

Nitric Oxide Signaling in Cardiac Development, Function and Disease

A thesis presented for the degree of Doctor of Philosophy
by

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Declaration of Originality

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Abstract

A variety of roles have been attributed to Nitric Oxide (NO) in the cardiovascular system. During development, exposure of mouse embryonic stem cells to high levels of NO has been shown to promote cardiomyocyte differentiation through activation of transcription factors such as *Nkx2.5*. In adults, NO has been implicated in control of blood pressure and regulation of cardiac contractility. In disease, it is evident that elevated levels of NO can have a protective effect during myocardial infarction.

ADMA and L-NMMA regulate NO biosynthesis by endogenously inhibiting Nitric Oxide Synthases (NOS). DDAH (1 and 2) enzymes metabolise ADMA in order to regulate the production of NO.

The aims of this PhD thesis are (1) to identify the role of NO levels during development of the cardiovascular system and (2) to identify the role of the *Ddah2* gene during an ischemia/reperfusion injury to the myocardium.

Treatment of mouse embryonic stem cells during differentiation with ADMA reduced NO biosynthesis and showed significantly lower expression of cardiac associated markers and endothelial cell markers. This decrease in cardiac differentiation was accompanied by an increase in *Runx1*, an important hematopoietic marker. Our findings suggest that reduced NO levels guide differentiating cells towards haematopoiesis by parallel reduction of endothelial and cardiac cell formation.

Global *Ddah2*^{-/-} mouse hearts that underwent a global ischemia/reperfusion injury showed significantly higher tissue survival when compared to WT hearts. Gene expression of iNOS and eNOS genes was elevated in *Ddah2*^{-/-} hearts along with other cellular survival markers. In an attempt to repeat our observations, WT and *Ddah2*^{-/-} adult cardiomyocytes were isolated and underwent a hypoxia/normoxia treatment. Analysis of cardiomyocytes, showed no increase in hypoxia associated markers such as *Glut1* and *Ddah1* gene suggesting that either *Ddah2*^{-/-} cardiomyocytes do not sense hypoxia or that they do not need to induce a hypoxic response to low oxygen levels.

This work provides novel insights into the role of NO in cardiac differentiation and *Ddah2* gene as a potential therapeutic target for ischemia/reperfusion injuries of the myocardium.

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Abbreviations

ADMA	Asymmetric Dimethylarginine
AGM	Aorta/Gonad Mesonephros
AV	Atrioventricular Node
BCA	Bicinchonic Acid
BDM	2,3-Butanedione Monoxime
BHF	British Heart Foundation
BH₄	Tetrahydrobiopterin
BMP	Bone Morphogenic Protein
BSA	Bovine Serum Albumin
CAD	Coronary Artery Disease
cDNA	Complementary DNA
cGMP	Cyclic Guanosine Monophosphate
CHD	Coronary Heart Disease
CT	X-Ray Computed Tomography
CVD	Cardiovascular Disease
DDAH	Dimethylarginine Dimethylaminohydrolases
DETA-NO	Diethylenetriamine NO adduct
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPC	Days post coitum
EB	Embryoid Body
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic Acid
eNOS	Endothelial Nitric Oxide Synthase
ES	Embryonic Stem cells

FBS	Fetal Bovine Serum
FGF	Fibroblast Growth Factor
GRK	G protein-coupled receptor kinase
Gy	Gray
HDL	High Density Lipoprotein
Hz	Hertz
iNOS	Inducible Nitric Oxide Synthase
iPS	Induced Pluripotent Stem Cells
JAK	Janus-Activated Kinase
LAD	Left Anterior Descending Artery
LDL	Low Density Lipoprotein
LIF	Leukemia Inhibition Factor
L-NAME	L-N ^ω -Nitroarginine Methyl Ester
L-NMMA	Monomethylarginine
MEFs	Mouse Embryonic Fibroblasts
MHC III	Myosin Heavy Chain III
MI	Myocardial Infarction
MRI	Magnetic Resonance Imaging
mRNA	messenger Ribonucleic Acid
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NHS	National Health System
nNOS	Neuronal Nitric Oxide Synthase
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
PBS	Phosphate Buffered Saline
PCI	Percutaneous Coronary Intervention

