	0.743	0	0.094627
Psd3	0.791877	0.672466	0.722104	0	0.153366
Triqk	1.010693	0.856923	0.827442	0	0.080473
Adcy7	0.761794	0.732073	0.626899	0	0.177528
Spice1	0.428322	0.34854	0.469787	0	0.053848
Ralgapa2	0.427756	0.488614	0.383404	0.09394	0.1648
Slc16a7	0.536624	0.393582	0.516856	0	0.0754

gene	Fast rewarming			Slow rewarming	
Puf60	32.197	24.49	27.758	60.8509	52.38734
Coro6	18.911	16.48	16.529	46.3893	45.02715
Eif3b	17.838	14.54	17.938	38.904	38.3246
Hdlbp	21.763	18.95	17.553	39.5402	40.94926
Tmem120a	16.419	14.47	19.083	38.0052	32.68282
Fam213b	8.9102	11.22	11.687	27.7474	30.97668
Sugp1	5.2548	8.03	8.5616	18.1663	22.43316
Obscn	7.5963	5.279	6.1071	15.1709	19.08679
Pus1	7.7195	6.532	6.3263	15.2938	13.91573
Tacc2	4.4628	4.444	5.9689	10.5075	12.66005
Podxl2	4.3998	4.779	3.5891	10.2552	12.37981
Cbx6	4.9475	4.583	3.9077	8.86746	10.71254
Sppl2b	4.8304	4.777	4.1943	11.0239	9.025077
Sh2b1	5.1596	4.607	5.4323	12.3712	10.34051
Upf1	5.1659	3.746	4.1504	12.7149	12.78237
Klhl22	4.2137	4.203	4.4377	8.06922	9.177552
LOC690617	3.1333	2.765	4.0661	10.1734	9.211537
Smg5	4.3598	3.84	4.2566	8.59316	10.19004
Taf6l	2.3794	2.278	2.7801	7.68134	8.219732
RGD1304963	2.0402	3.205	3.5152	8.53077	9.746853
Gbf1	3.1605	2.93	3.0139	6.19106	6.950707
Dgcr14	2.2861	1.766	3.3139	6.22543	7.138141
Gcgr	1.5249	2.582	2.0707	4.91751	4.920504
Zfp775	1.9397	2.384	2.1233	4.7104	4.49
AC142126.2	0	0.941	1.4045	8.99279	6.761212
Ubr4	2.3836	2.011	2.1951	5.02297	4.55414
Zfp865	1.922	1.602	2.4023	5.86349	5.249744
Megf8	1.1939	2.006	1.9284	4.42615	3.817846
Slc25a23	0	1.178	1.9671	6.48858	6.249494
Slc39a5	1.45	1.119	1.2624	5.99731	4.835813
Ptcd1	1.6525	1.873	1.8511	4.97742	4.027067
Dhx37	0.9687	1.03	1.1336	2.31443	2.532194
Cdan1	0.8339	0.811	1.0965	2.89547	2.705781
Ccdc136	0.9681	0.553	0.6004	2.63635	2.071605
Lime1	0.8196	0.879	1.22	1.95288	2.088209
Pak6	0.4456	0.519	0.8516	2.51675	2.260147
Reps1	0.4626	0.448	0.6751	1.40805	1.321749
AABR07024058.4	0.0308	0.061	0.0611	2.59902	3.824089
Frmpd1	0	0.07	0.244	1.02833	1.244629
AABR07040944.1	0	0.085	0.2542	1.16274	1.087897

Figure 4: Heat map of differentially expressed genes after fast vs slow rewarming.

Samples from fast rewarming group or slow rewarming group are compared for FPKMs of sequenced genes. Heat map is generated so red color reflects higher FPKMs, while yellow is lower FPKMs. (A) Heat map of the Up-regulated genes in fast rewarming, (B) Heat map of the down-regulated genes. The list includes metabolic, signaling, and apoptotic genes.

Figure 5: Go terms for up regulated genes in fast rewarming compared to slow rewarming.

The GO analysis shows significant up regulation of intracellular signaling, response to stimulus and stress, ubiquitination, and MAPK signaling pathways after fast rewarming compared to slow rewarming.

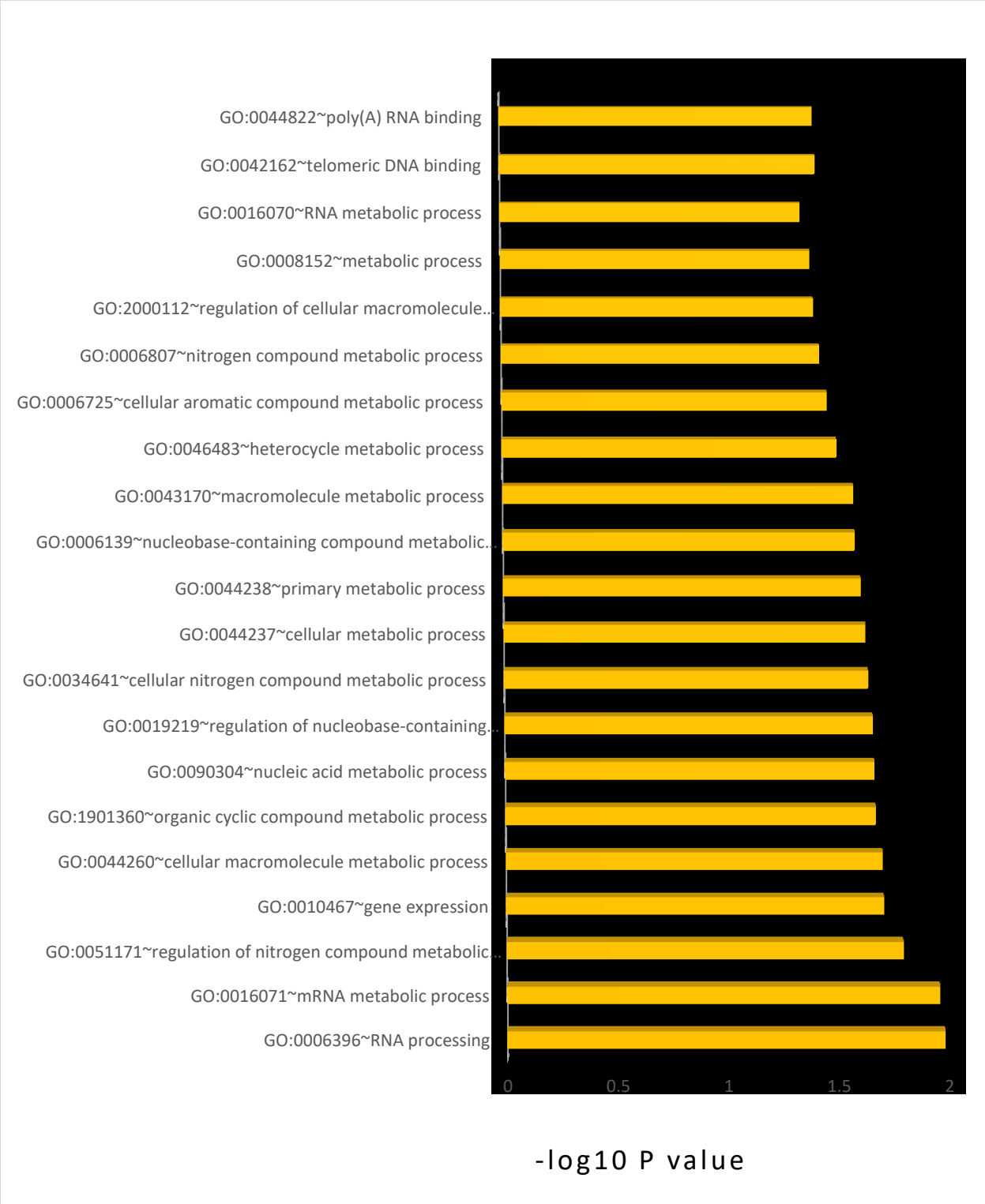

Figure 6: Go terms for down regulated genes in fast rewarming compared to slow rewarming.
The GO analysis shows significant down regulation of many metabolic and biosynthetic pathways after fast rewarming compared to slow rewarming.

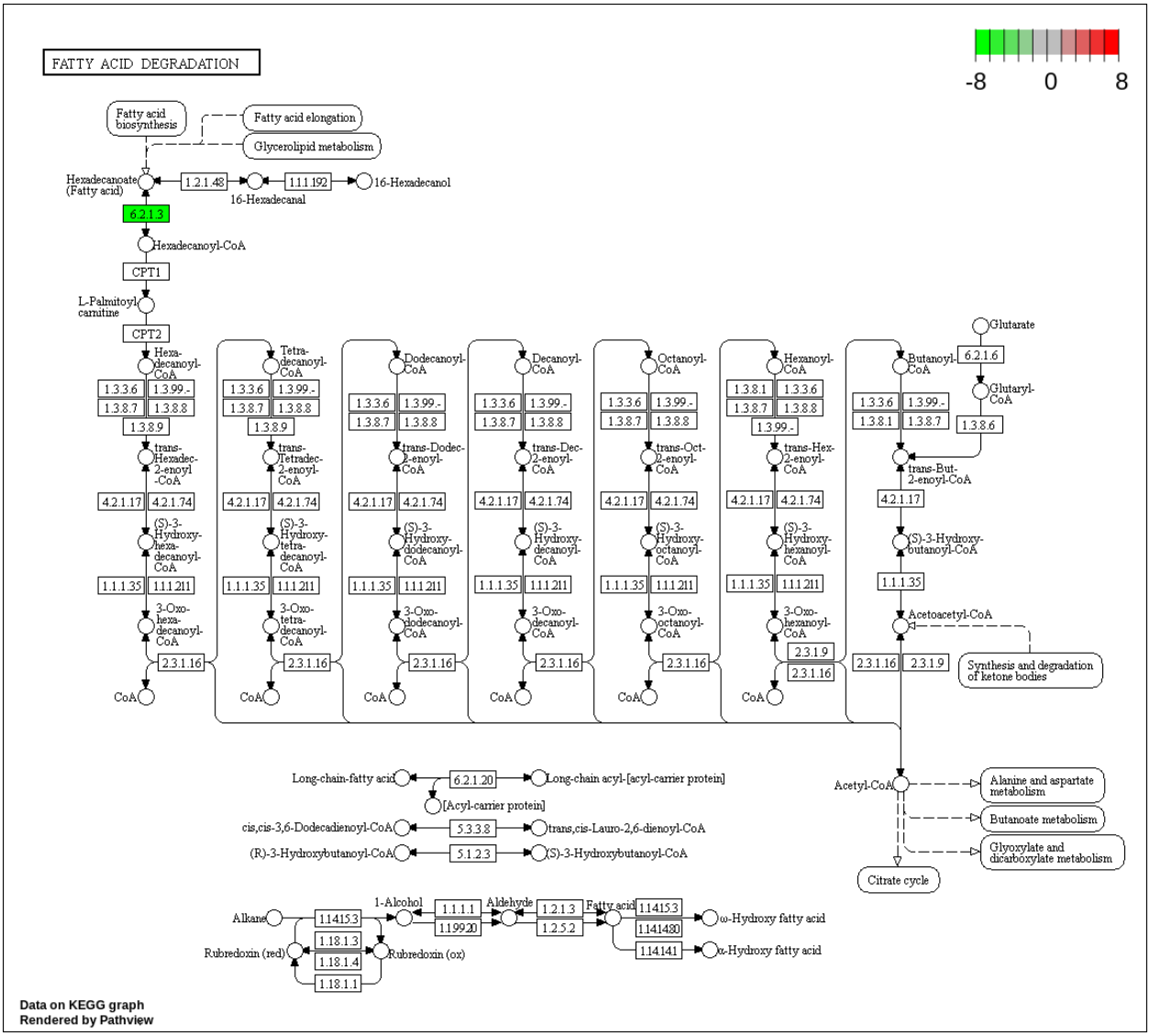

Figure 7: Differentially expressed genes related to fatty acids degradation after fast compared to slow rewarming presented on KEGG graph rendered by Pathview.
The KEGG graph of the fatty acid degradation shows 8 folds down-regulation of the long chain fatty acids ligase which is the rate limiting step of fatty acids oxidation after fast rewarming compared to slow rewarming.

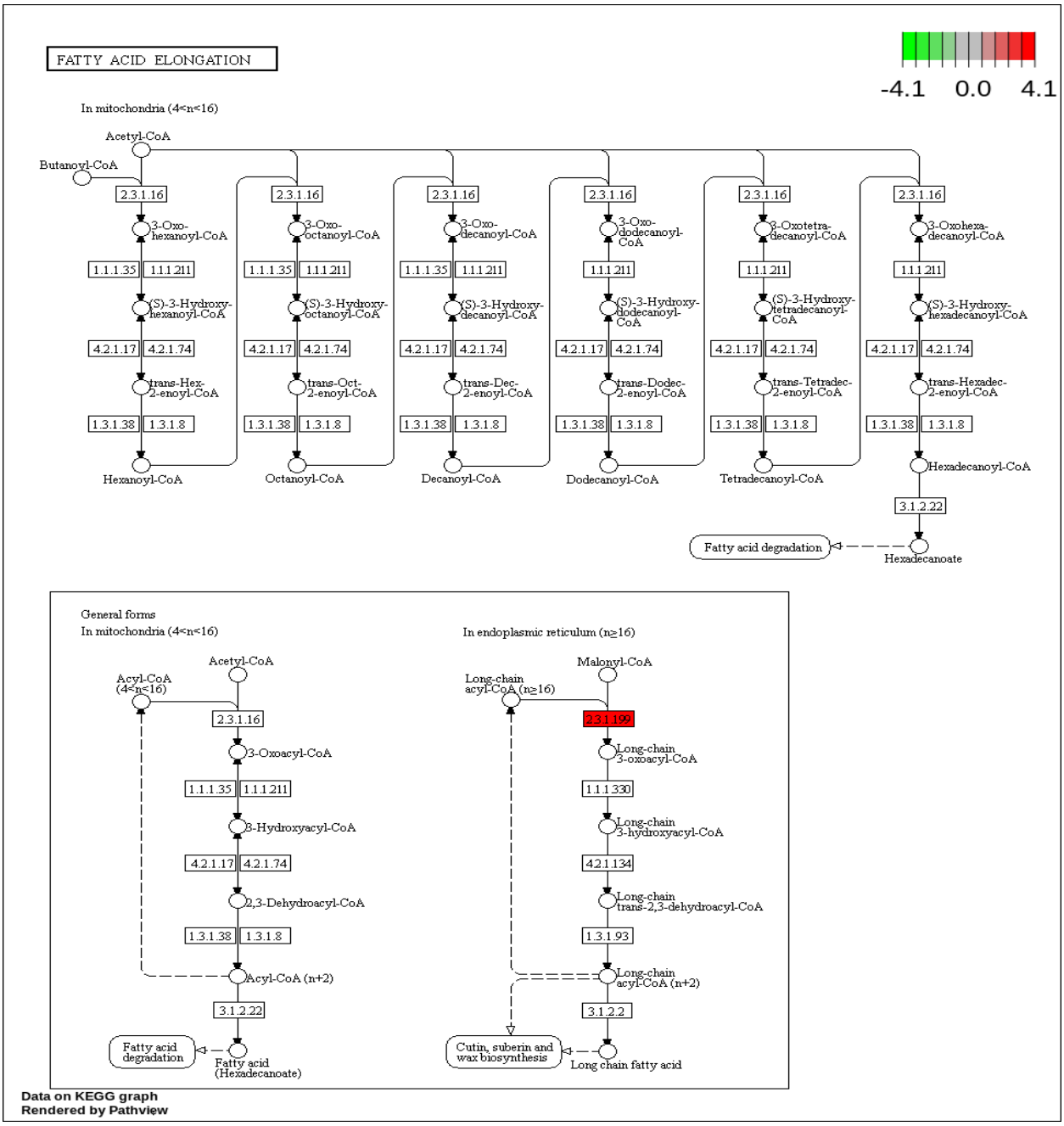

Figure 8: Differentially expressed genes related to fatty acids elongation after fast compared to slow rewarming presented on KEGG graph rendered by Pathview.
The KEGG graph shows 4 folds up-regulation of the very-long-chain 3-oxoacyle-COA synthase which is the rate limiting step of endoplasmic fatty acids elongation after fast rewarming compared to slow rewarming.

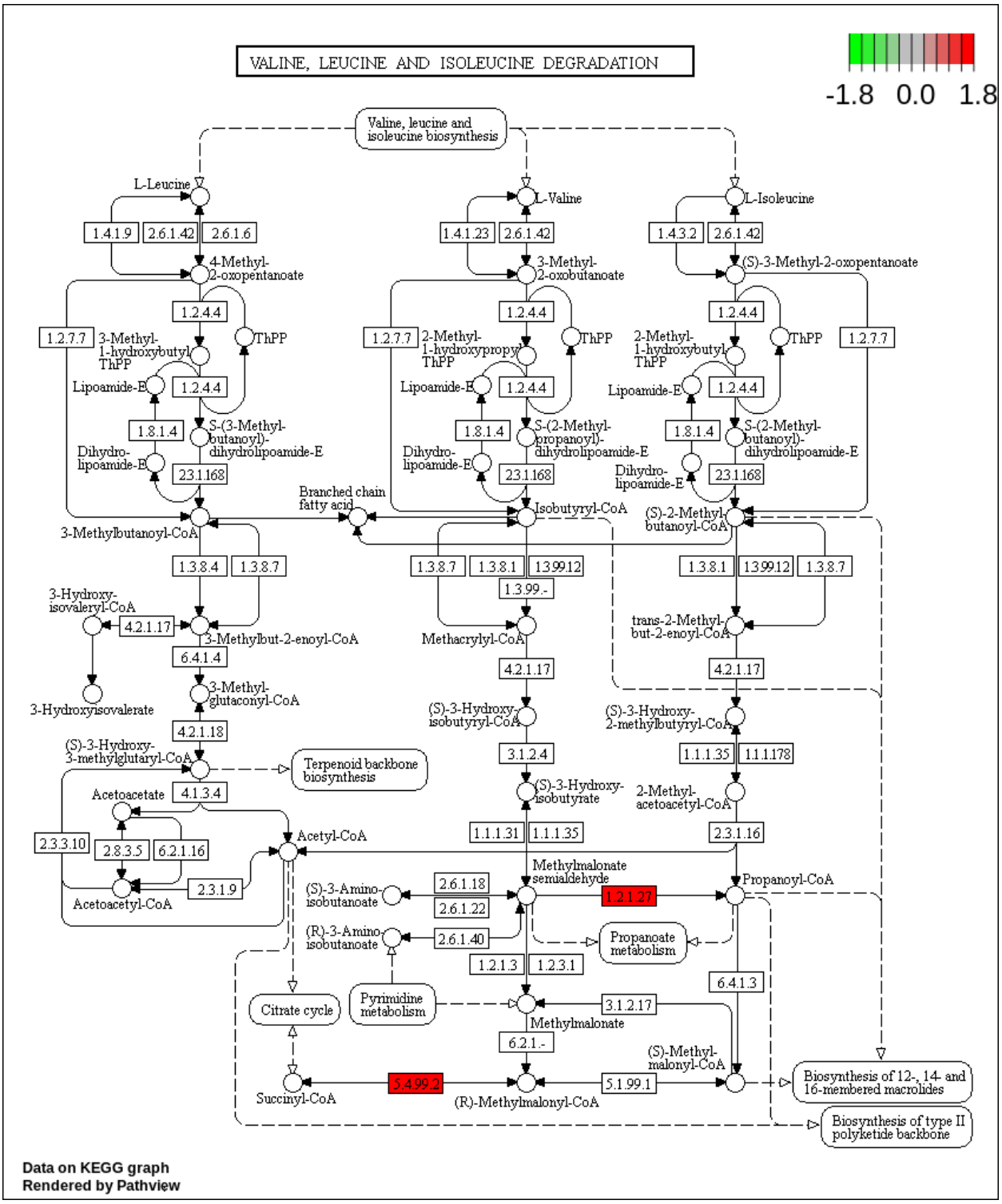

Figure 9: Differentially expressed genes related to branched chain amino acids degradation after fast compared to slow rewarming presented on KEGG graph rendered by Pathview.
The KEGG graph shows 1.8 folds up-regulation of methylmalonyl-COA mutase and methylmalonate-semialdhyde dehydrogenase which are the rate limiting enzymes for branched chain amino acids metabolism, after fast rewarming compared to slow rewarming.

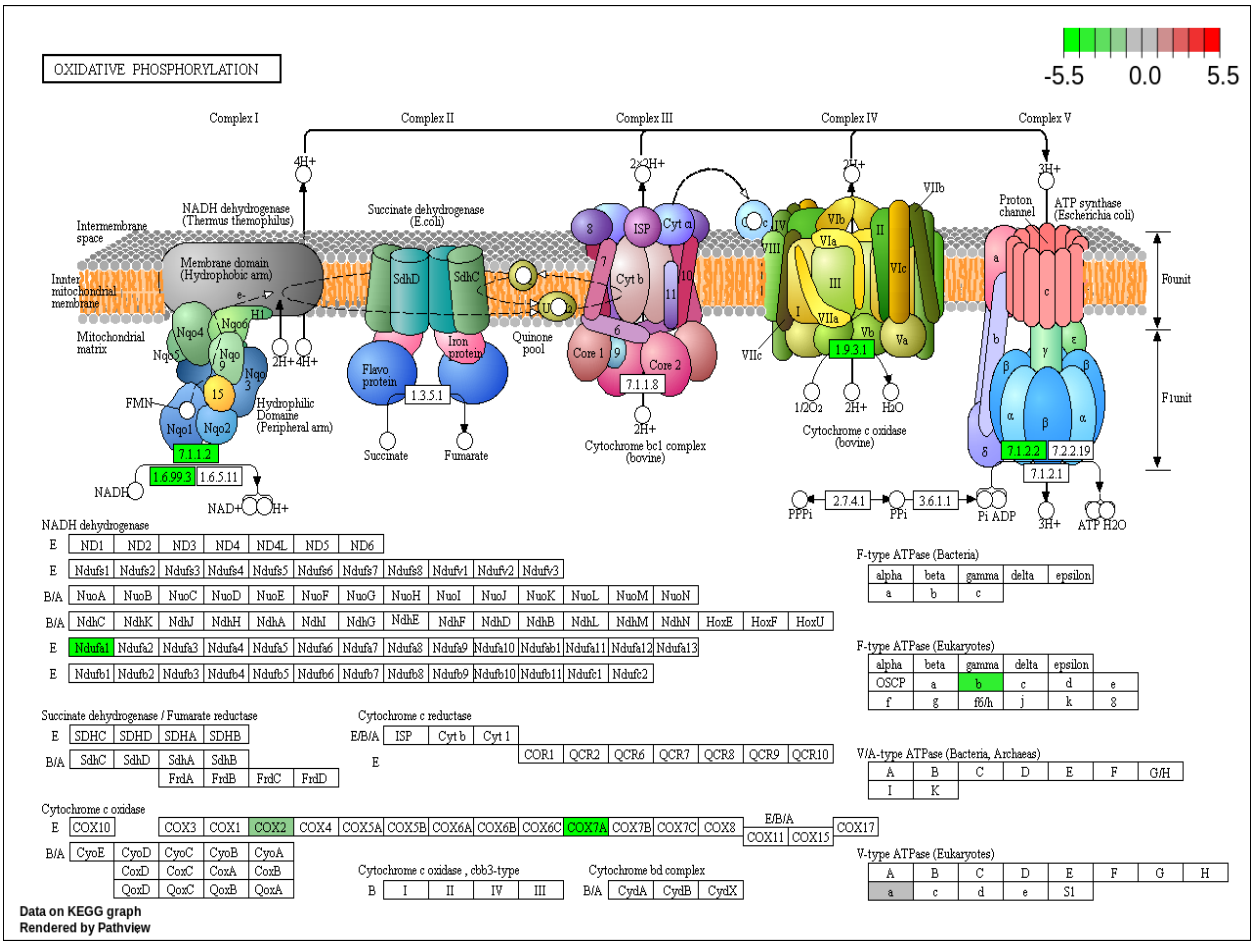

Figure 10: Differentially expressed genes related to electron transport chain after fast compared to slow rewarming presented on KEGG graph rendered by Pathview.
The KEGG graph shows 5 folds down-regulation of subunits of complex I, IV and V of respiratory chain after fast rewarming compared to slow rewarming.

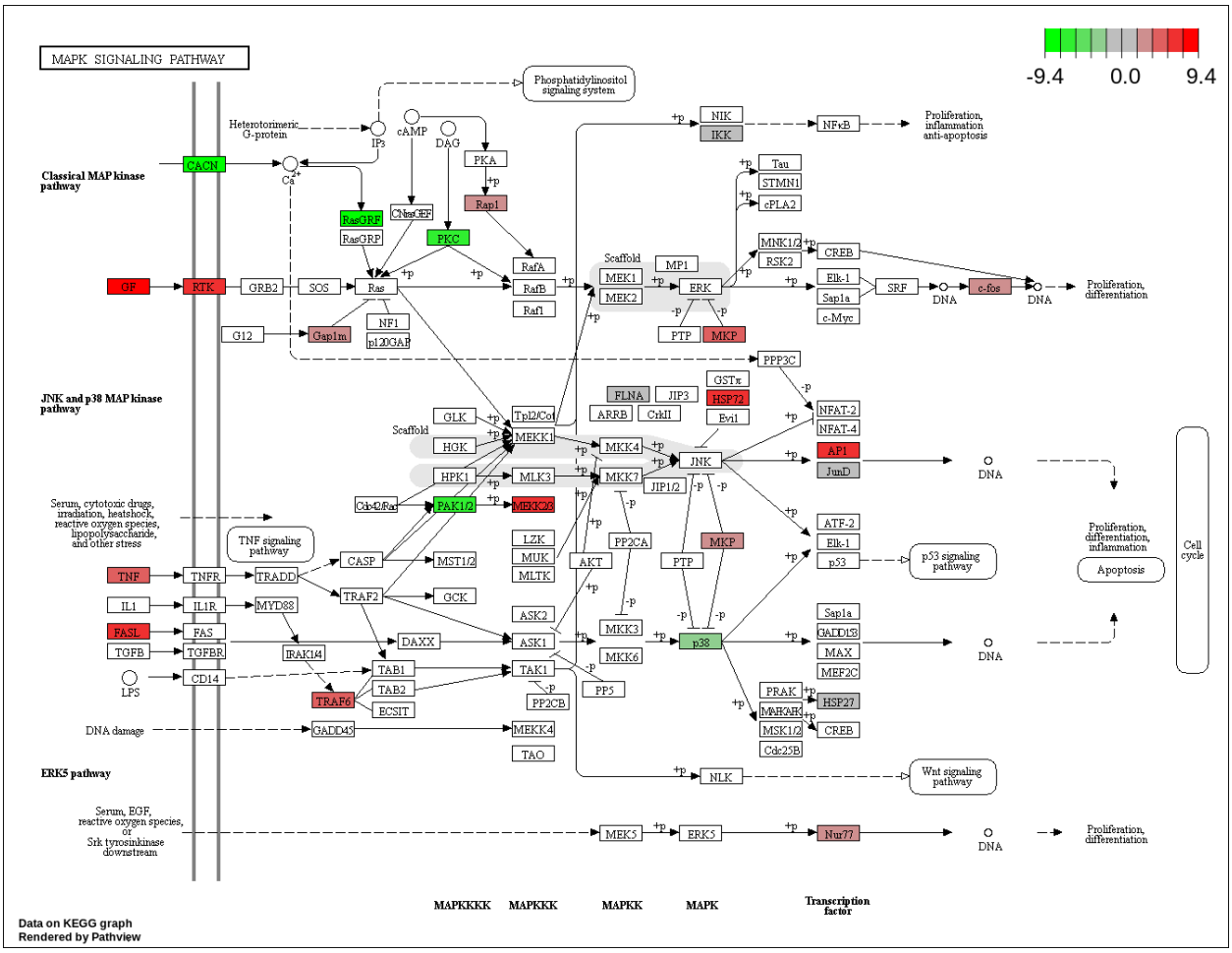

Figure 11: Differentially expressed genes related to MAPK signaling pathway after fast compared to slow rewarming presented on KEGG graph rendered by Pathview.
The KEGG graph shows up to 9 folds up-regulation of FAS-L, AP-1, TNF, and TRAF6 among many other targets after fast rewarming compared to slow rewarming which favors apoptosis.

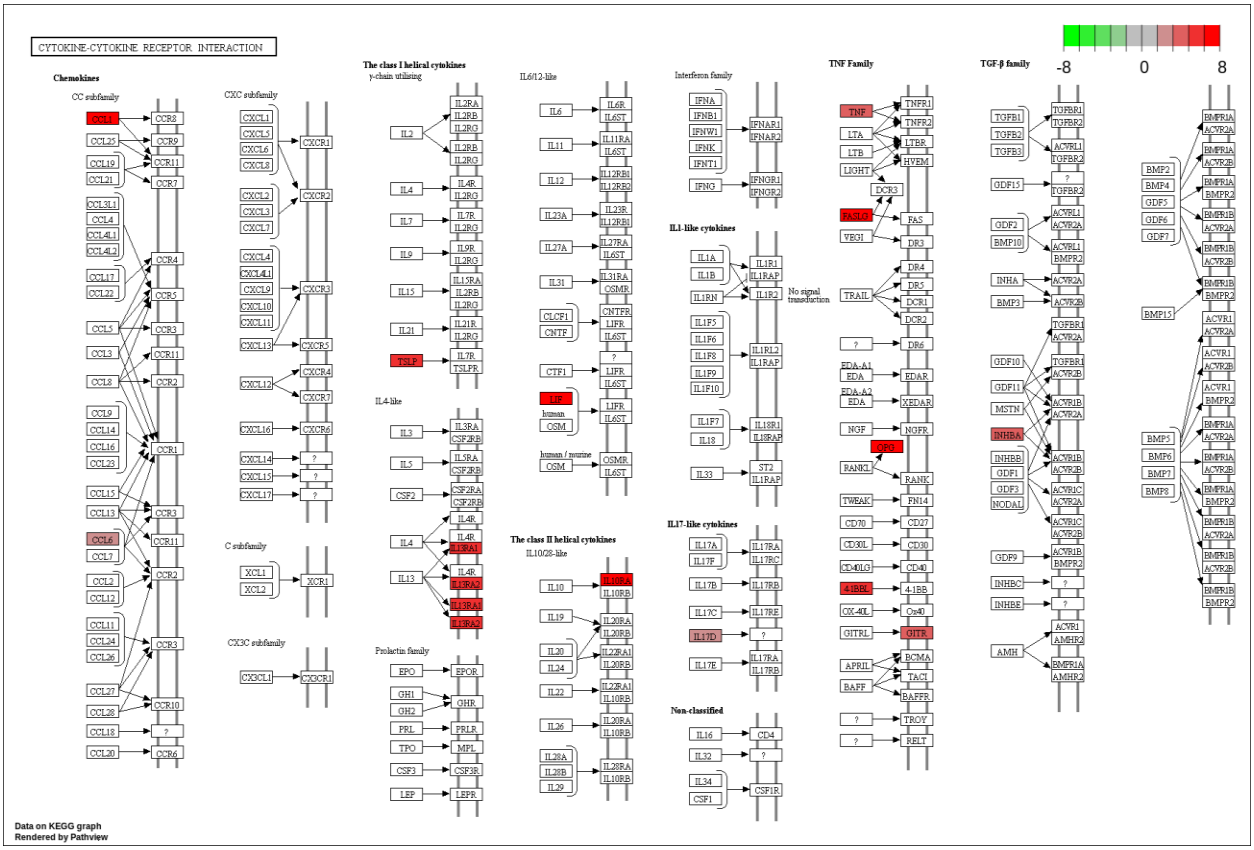

Figure 12: Differentially expressed genes related to cytokines signaling after fast compared to slow rewarming presented on KEGG graph rendered by Pathview.
The KEGG graph shows up to 8 folds up-regulation of CCL1, ILRs, TNF, and FASLG among many other targets after fast rewarming compared to slow rewarming which favors inflammation.