PKG	Protein kinase G
PRMTs	Protein Methyltransferases
P/S	Penicillin/Streptomycin
RIPA	Radioimmunoprecipitation Assay
RISK	Reperfusion Injury Kinase System
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
rRNA	ribosomal RNA
RT-PCR	Real-Time Polymerase Chain Reaction
SA	Sinoatrial Node
SDMA	Symmetric Dimethylarginine
SEM	Standard Error of the Mean
sGC	Soluble Guanylylcyclase
SNAP	S-Nitro so-N-Acetyl-D, L-Penicillamine
SR	Sarcoplasmic Reticulum
STAT	Signal Transducer and Activator of Transcription
TNFα	Tumor Necrosis Factor α
UK	United Kingdom
USA	United States of America
VCAM-1	Vascular Cell Adhesion Molecule 1
WT	Wild Type

1. Introduction

1.1 The Nitric Oxide Signaling Pathway

1.1.1 Nitric Oxide

Nitric oxide (NO) is an important signaling molecule that has been implicated in a variety of physiological and pathophysiological processes. NO is a simple, highly reactive molecule consisting of nitrogen and oxygen and it is characterized as a free radical due to one unpaired electron in its bonding structure. The first role attributed to NO was the regulation of vascular tone through induction of arterial smooth muscle relaxation (Moncada, Radomski, & Palmer, 1988).

Since its discovery, NO has been found to be implicated in regulation of different cellular pathways (Dimmeler et al., 1999; Lima et al., 2010; Mora-Castilla et al., 2010). Its main cellular signaling target is the activation of soluble guanylate cyclase (sGC) to increase the synthesis of the secondary messenger cyclic guanosine monophosphate (cGMP) (Moncada & Higgs, 1991). NO has also been implicated in influencing protein gain or loss of function via S-nitrosation or nitrosylation (Vallance & Leiper, 2002).

Finally, NO has been shown to regulate a high number of physiological processes in the cardiovascular, immune and neuronal systems (Casadei & Sears, 2003; Iannone et al., 2014; Parmentier et al., 1999). In health and disease, NO can be beneficial or harmful depending upon factors such as time, amount and site of production (Bolli, 2001; Mullanikal & Toledo-Pereyra, 2007; Shaul, 2002).

1.1.2 Nitric Oxide Production

Nitric oxide synthases (NOS) are the enzymes responsible for the production of NO from the conversion of L-arginine to L-citrulline (Moncada et al., 1988). In addition to the substrate L-arginine, an array of co-factors is also important for NO production including; oxygen, nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin (BH₄), calmodulin and FAD (Andrew & Mayer, 1999) (Figure 1). Three isoforms of NOS have been identified: the endothelial NOS (NOS-3), the neuronal NOS (NOS-1) and inducible NOS (NOS-2) (Knowles & Moncada, 1994). The enzymes were named after the tissue or the circumstances under which they were identified.