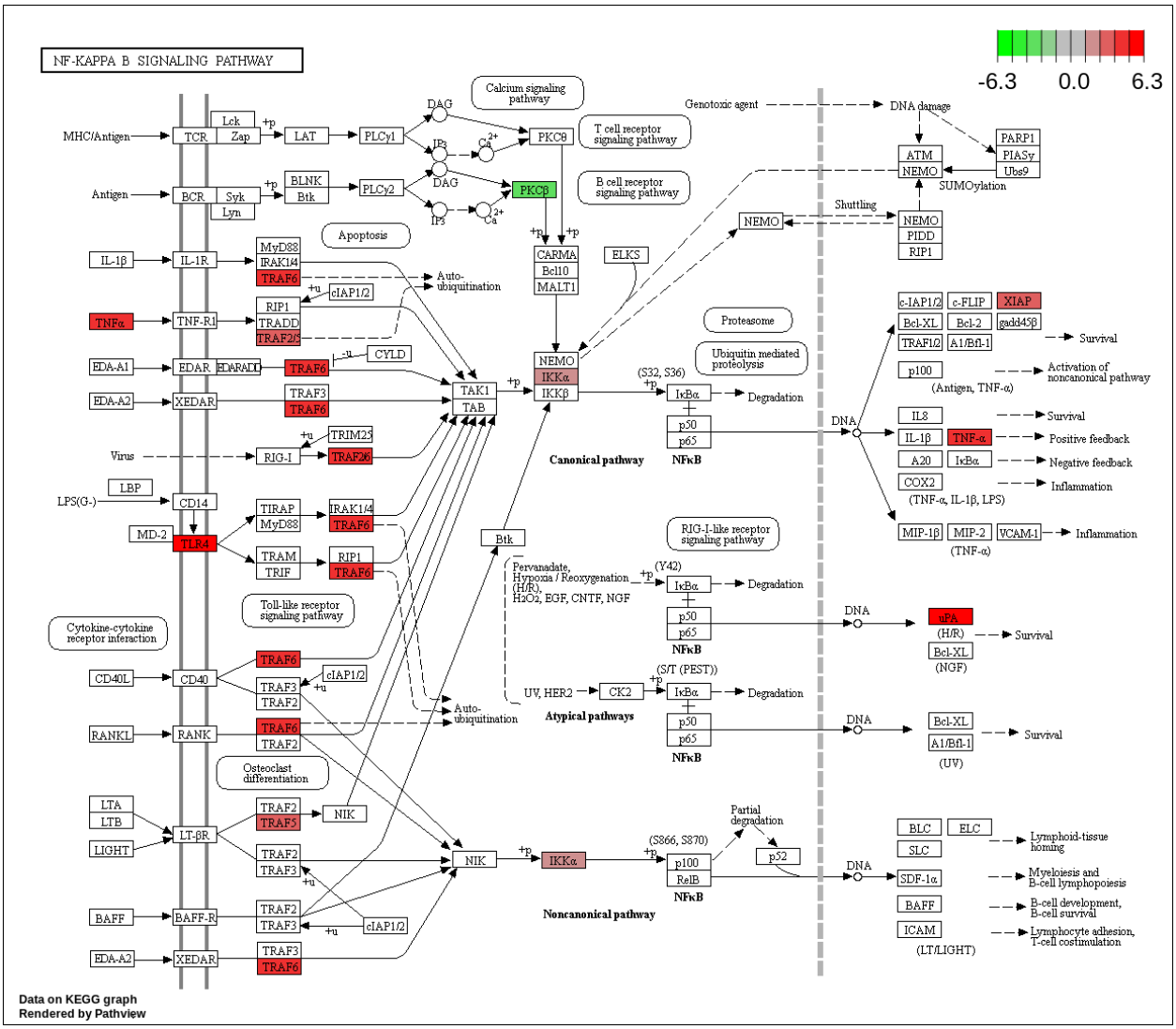

Figure 13: Differentially expressed genes related to NF-κB signaling after fast compared to slow rewarming presented on KEGG graph rendered by Pathview.
The KEGG graph shows up to 6 folds up-regulation of TNFα, and TRAFs 6 among other targets and down-regulation of PKCβ after fast rewarming compared to slow rewarming which favors inflammatory response.

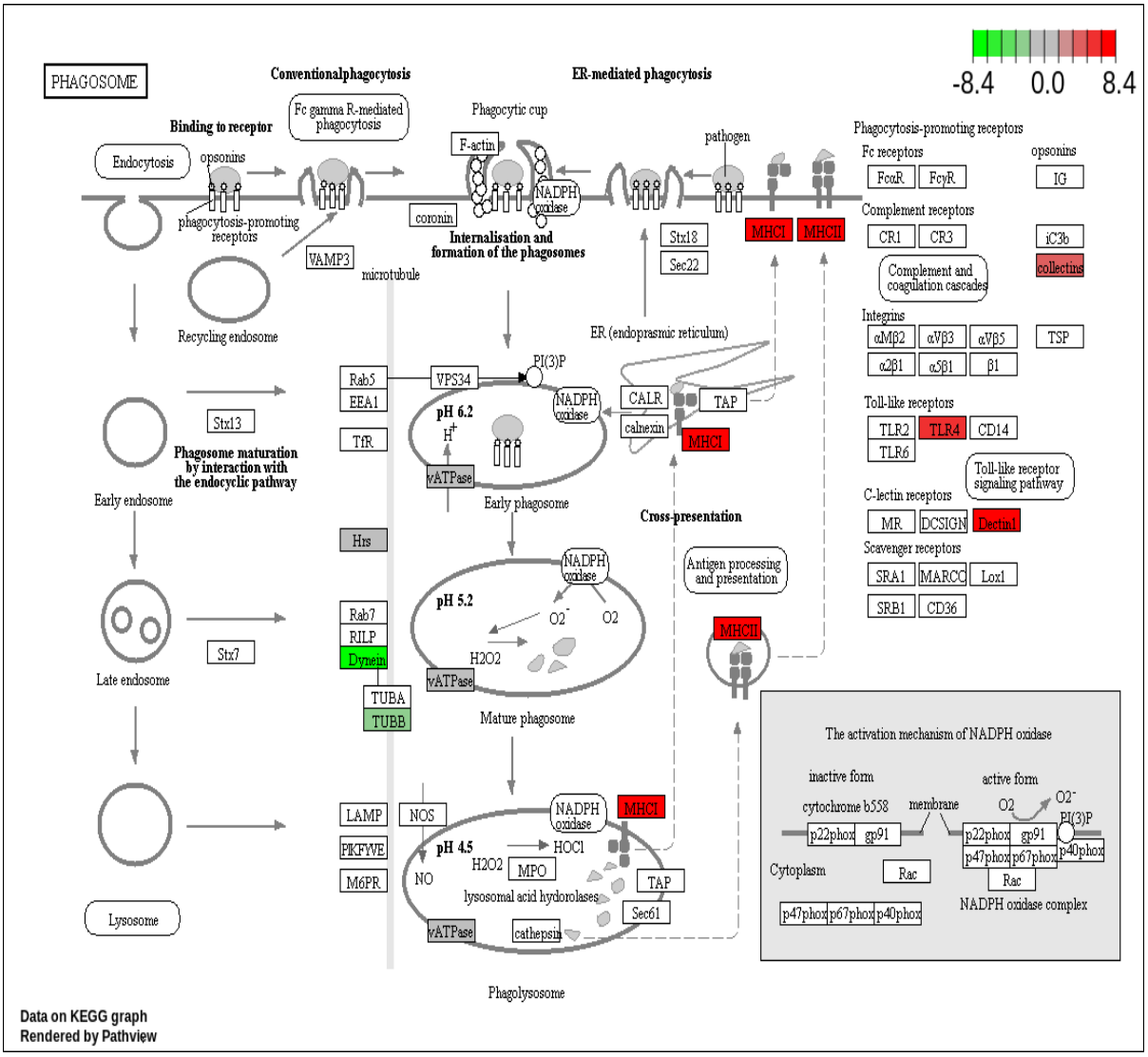

Figure 14: Differentially expressed genes related to phagocytosis after fast compared to slow rewarming presented on KEGG graph rendered by Pathview.
The KEGG graph shows up to 8.4 folds up-regulation of phagocytic receptors including MHCI, MHCII, TLRs, and Dectin1 after fast rewarming compared to slow rewarming which favors phagocytosis activation.

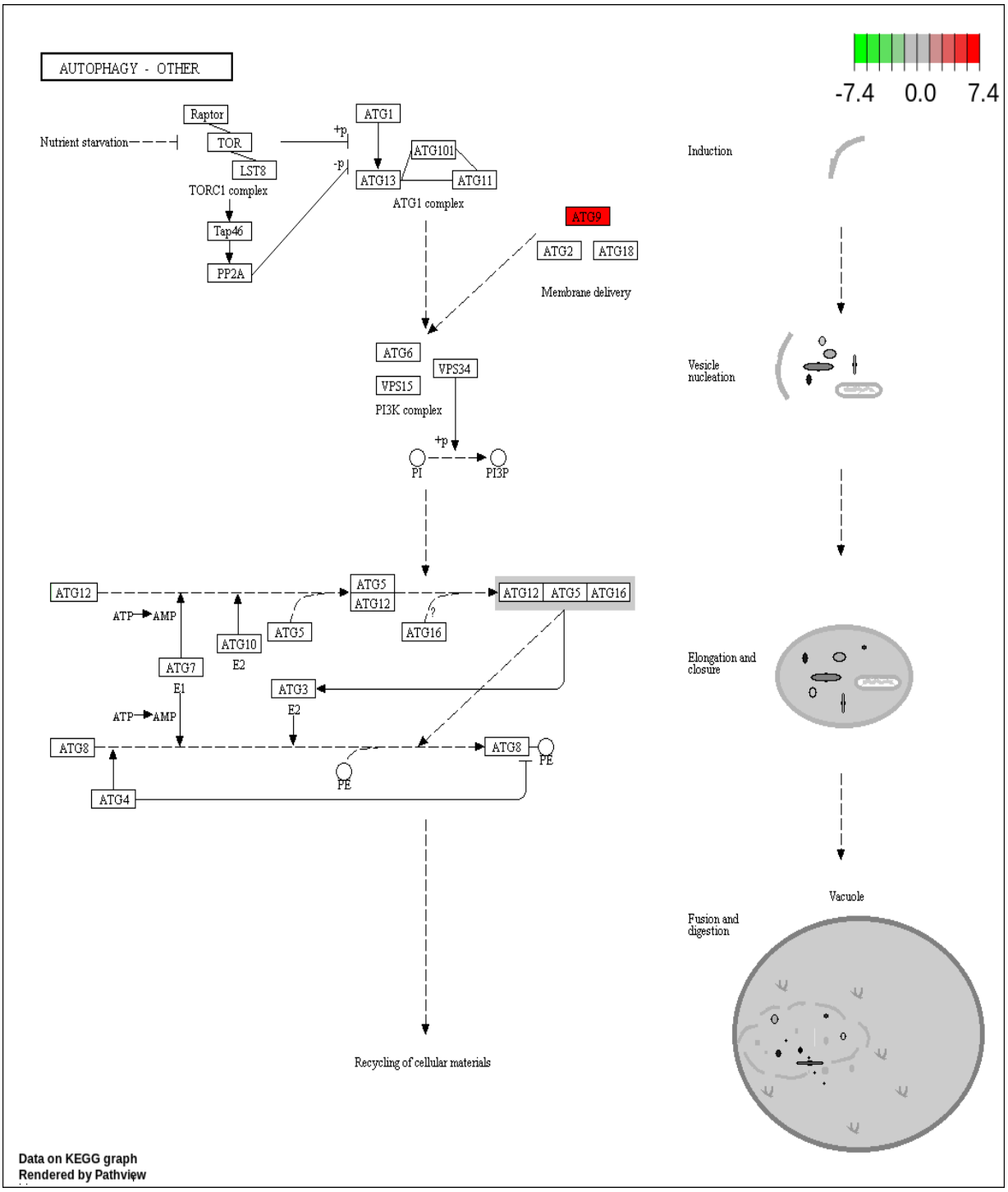

Figure 15: Differentially expressed genes related to autophagy after fast compared to slow rewarming presented on KEGG graph rendered by Pathview.
The KEGG graph shows 7.4 folds up-regulation of ATG9 after fast rewarming compared to slow rewarming which is key for induction of autophagy.

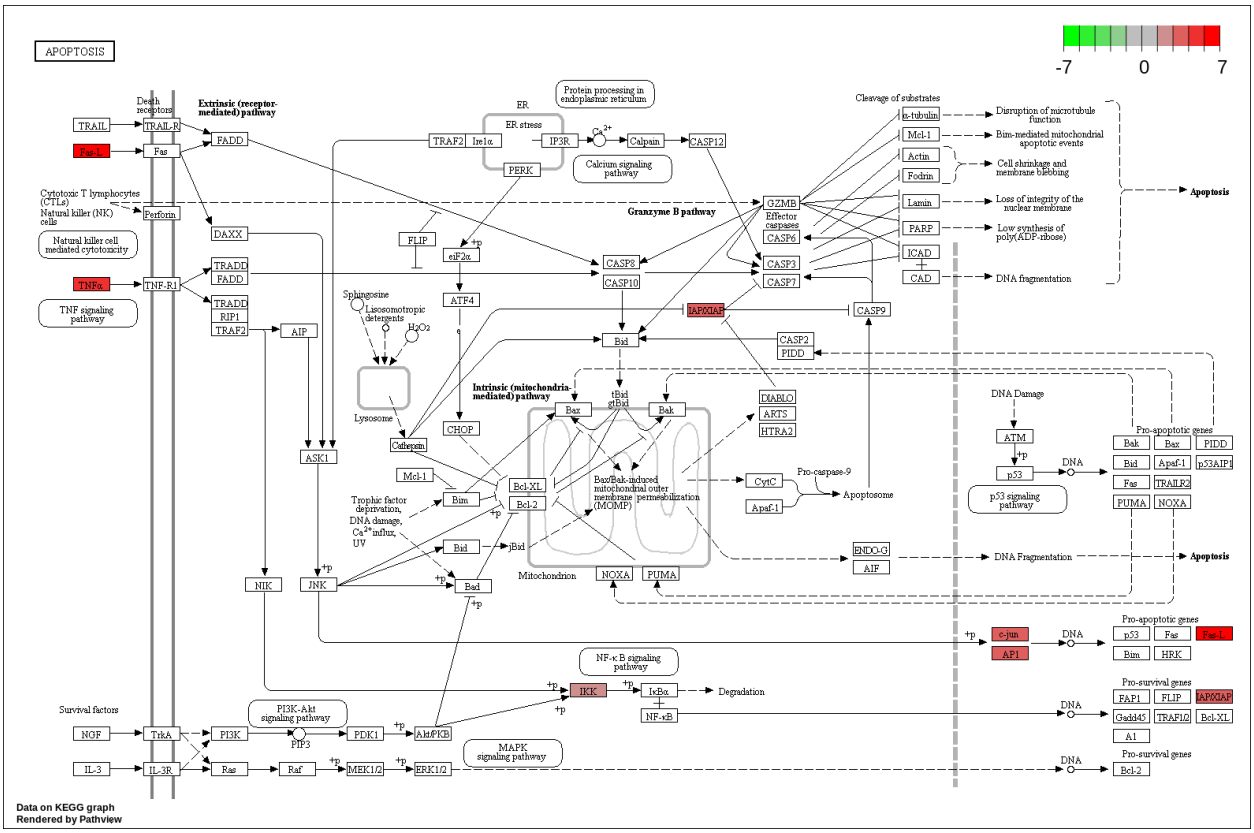

Figure 16: Differentially expressed genes related to apoptosis activation after fast compared to slow rewarming presented on KEGG graph rendered by Pathview.

The KEGG graph shows up to 7 folds up-regulation of FAS-L, and TNFα among many other targets after fast rewarming compared to slow rewarming which favors apoptosis.

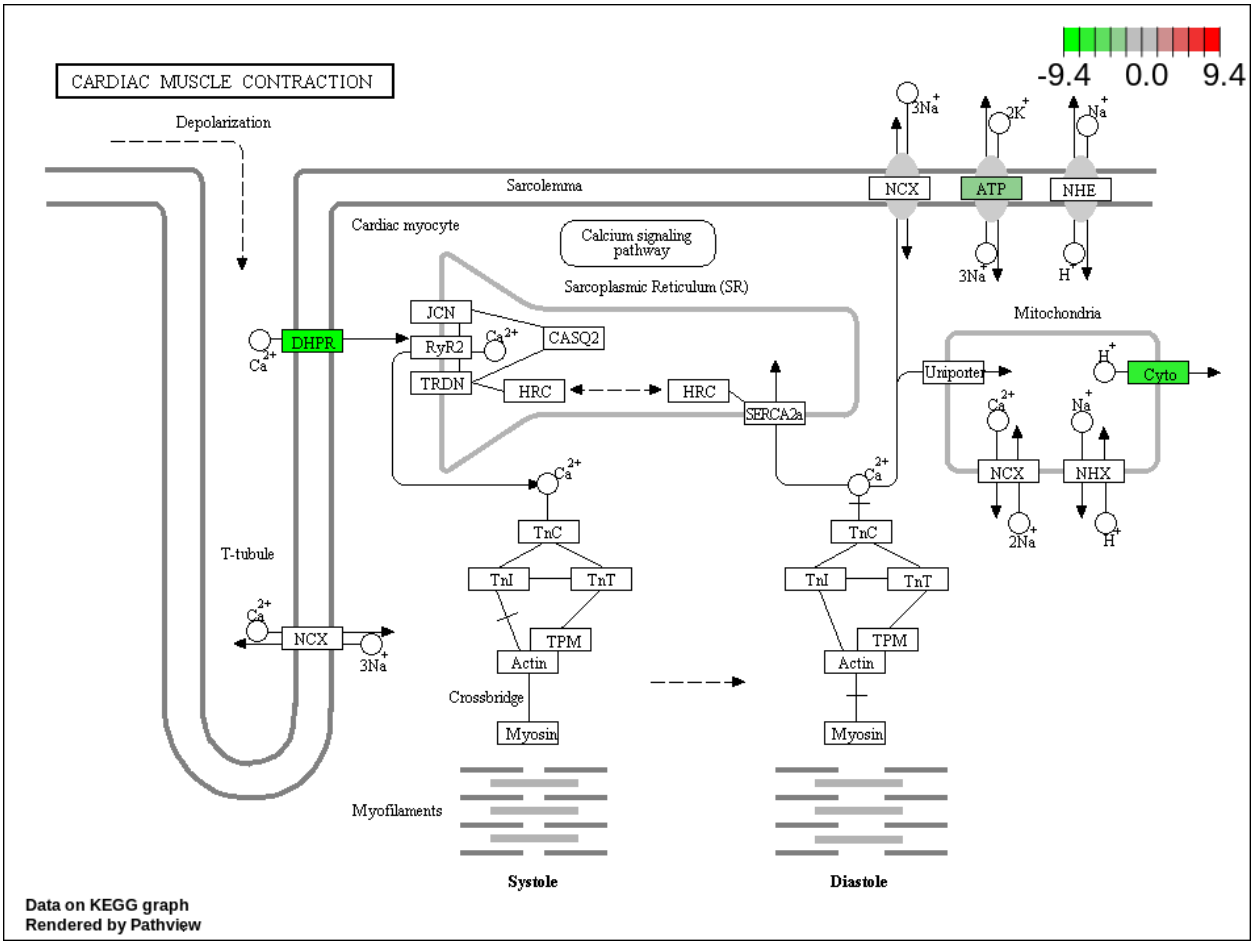

Figure 17: Differentially expressed genes related to cardiac muscle contraction after fast compared to slow rewarming presented on KEGG graph rendered by Pathview.

The KEGG graph shows up to 9.4 folds down-regulation of DHPR, Na^+/K^+ ATPase and mitochondrial proton pump after fast rewarming compared to slow rewarming which reflects intracellular electrolytes disturbance.

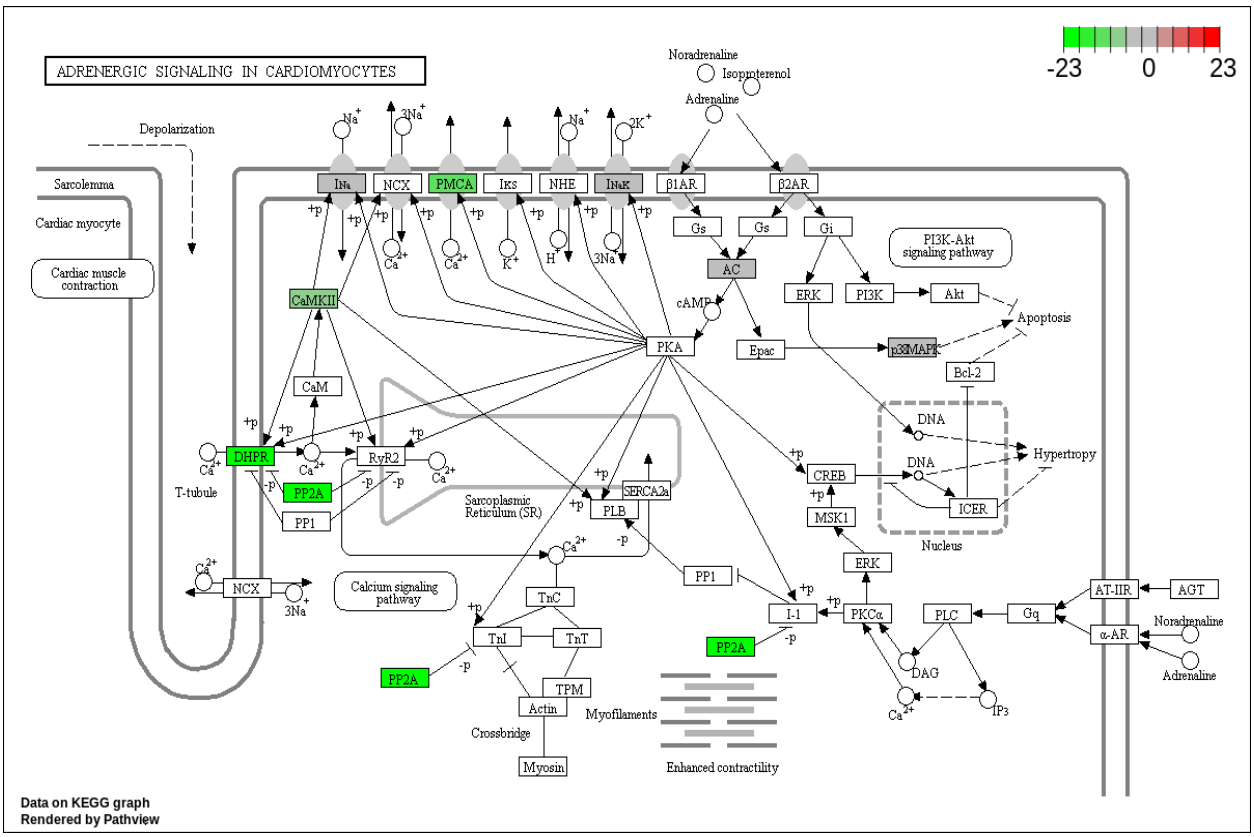

Figure 18: Differentially expressed genes related to adrenergic signaling in cardiomyocytes after fast compared to slow rewarming presented on KEGG graph rendered by Pathview.

The KEGG graph shows up to 23 folds down-regulation of DHPR, PP2A, and PMCA after fast rewarming compared to slow rewarming which reflects impaired calcium handling.

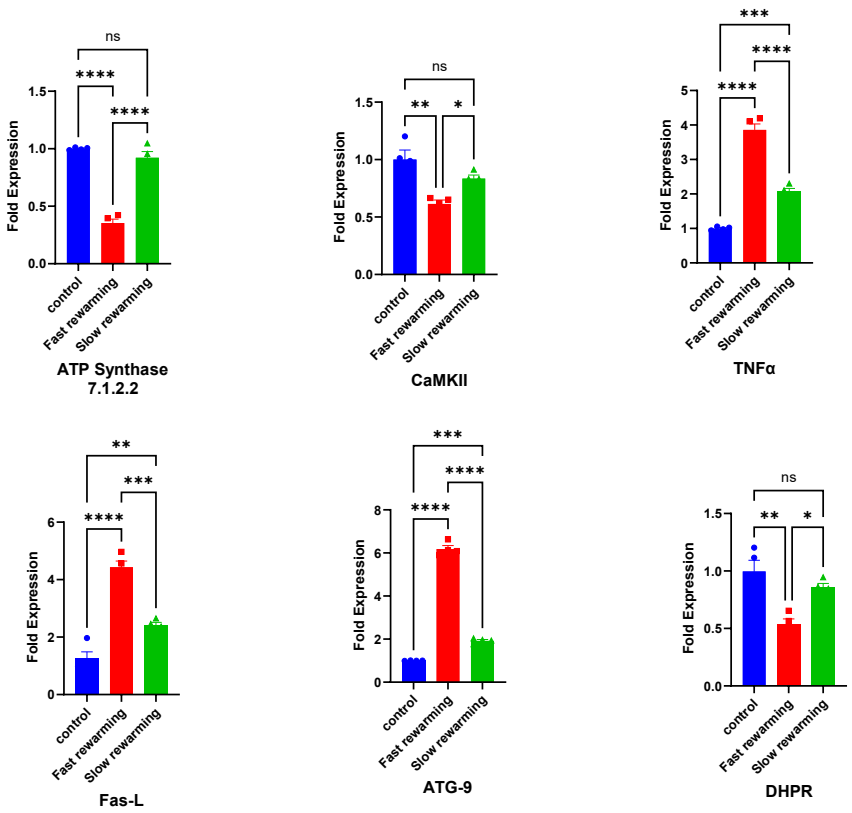

Figure 19: RT-PCR analysis of different targets from negative control, fast, and slow rewarming samples.

The RT-PCR confirms that fast rewarming is associated with significant down-regulation of ATP synthase subunit, CAMKII, and DHPR and up-regulation of TNFα, FAS-L and ATG9 (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n=4 per group).

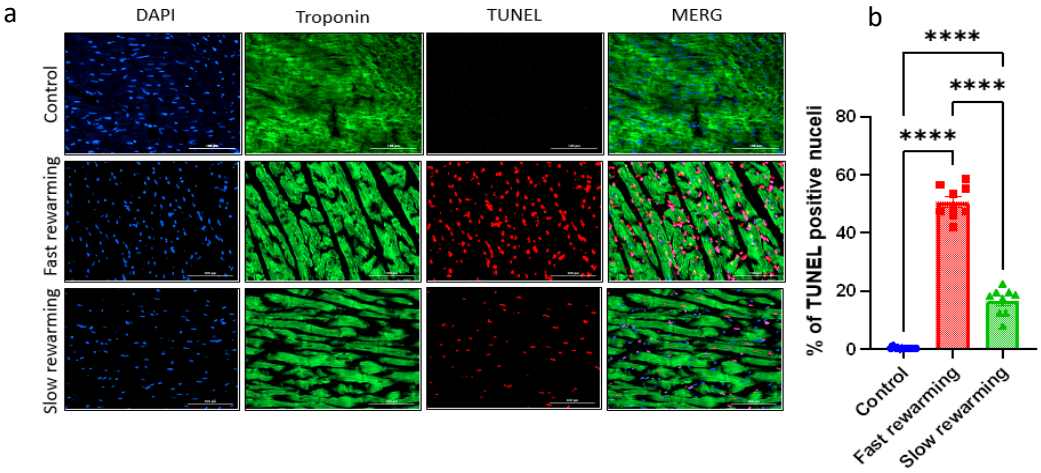

Figure 20: slow rewarming leads to less apoptosis events compared to fast rewarming.

(a) Representative images of ventricular muscles stained with antibody against Troponin-T (green), tunnel positive nuclei (red) and nuclei (blue), scale bar: 100 µm. (b) graph bar represents the percentage of TUNEL positive cardiomyocyte nuclei (****p<0.0001, n=8 per group).

5.3. Discussion

Preserving myocardial viability and cardiac functions after cardiac arrest, cardiac surgery, or pathological insult, have been issues of extensive research for decades. Despite the thorough clinical research done, many questions are still open for basic science to answer. Most of the molecular determinants of cardiac response to injury and resuscitation are not fully depicted.

In this study, we tried to highlight the main transcriptomic changes in the cardiac muscle cells in the sitting of hypothermic cardiac arrest followed by different rates of rewarming during resuscitation. The rate of rewarming post cardiac arrest has been discussed extensively in clinical research. However, ~~a~~ little is known about the transcriptional and molecular remolding associated with different rates of rewarming post cardiac arrest. The rate of rewarming after accidental or therapeutic hypothermia has been reported to affect the outcome of resuscitation. Slower rate of rewarming but not too slow rates may be more beneficial (88, 144). In a focused update endorsed by the Commission for Mountain Emergency Medicine, the rate of rewarming was shown to impact both the short and long term consequences after hypothermia and resuscitation as slower rewarming results in more favorable outcome (145). Hifumi et al showed that the longer rewarming is associated with favorable neurological outcome after out of hospital cardiac arrest (146). Nielson and his colleagues, have concluded that the rate of rewarming may not be a significant determinant of outcome after out-of-hospital cardiac arrest (147). We think that their conclusion may have been impacted by the heterogeneous nature of the sample they used. In a retrospective study of the data available through the International Hypothermia Registry, Walpoth and his colleagues showed that accidental hypothermic cardiac arrest is a heterogeneous entity with many factors that may impact the treatment outcome and suggested that the slower rewarming post cardiac arrest may be beneficial (148).

In this study we investigated the effects of different rates of rewarming post hypothermic cardiac arrest. Our results showed that slower rewarming post cardiac arrest results in significant reduction in the apoptotic events in cardiomyocytes. Bulk transcriptomic analysis identifies the key pathways affected by the rates of

rewarming to be: 1- metabolic pathways, 2- pro-inflammatory, pro-apoptotic, and phagocytic pathways, 3- calcium handling and contraction pathways.

In general, hypothermia is known to cause a global slowing down of metabolism (149-152). Brain is the most sensitive organ to oxygen deprivation and lack of ATP, thus, most of studies on hypothermia focused on cerebral metabolic shifts (149, 153, 154). Similar slowing down of metabolism is expected in all organs including the heart (155). Luckily, there is dampening of energy demands and ATP requirements in the setting of hypothermic cardiac arrest. This allows organs to escape the deleterious effects of the slowing the metabolism (155, 156). During rewarming and restoration of circulation, organs' requirements of energy increased and thus, the regain of normal rate of metabolism and matching blood and oxygen supply is of crucial (157-160). We have shown that fast rewarming post hypothermic cardiac arrest is associated with lower expression of metabolic genes which may indicate lower ATP production capacity. This might explain the worse outcome after fast rewarming compared to slow rewarming.

Our transcriptome analysis shows that rapid rewarming is associated with decrease expression of long chain fatty acyl-CoA ligase which is the rate limiting step of fatty acid oxidation (161). The heart is dependent on fatty acids oxidation for energy production (162). Metabolism shifting away from fatty acids oxidation is a main character of many cardiac pathologies and may be one of the underlying mechanisms of worse outcome after rapid rewarming (163).

Our findings also show rapid rewarming is associated with increased expression of very-long-chain 3-oxoacyl-CoA synthase, an enzyme responsible for microsomal fatty acids elongation, which indicate accumulation of intracellular lipids and cellular injury (164). As well, the increased expression of both methylmalonate-semialdhyde dehydrogenase, and methylmalonyl-CoA mutase indicates increased metabolism of amino acids and activated gluconeogenesis as an alternative pathway for energy production (165, 166). Rapid rewarming was also associated with lower expression of subunits of the electron transport chain complexes which indicates shift from the aerobic oxidation to anaerobic glycolysis for energy production (167, 168). This full set of gene expression in the metabolic pathways

is a hall mark of cellular energy, which may explain the worse clinical outcome after rapid rewarming (169, 170).

The transcriptomic changes after fast rewarming compared to slow rewarming reflects the full blown picture of activation of pro-inflammatory, pro-apoptotic, and pro-phagocytic responses (171). Overexpression of TNF, FASL, TRAF-6, and AP-1 reflects activation of external apoptotic signaling (172). Overexpression of CCL1, TSLP, LIF, FASL and ILRs indicate activated cytokines response (173). The increased expression of major histocompatibility molecules I and II, Collectins, and Dectins reflects the increased immune surveillance (174-177). While, CD94 and NKp30 expression is associated with natural killer cells mediated cytotoxicity (178, 179). The overexpression of ATG9 reflects enhanced autophagosome formation and expansion (180, 181). This may reflect increased cycling of intracellular components (182), or it can be related to the accumulation of intracellular lipid droplets (183). All these changes are contributing to increased myocardial injury and thus, worsen the outcome (184-186).

Finally, fast rewarming was associated with lower expression of DHPR, CaMKII, proton transporters, and Na+/K+ exchanger, which may indicate impaired calcium handling and altered cellular homeostasis (187). The downregulation of pyrophosphatase PPA2 is known to be associated with poor outcome (188-190).

This set of findings explains the increase in the percentage of TUNEL positive nuclei in the heart tissues exposed to fast rewarming compared to slow rewarming. The molecular fingerprint of slow rewarming clearly signifies the importance of the rate of rewarming during resuscitation and after hypothermia, and highlights many signaling pathways that might be targeted in the future to improve the outcome of resuscitation.

A key limitation to the current study is the lack of functional assessment of the resuscitated hearts with different rates of rewarming after hypothermic cardiac arrest. While it is not clear if the change in gene expression will be reflected immediately on the cardiac functions, these changes might have many long-term effects. It is difficult to keep the experimental rats for longer times after resuscitation due to many technical and ethical concerns, So, long-term

consequences of the reported transcriptomic shifts are not reported in the current study. A follow-up study is recommended for assessment of both short-term and long-term clinical outcomes after addressing of the technical concerns of long term care of experimental animals after cardiac arrest. Finally, it is worth studying the effects of slower rates of rewarming than those used in our study.

6. Model of cardiac arrest followed by NO supplementation:

Nitric oxide is already available in intensive care and operating room but limited to inhaled use only, in acute pulmonary hypertension and in post-cardiotomy right ventricular dysfunction. Depending on the demonstrated anti-inflammatory and anti-apoptotic effects of NO donor drugs, it is considered highly probable that the administration of NO medical gas inside the oxygenator during extracorporeal life support (ECLS), may lead to a reduction in hypoxic-ischemic damage and an improvement in post-arrest cardiac function during resuscitation with ECLS. This hypothesis, if proven right, could govern the possibility of use of NO medical gas and extend its uses. So, we aimed to investigate the effect of NO inhalation through ECLS during ischemia-reperfusion damage.

This study proposes the use of the medical gas nitric oxide (NO) through ECLS. We conducted this experiment to study the transcriptomic changes in cardiomyocytes during ECLS in the absence and presence of NO.

6.1. Surgical Methods

Surgical protocols were followed as mentioned in section 4.1. At the time of cardiac arrest, the rats were randomized into two groups: CPB conducted normally in normothermia for 1 h (Control group, n = 10), CPB supplemented with NO 20 ppm (CPB-NO) administered through oxygenator for 1h (n = 10).

In subjects treated with NO, the INOblender dispenser was also prepared with its INOMAX detector. To proceed with this operation, the Oxygen supply line was connected with 90% FiO2 and the Nitric Oxide cylinder operated at 20 ppm to the mixer. This concentration was efficiently used for pediatric congenital heart diseases surgeries. The NO source was conducted to the oxygenator of the ECLS circuit by mixing it with oxygen using INOblender. For the control group, the oxygen supply line was connected directly to the oxygenator. After 60 minutes of reperfusion via CPB the rat was sacrificed and the cardiac tissues snap frozen in liquid nitrogen for future analysis.

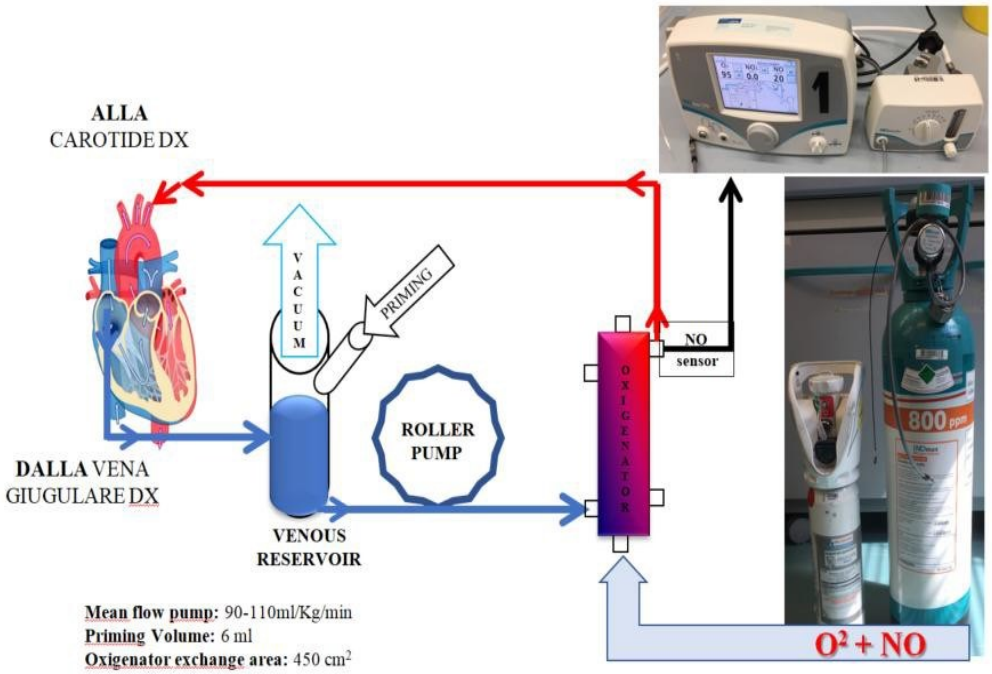

Figure 21: Schematic presentation of the extracorporeal circulation circuit with NO supplementation to the oxygenator.

6.2. Results:

6.2.1 Bulk RNA sequencing to characterize the general transcriptional changes accompanied NO treatment:

Previous studies have showed a neuroprotective effect of the NO during cardiac arrest at both the clinical and molecular levels. In this study we are investigating whether NO supplementation during resuscitation would results in a cardio-protective effect at the molecular and genetic level. In the current study, the rats were randomized into two groups: CPB conducted normally in normothermia for 1 h (Control group, n = 10), CPB supplemented with NO 20 ppm (CPB-NO) administered through oxygenator for 1h (n = 10). Hearts were snap frozen in liquid nitrogen immediately after the experiment and RNA was isolated afterwards. RNA Sequencing reviled more than 550 genes are differentially expressed in NO treated group (NO-CPB) compared to the control group (CPB only). GO term enrichment shows NO-CPB group showed up regulation of pathways related to inflammation, immune response, cell cycle arrest, response to stress, and apoptosis activation

(figure 22). NO-CPB group showed downregulation of the metabolic genes (figure 23).

6.2.2 Pathway analysis using Pathview software to highlight the main molecular pathways that been affected by the profound transcriptional changes:

To Further understand the key transcriptomic changes and depict the major affected pathways, Pathview software analysis was used and KEGG graphs were generated for all the differentially expressed genes. Metabolic, signaling, inflammatory, apoptotic, phagocytic, and autonomic pathways among other molecular pathways were analyzed and fold expression of the related genes presented on the respective KEGG graph. The analysis showed that NO-CPB is associated with complex metabolic derangement as indicated by down regulation of genes related to fatty acids metabolism (figures 24, 25), amino acids metabolism (figure 26), and all complexes of the electron transport chain (figure 27).

NO-CPB results in increased expression of the genes involved in pro-inflammatory and pro-apoptotic signaling pathways compared to CPB only. NO-CPB resulted in up regulation of genes related to IL1, MAPK, TNF and NF-KB signaling (figures 28, 30). NO treatment showed increased expression of the genes involved in proteasome subunits (figure 29), and Toll-like receptors (TLR) (figure 31).

On the other hand, NO-CPB treatment downregulated of myosin light chain kinase (MLCK) enzyme expression, which is responsible for phosphorylation of myosin light chains and enhancement of muscle contraction. This effect is supposed to be associated with enhanced smooth muscle relaxation (figure 32). Moreover, NO-CPB resulted in lower expression of sympathetic receptors including α-adrenergic receptors (α-AR) and β1adrenergic receptors (β1-AR) and higher expression of β-2adrenergic receptors (β2-AR). NO treatment has led to lower expression of most of calcium handling genes including di-hydropyridine receptors (DHPR), smooth endoplasmic reticulum calcium uptake (SERCA2a) and ryanodine receptors (RYR2) which is supposed to add to cardiac muscle relaxation (figures 33, 34).

6.2.3 RT-PCR confirms the effects of NO treatment on metabolic, inflammatory, and apoptotic pathways:

To confirm our findings from the bulk-RNA sequencing data, we conducted RT-PCR analysis of the key differentially expressed targets genes for samples from negative control (hearts that did not subjected to cardiopulmonary bypass), NO-CPB and CPB-only. RT-PCR confirmed the bulk RNA sequencing findings with NO-CPB found to have increased fold expression of the inflammatory markers including cyclo-oxygenase-2 (COX-2) (1.5-fold change), IL-1 (5-fold change), NFκB (5-fold change), and β2-AR (2.7-fold change), while NO treatment has led to normalization of the expression of both α1-AR and β1-AR compared to CPB only (figure 35).

6.2.4 TUNEL assay shows increased apoptotic markers in tissues treated with NO:

Finally, to identify the effect of these transcriptomic changes on tissue viability, we conducted TUNEL assay for assessment of the apoptotic nuclei. Tissues exposed to NO has showed significant increase in the TUNEL positive nuclei compared to CPB only (46%±3 vs 27%±2) (figure 20). This clearly indicates that the transcriptomic changes associated with NO treatment lead to more tissue injury and apoptosis compared to controls.

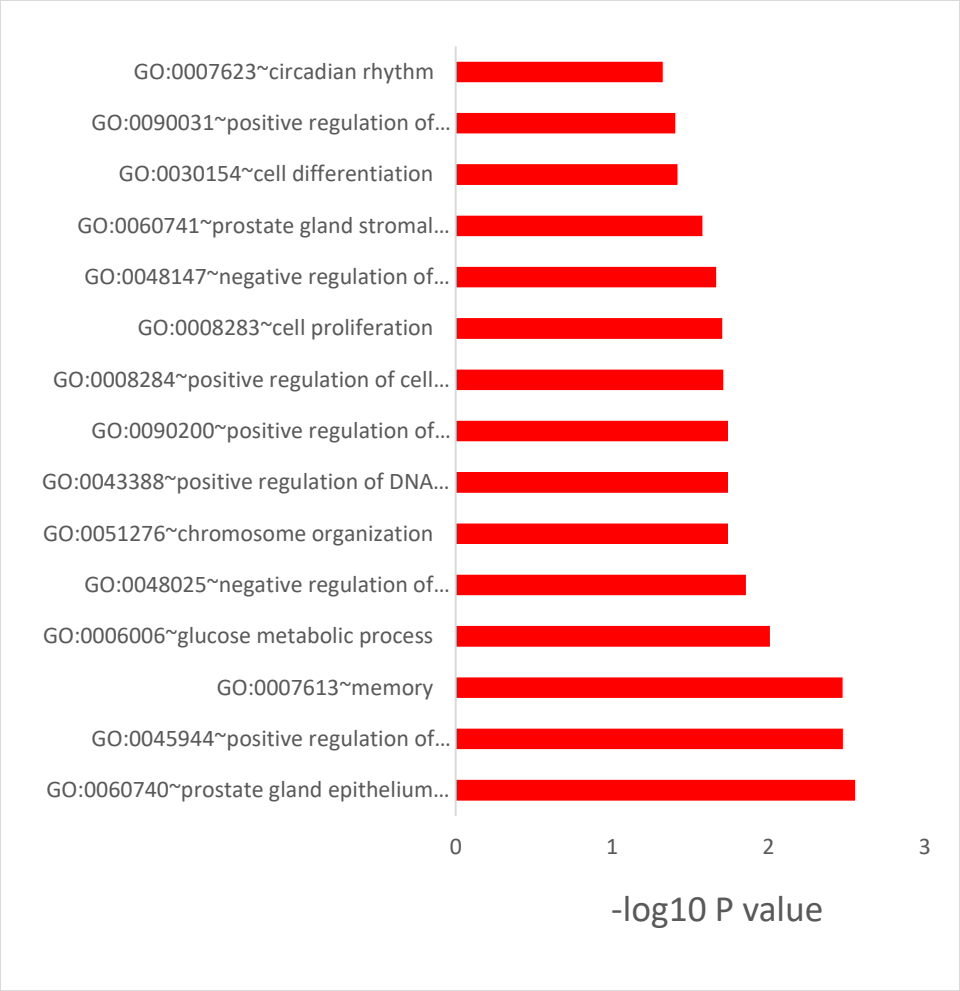

Figure 22: Go terms for up regulated genes in NO-CPB compared to CBP only. The GO analysis shows significant up regulation of inflammation, immune response, cell cycle arrest, response to stress, and apoptosis pathways in NO-CPB compared to CPB only.

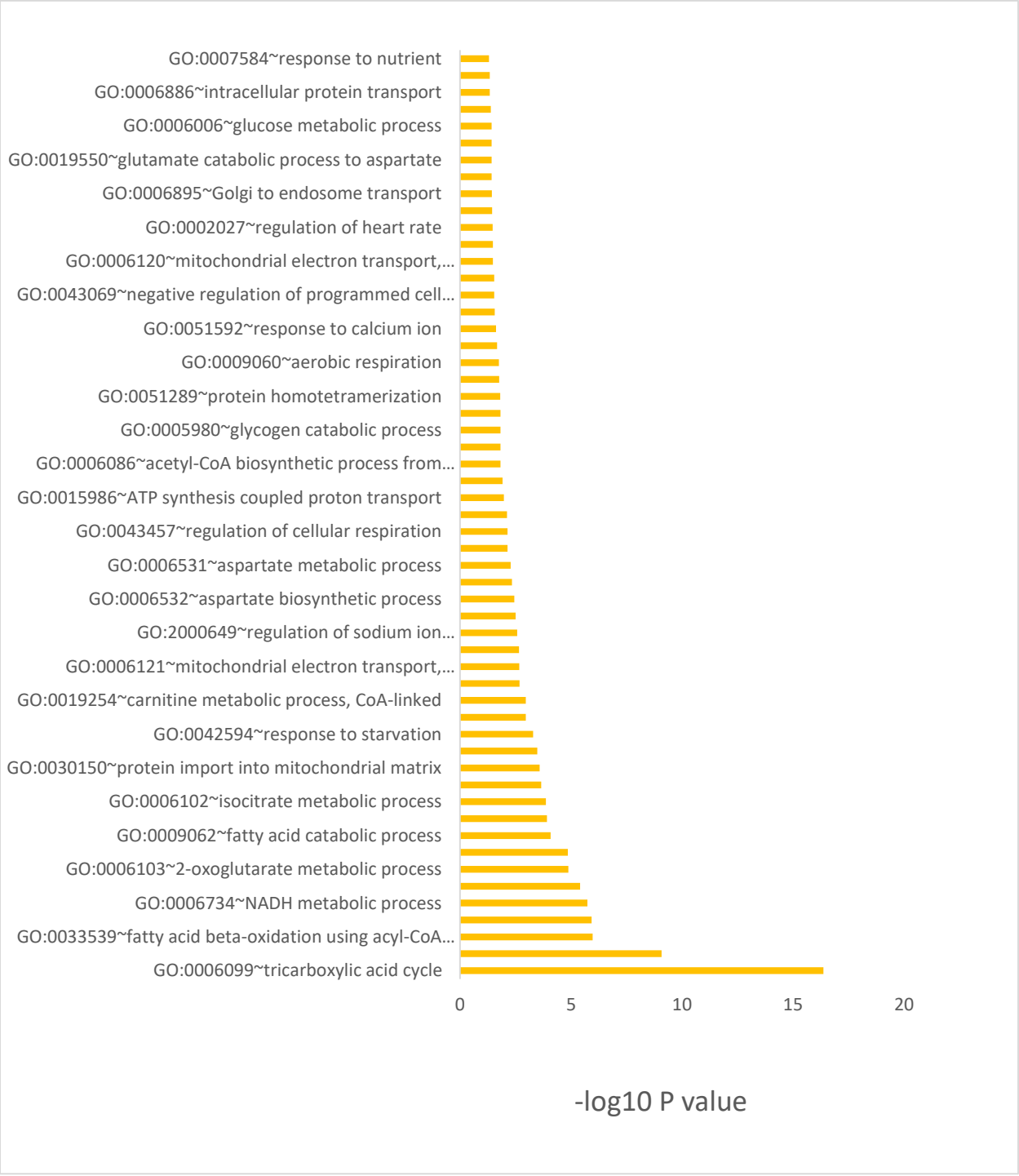

Figure 23: Go terms for down regulated genes in NO-CPB compared to CBP only. The GO analysis shows significant down regulation of most metabolic pathways in NO-CPB compared to CPB only.

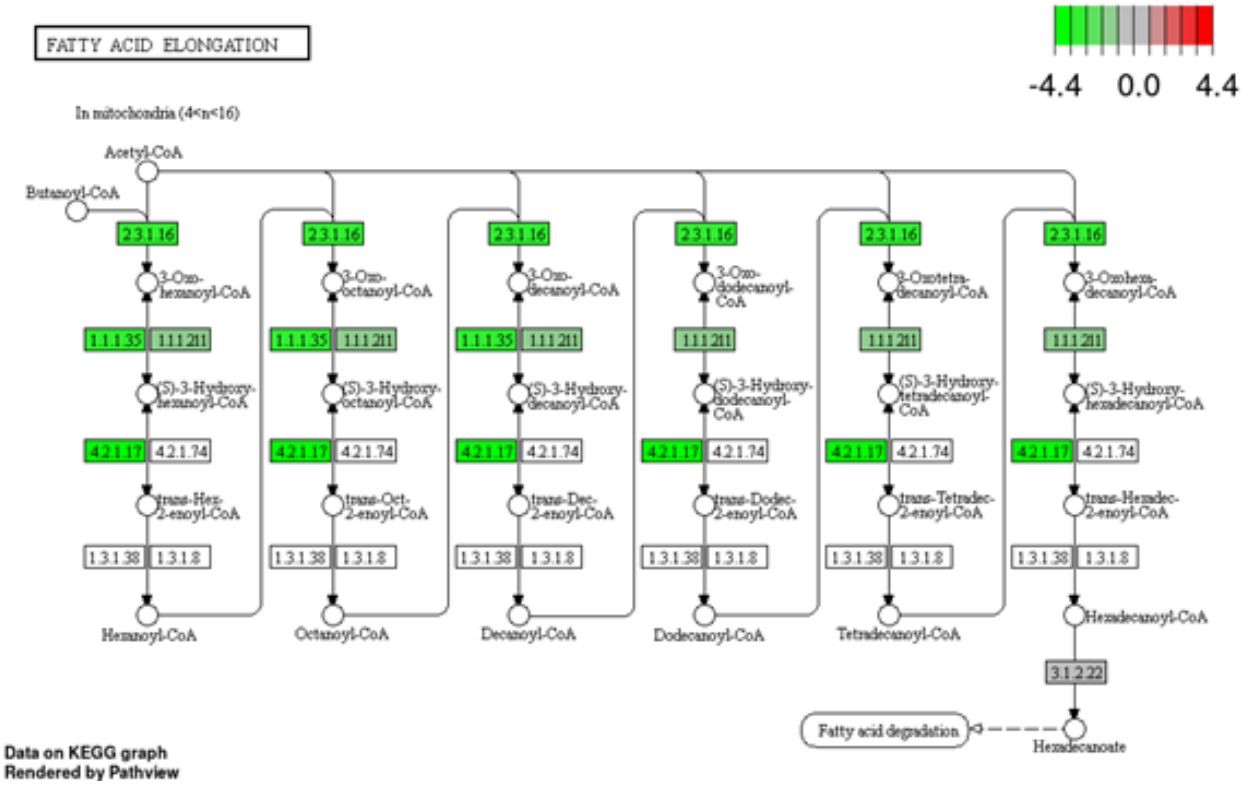

Figure 24: Differentially expressed genes related to fatty acids elongation after NO-CBP compared to CBP only presented on KEGG graph rendered by Pathview. The KEGG graph shows 4.4 folds down-regulation of most of the enzymes involved in fatty acids elongation in NO-CPB compared to CPB only.

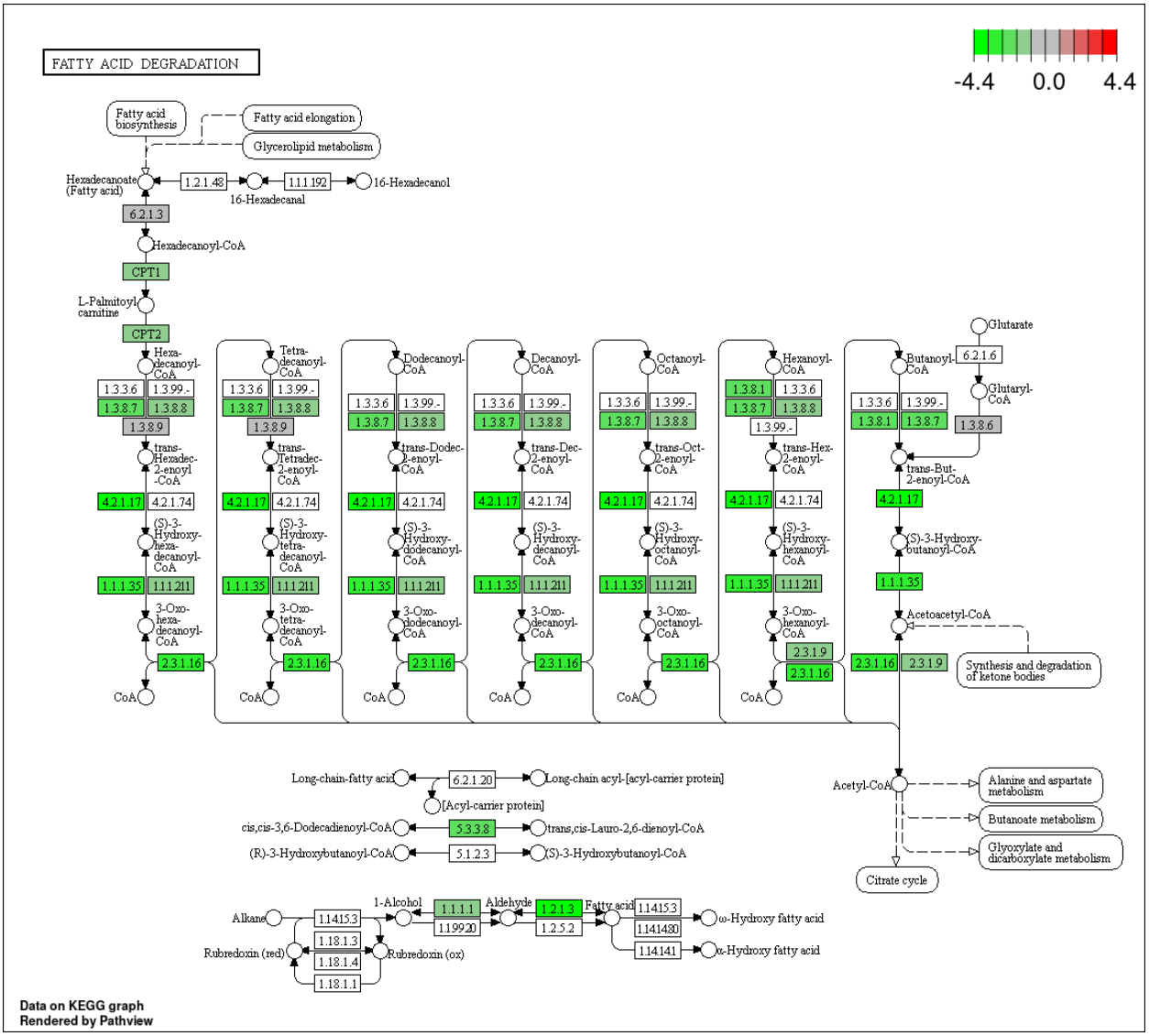

Figure 25: Differentially expressed genes related to fatty acids degradation after NO-CBP compared to CBP only presented on KEGG graph rendered by Pathview. The KEGG graph shows 4.4 folds down-regulation of all the enzymes involved in fatty acids degradation in NO-CPB compared to CPB only.

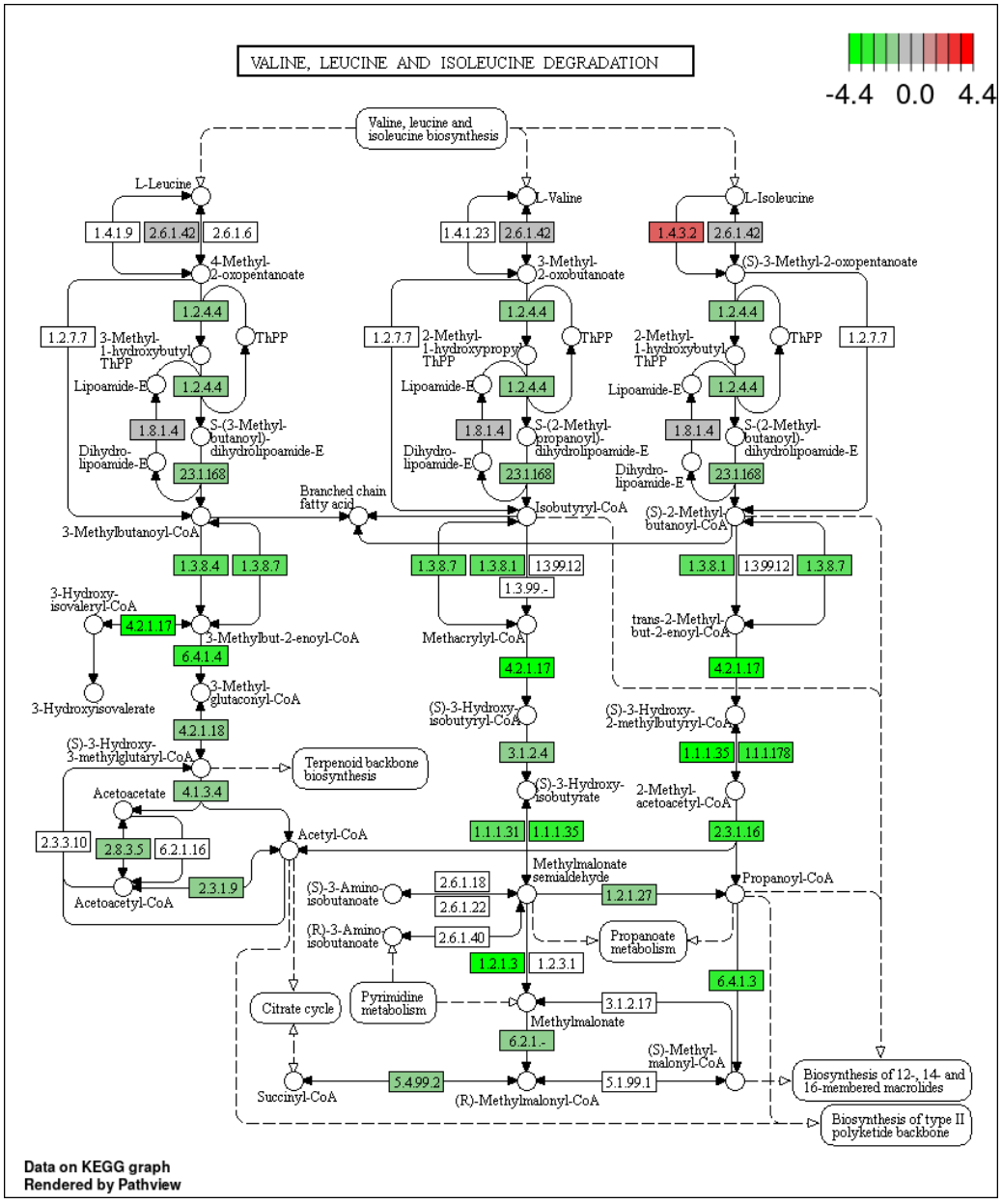

Figure 26: Differentially expressed genes related to branched chain amino acids degradation after NO-CBP compared to CBP only presented on KEGG graph rendered by Pathview.

The KEGG graph shows 4.4-folds down-regulation of most of the enzymes involved in branched chain amino acids metabolism in NO-CPB compared to CPB only.

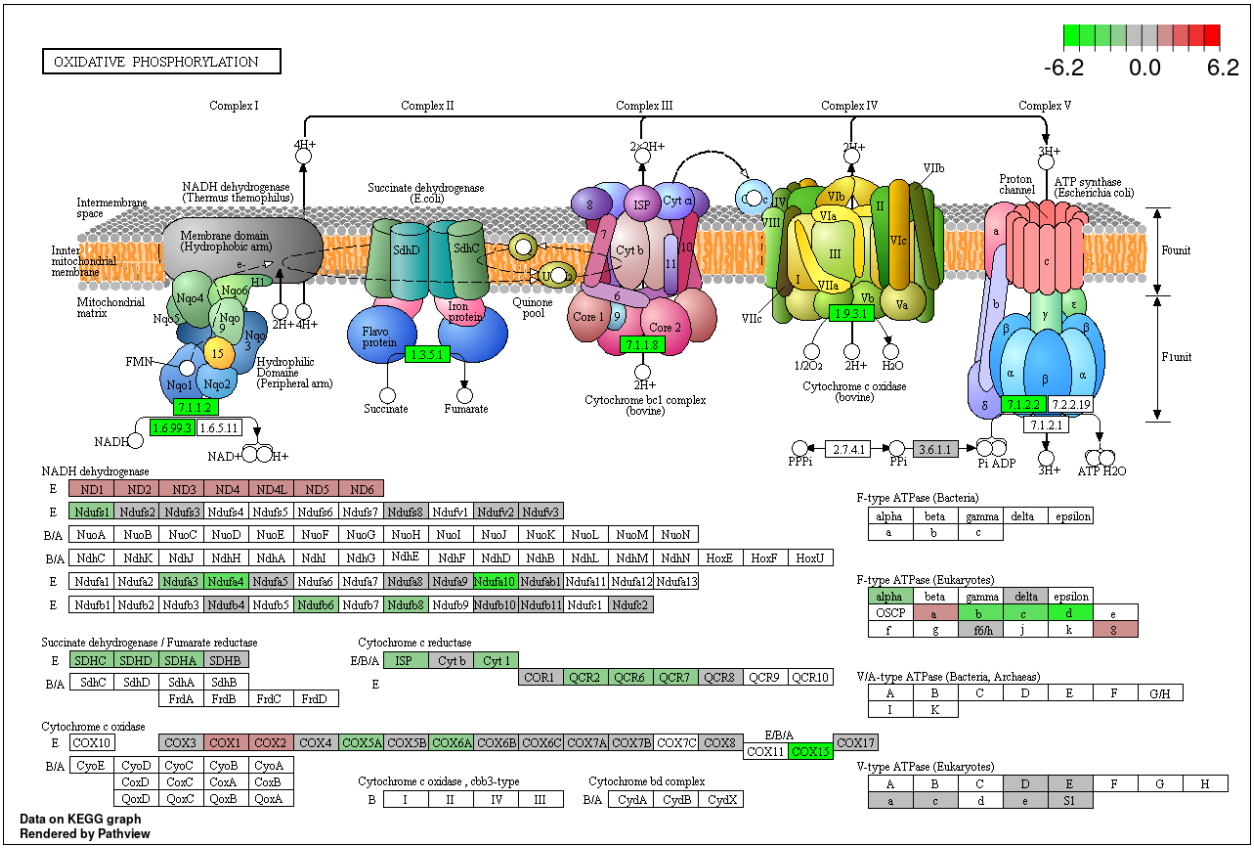

Figure 27: Differentially expressed genes related to electron transport chain after NO-CBP compared to CBP only presented on KEGG graph rendered by Pathview. The KEGG graph shows 6.2 folds down-regulation of the subunits of all complexes of the respiratory chain in NO-CPB compared to CPB only.

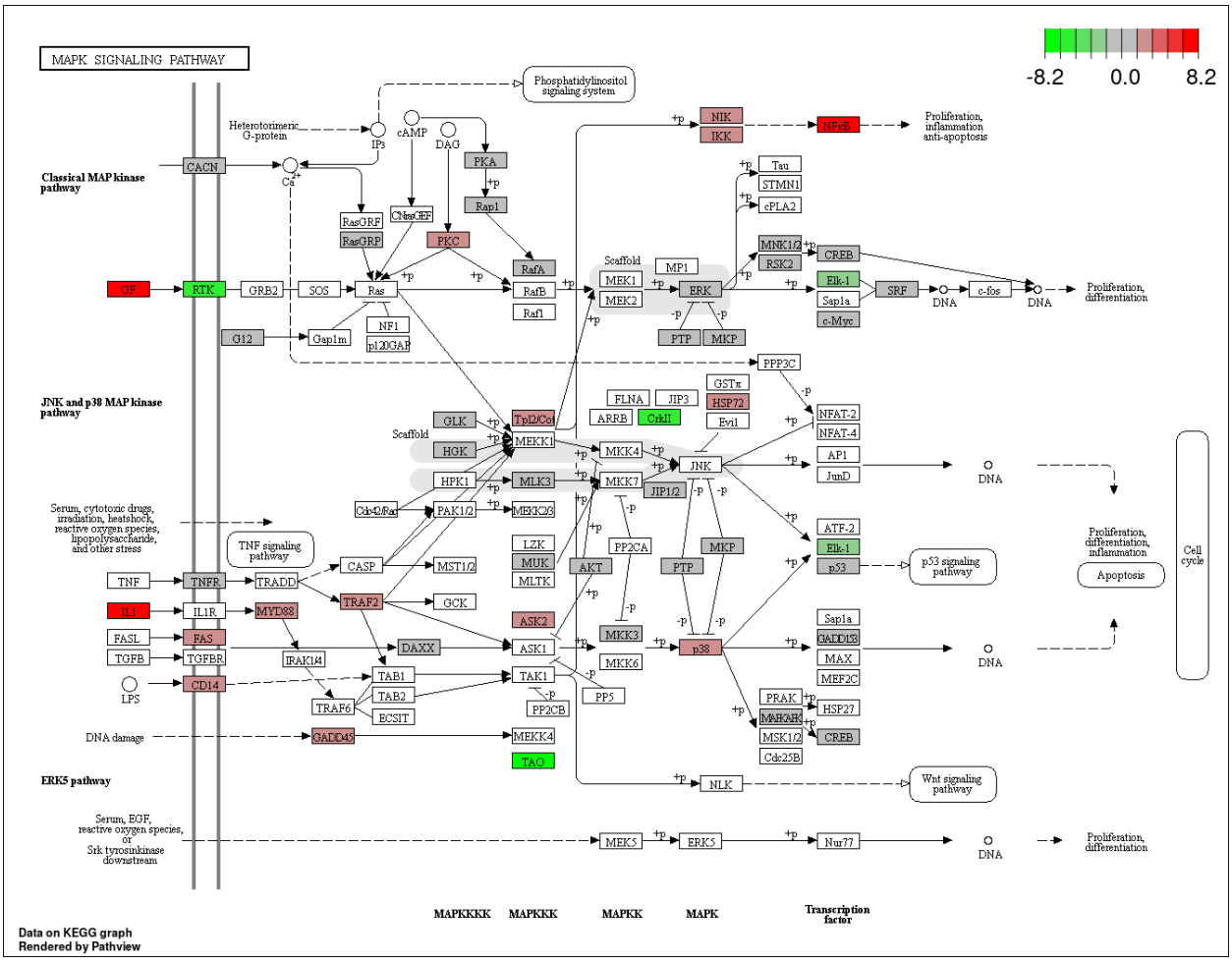

Figure 28: Differentially expressed genes related to MAPK signaling pathway after NO-CBP compared to CBP only presented on KEGG graph rendered by Pathview.

The KEGG graph shows up to 8.2 folds up-regulation of IL-1, FAS, CD14, NFκβ, and TRAF2 among many other targets after NO-CPB compared to CPB only which favors apoptosis.

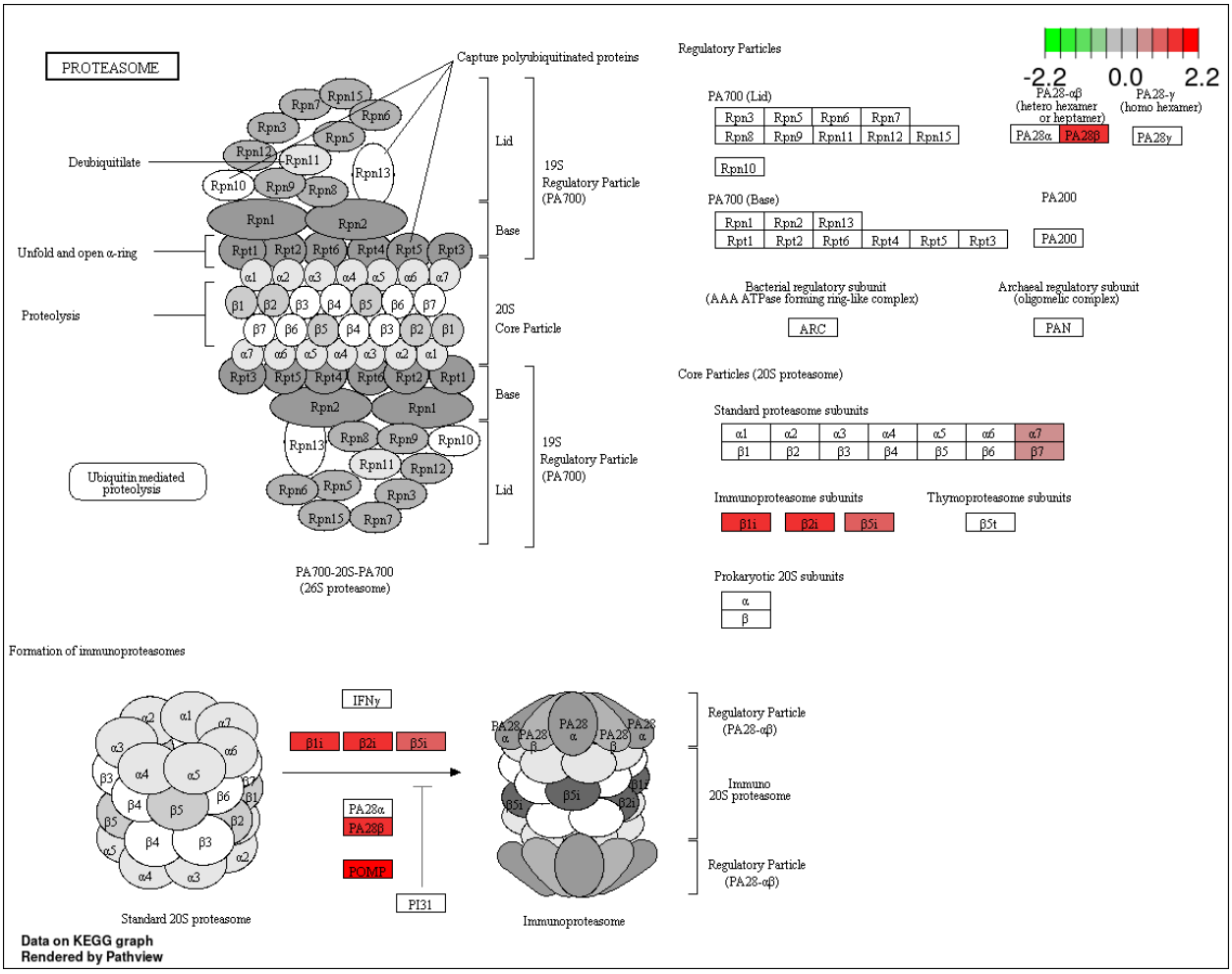

Figure 29: Differentially expressed genes related to proteasome formation after NO-CBP compared to CBP only presented on KEGG graph rendered by Pathview. The KEGG graph shows up to 2.2 folds up-regulation of the immunoproteosome subunits after NO-CPB compared to CPB only which favors proteolysis.

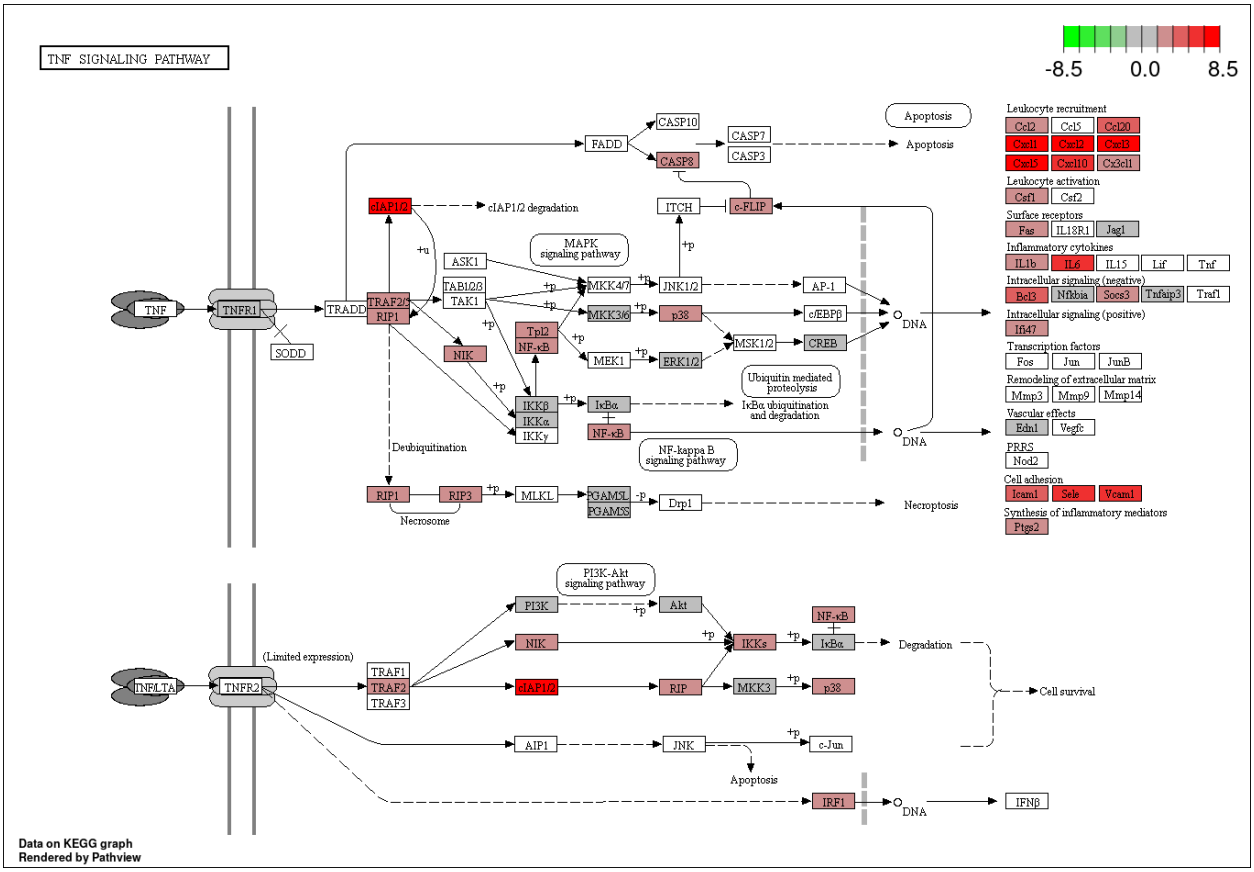

Figure 30: Differentially expressed genes related to TNF signaling after NO-CBP compared to CBP only presented on KEGG graph rendered by Pathview.

The KEGG graph shows up to 8.5 folds up-regulation of RIPs, ILs, NFκβ, BCL3 and TRAFs among other targets after NO-CPB compared to CPB only which favors inflammatory response and apoptosis.

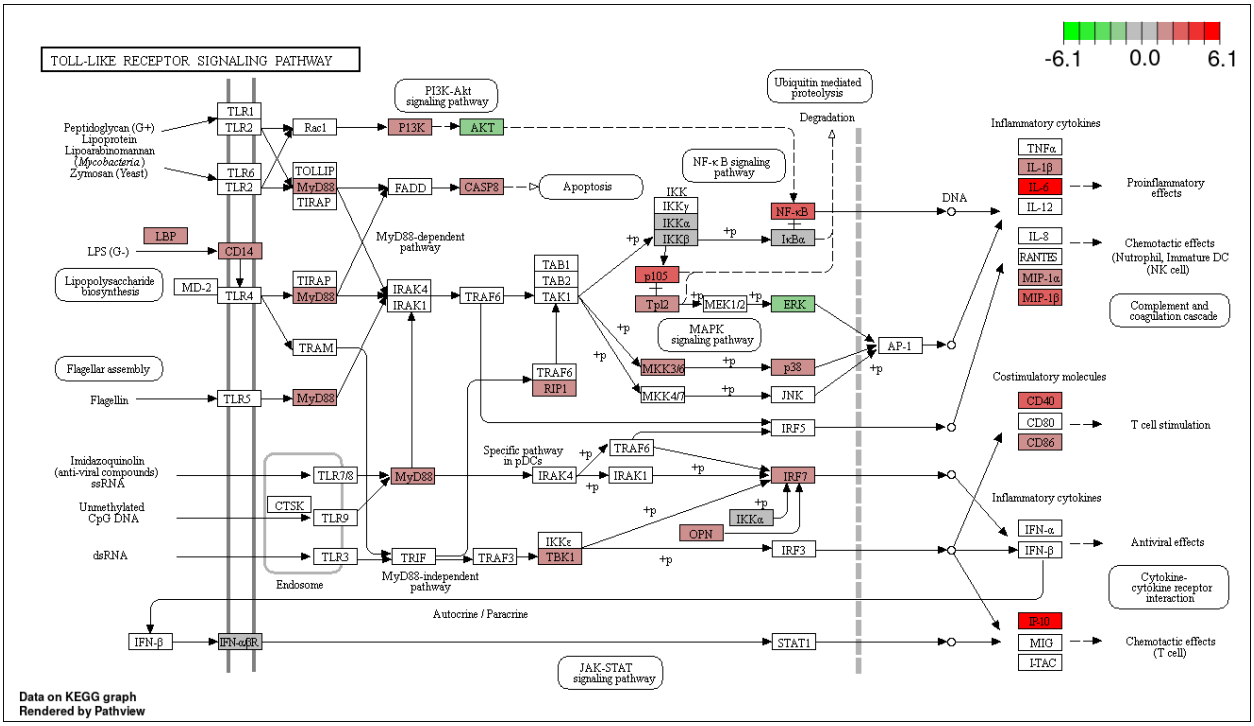

Figure 31: Differentially expressed genes related to Toll-like receptor signaling pathway after NO-CBP compared to CBP only presented on KEGG graph rendered by Pathview.

The KEGG graph shows up to 6.1 folds up-regulation of LBP, CD14, CD40, CD86, MIPs, and ILs among other targets after NO-CPB compared to CPB only which favors inflammatory response and chemotaxis.

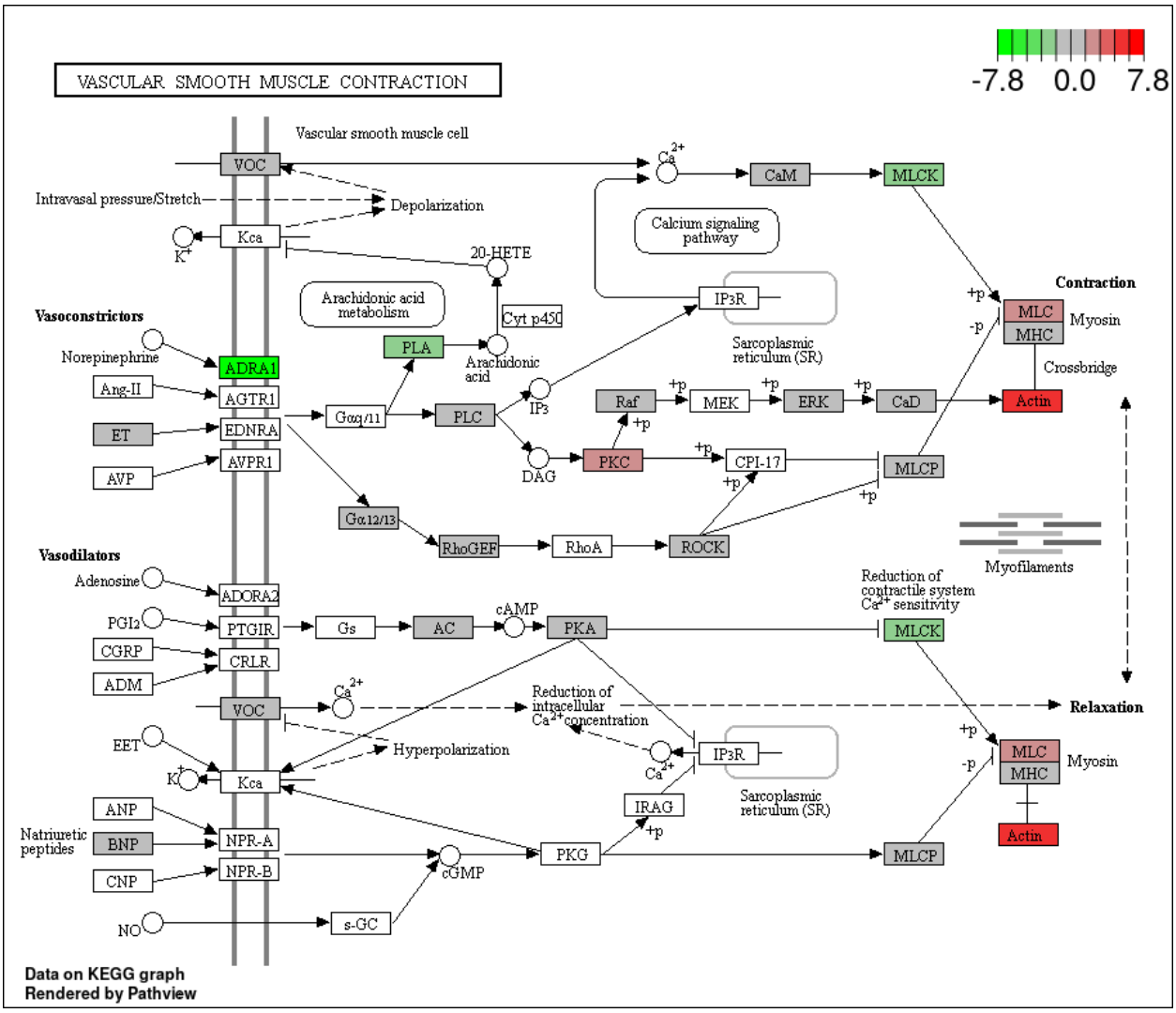

Figure 32: Differentially expressed genes related to vascular smooth muscle contraction after NO-CBP compared to CBP only presented on KEGG graph rendered by Pathview.

The KEGG graph shows up to 7.8 folds down-regulation of ADRA1 after NO-CPB compared to CPB only which favors smooth muscle relaxation.

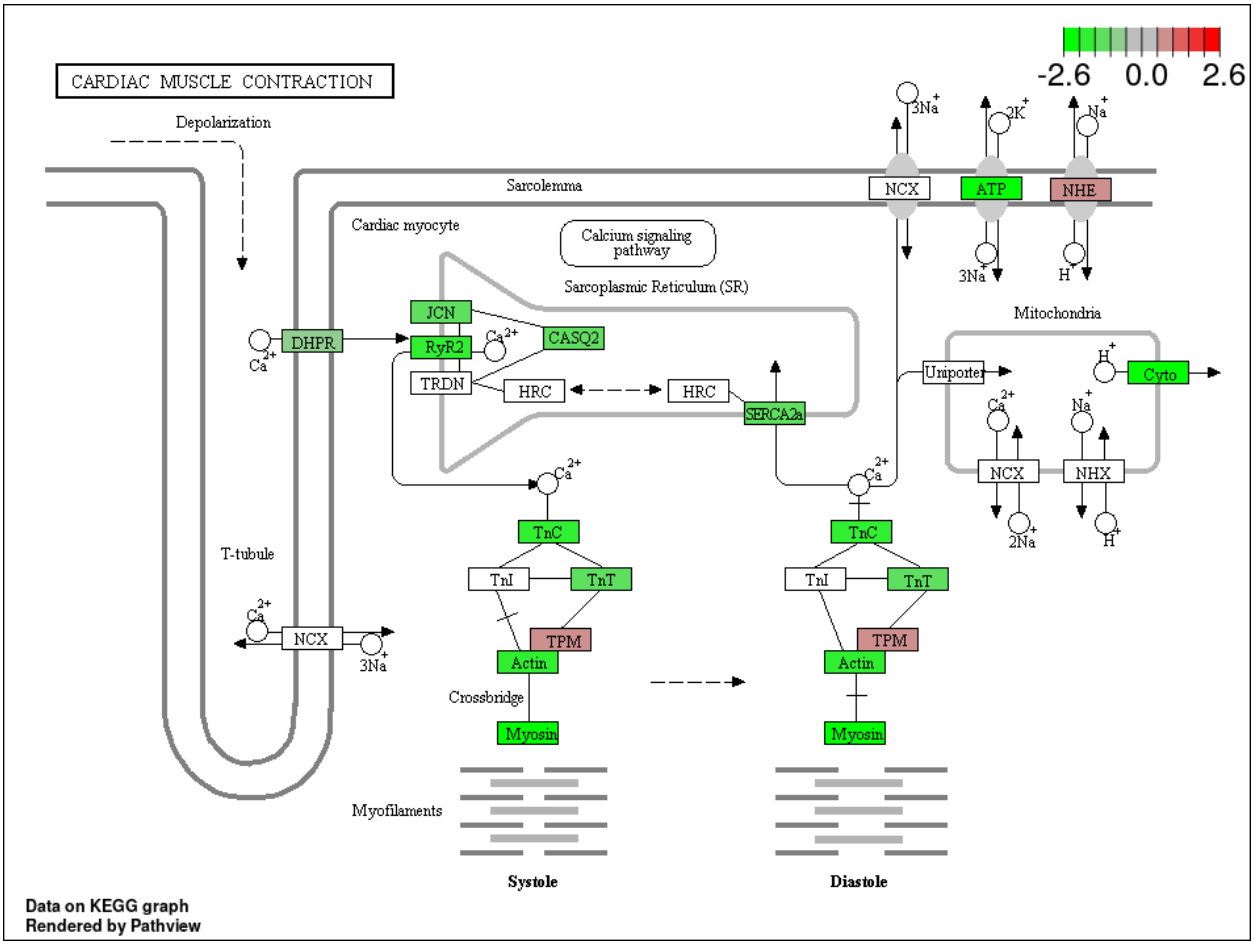

Figure 33: Differentially expressed genes related to cardiac muscle contraction after NO-CBP compared to CBP only presented on KEGG graph rendered by Pathview.

The KEGG graph shows up to 2.6 folds down-regulation of DHPR, RyR2, CASQ2, SERCA2a, TnC, and TnT after NO-CPB compared to CPB only which favors cardiac muscle relaxation.

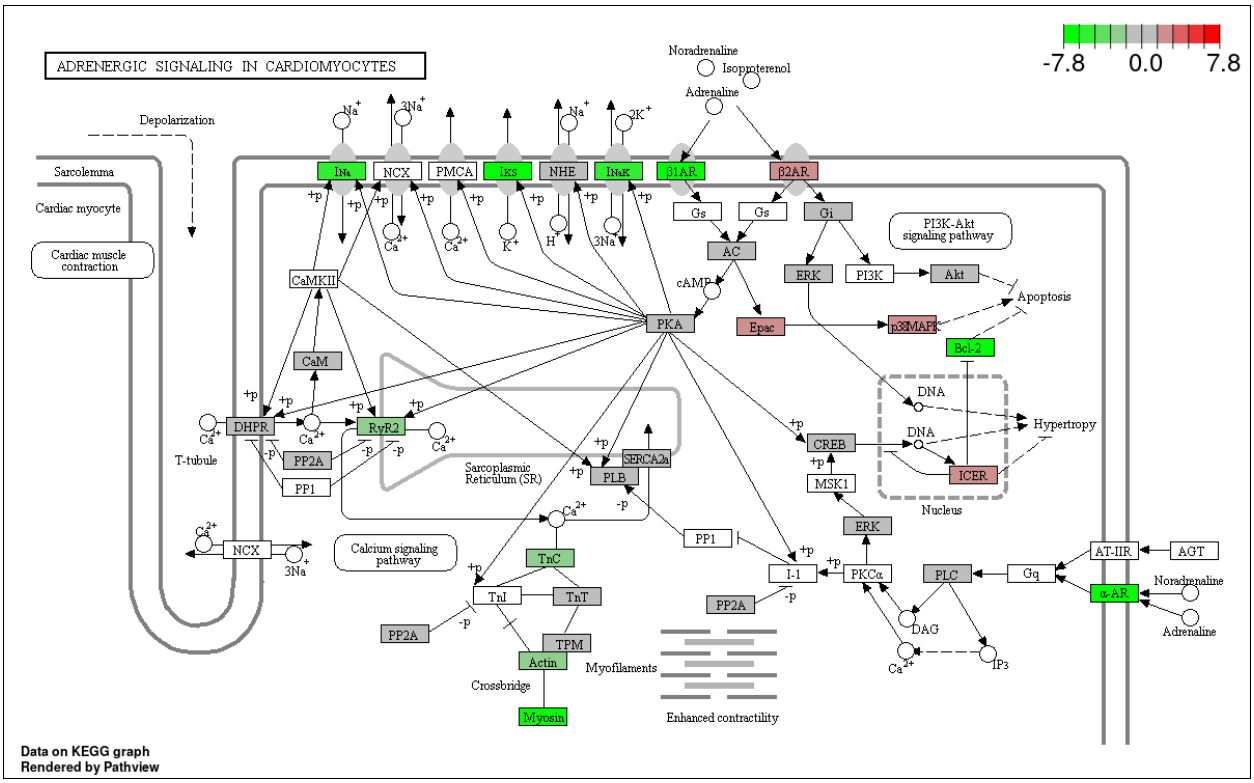

Figure 34: Differentially expressed genes related to adrenergic signaling in cardiomyocytes after NO-CBP compared to CBP only presented on KEGG graph rendered by Pathview.

The KEGG graph shows up to 7.8 folds down-regulation of β1AR and up-regulation of β2AR after NO-CPB compared to CPB only which favors attenuation of adrenergic signaling in cardiomyocytes.

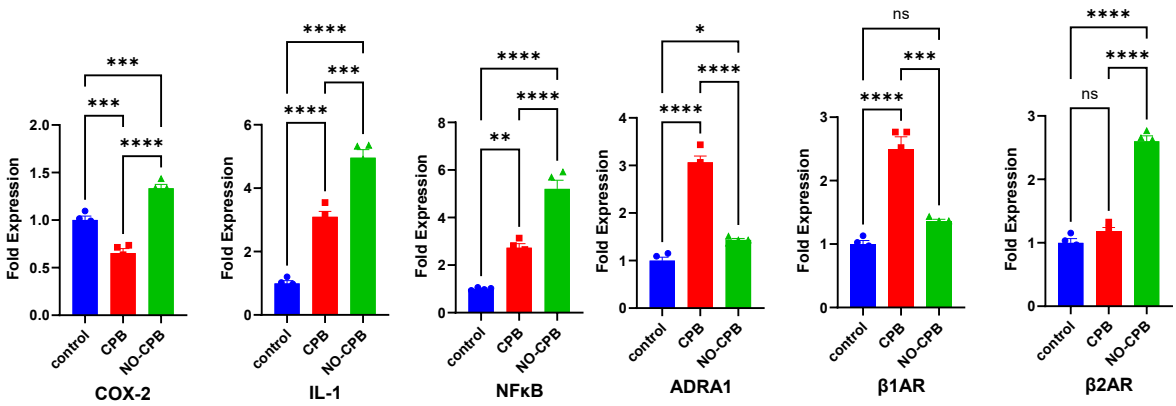

Figure 35: RT-PCR analysis of inflammatory and sympathetic receptor genes from negative control, CPB, and NO-CPB samples.

The RT-PCR confirms that NO treatment has led to significant up-regulation of inflammatory markers including COX-2, IL-1, and NFκB, and the β2AR as well. NO treatment has led to significant down-regulation of ADRA1, and β1AR (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, n=4 per group).

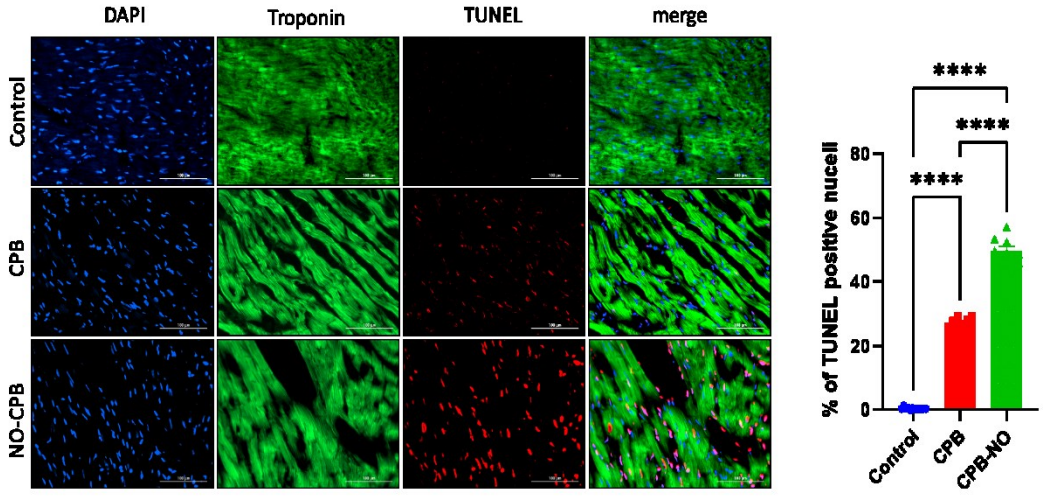

Figure 36: NO treatment has led to higher apoptosis events compared to CPB only. (a) Representative images of ventricular muscles stained with antibody against Troponin-T (green), tunnel positive nuclei (red) and nuclei (blue), scale bar: 100 µm. (b) graph bar represents the percentage of TUNEL positive cardiomyocyte nuclei (****p<0.0001, n=8 per group).

6.3. Discussion

Nitric oxide is an effective supplement for many purposes including neuroprotection during cardiac and major vascular surgeries, as well pulmonary hypertension management (191-193). Some neuroprotective effects for NO in normal and failing hearts has been reported which leads to the hypothesis that NO supplementation may have some therapeutic and cardio-protective effects during resuscitation (194). The cardio-protective effects in the setting of accidental or surgical cardiac arrest has never been studied and a little is known about the molecular effects of NO treatment in that sitting.

The reports about cardiac effects of NO treatments are controversial. Bocchi et al. showed that inhalation of NO leads to development of pulmonary edema in severe heart failure cases (195). Bocchi et al. explained their findings as to be related to increased right ventricular work with acute decompensation and development of acute heart failure (195). Moreover, Semigran et al. showed that NO inhalation increases left ventricular load by unknown mechanism (196). In the current study, we investigated the transcriptomic changes in cardiomyocytes after NO supplementation via extracorporeal life support in experimental rat models of cardiac arrest.

Our data shows that nitric oxide supplementation with ECLS induces complex metabolic derangement as indicated by downregulation of genes involved in lipid metabolism including both fatty acids oxidation and elongation pathways, down regulation of branched chain amino acids metabolism, and down regulation of respiratory chain complexes. Nitric oxide has pleotropic effects on cardiac cell biology. Physiologic level of NO production enhances fatty acids metabolism, lower glucose utilization and decrease oxygen consumption (197-199). Cytotoxic concentrations of NO, as in heart failure and experimental models of increased nitric oxide synthase, has been reported to inhibit fatty acids metabolism, increase glucose utilization, lower oxygen consumption and ATP production, and increase reactive oxygen species production (200-202). Our data is in line with the cytotoxic effect of NO.

Also, our data shows NO supplementation is associated with the profound

transcriptomic activation of pro-inflammatory and pro-apoptotic signaling pathways including: MAPK signaling pathway, TNF signaling pathway, TLR signaling pathways, and immune-proteasome formation. NO contributes to myocytes loss by conferring an oxidative stress and triggering of apoptosis at pathological concentrations, (203-207). Excess NO leads to formation of reactive nitrogen species (RNS) which contributes to the cell damage (192). NO mediates the negative inotropic effects of the cytokines (208, 209). In our study, NO directly and actively induce the transcription of inflammatory and apoptotic signaling genes. This pathological activation of inflammation and apoptosis might be mediated by post-translational modification of cellular proteins through nitrosation of their thiol groups (210-217).

Finally, our analysis shows that NO supplementation downregulates α1, β1 -adrenergic receptors and contractile genes, while upregulate β2-adrenergic receptors. These findings are in line with previous reports. It has been previously reported that NO is responsible for α-adrenergic receptors hypo-responsiveness (218). NO is known to modulate the response to β2-adrenergic receptors stimulation (197, 219). NO has been reported to protect the heart against excessive catecholaminergic stimulation producing negative inotropic and chronotropic effects, and reinforcing the vagal tone in the heart (197, 220, 221). So, NO is well known to maintain the balance between the adrenergic and cholinergic tone on the myocardium (222). Accordingly, increased tissue level of NO has been proposed as a strategy for cardio-protection and/or treatment of different cardiac insults (223-226).

Despite the beneficial effects of NO on the adrenergic signaling on the heart, NO supplementation through ECLS has led to an increase in the percentage of apoptosis. This indicates that the cytotoxic and inflammatory effects of NO supplementation overweigh the protective effects. Although this may challenge the whole idea of the use of NO supplementation during cardiac and vascular surgeries, still some points are worth investigation.

A key limitation to the current study is the use of a single dose of supplemented NO through ECLS. Our previous work showed that NO 20 ppm supplementation

has neuroprotective effects in the setting of selective cerebral perfusion and aortic arch surgery {Linardi, 2021 #1}. Accordingly, this was the dose tested in the current study. However, it has been reported that different tissue levels of NO influence different signaling pathways (227-229). So, it is worth studying the effects of supplementation of lower concentrations of NO.

Another limitation to the current study is that the lack of functional assessment of the cardiac functions after resuscitation which we think will not influence the conclusions.

7. Final Conclusions:

Resuscitation post cardiac arrest, either accidental or induced, is a field of active research. Despite the solid clinical guidelines, some points remain unconcluded. Neuroprotection and cardio-protection are two main concerns of resuscitation sciences. While clinical research provides some answers, basic science tools add a lot of valuable insights. In our study, we sought to answer two main questions using basic science tools: 1- In the setting of hypothermic cardiac arrest, does slow rewarming provide more cardio-protection? 2- In the sitting of cardiac arrest and extracorporeal circulatory support, does nitric oxide provide any cardio-protection? Our genetic results showed that slow rewarming after hypothermic cardiac arrest provides more beneficial metabolic, inflammatory, and apoptotic shifts compared to fast rewarming. This is reflected to the myocardium in the term of lower apoptotic nuclei. In contrary, NO did not show the expected benefits. Despite showing genetic evidence of smooth muscle relaxation and sympathetic regulation, NO was linked to activation of inflammatory and apoptotic genes and downregulation of metabolic genes.

Hereby, we show that basic science tools, including sequencing, software algorithms for data analysis, molecular screening and immunopathology staining, these tools can be more valuable proving clinical hypothesis as in the case of first model, or disproving it as in the second model. While these tools should be included more in the design of experiments to answer future questions, it is highly recommended that this should be done in the context of clinical findings to support the basic science findings.

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