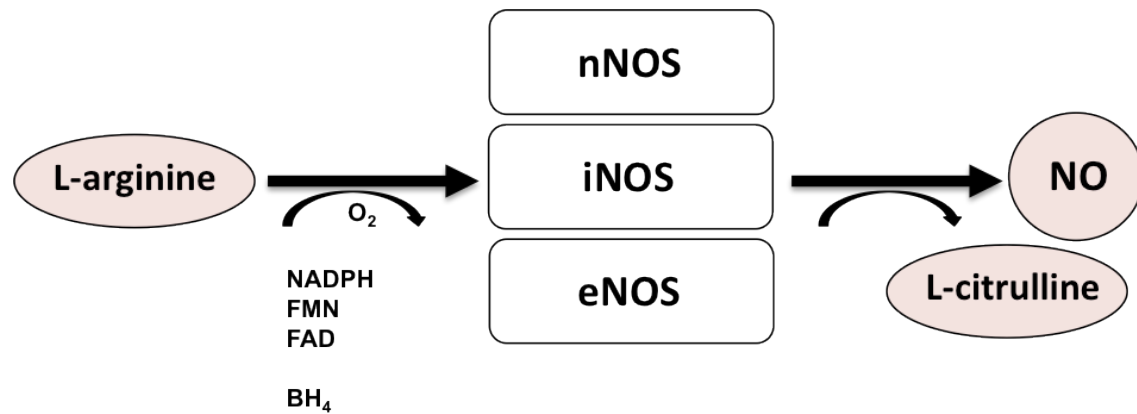


Figure 1. Production of Nitric Oxide (NO). NO production by the three isoforms of Nitric Oxide Synthases (NOS) requires L-arginine as a substrate and nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH₄) as co-factors. Figure was kindly provided by Dr. James Tomlinson.

eNOS is the predominant isoform expressed in the vascular endothelial cells (Salvador Moncada et al., 1988). Its role is the constitutive production of NO for the regulation of blood pressure via arterial smooth muscle relaxation (Andrew & Mayer, 1999). Pharmacological inhibition of eNOS enzyme, leads to vasoconstriction and hypertension (Vallance & Leiper, 2002). Also, it has been shown to regulate platelet adhesion and smooth muscle cell proliferation during vascular injury with eNOS^{-/-} mice exhibiting exaggerated smooth muscle replicative responses (Shesely et al., 1996).

nNOS is the second isoform that constitutively produces NO and that production of NO is calcium dependent (Tran, Leiper, & Vallance, 2003). Its main role is the production of NO in neurons as a neurotransmitter (Zhang & Snyder, 1995). In the heart, nNOS is located in the cardiac sarcoplasmic reticulum (SR) (Sears et al., 2003; Xu et al., 1999) and mitochondria (Elfering, Sarkela, & Giulivi, 2002).

The third isoform, iNOS, is the only inducible isoform to produce NO independently of [Ca²⁺] and in micromolar concentrations (in comparison to eNOS and nNOS that produce NO in nanomolar concentrations) (Aktan, 2004). Initially, iNOS was identified as an important host defense effector in the immune system implicated in inflammatory responses (Knowles & Moncada, 1994). iNOS is not present in resting cells (Knowles & Moncada, 1994). Inflammatory stimuli such as bacterial toxins and endogenous cytokines of the immune system, activate the transcription of the iNOS gene and the production of NO concentrations that are cytotoxic for the invading pathogens (Knowles et

al., 1990; MacMicking et al., 1995). NO production in this context therefore has an initial protective anti-microbial effect but this can subsequently contribute to vascular hyporeactivity and reduced organ perfusion (Kröncke, Fehsel, & Kolb-Bachofen, 1998). Finally, in response to cytokine signaling, the myocardium can also produce NO through the activation of the iNOS gene (Kröncke et al., 1998; Umar & van der Laarse, 2010).

1.1.3 Nitric Oxide Regulation

Since the discovery of NO and its roles in a variety of physiological and pathophysiological processes in the body, it was evident that manipulation of NO production could be an important pharmacological target (Leiper and Nandi, 2011; Parmentier et al., 1999; Vallance & Leiper, 2002).

Given the diverse roles of endogenous NO, its biosynthesis is intricately regulated through a number of mechanisms. Evidence suggests that NOS gene expression can be regulated at the level of transcription, post-transcription, translation and post-translation (Nathan & Xie, 1994). Furthermore, availability and co-localization of L-arginine and co-factors, also has been shown to regulate NO production (Nathan & Xie, 1994). Finally, and most relevant to this thesis, endogenous arginine analogues such as asymmetric dimethylarginine (ADMA) and L-N-monomethylarginine (L-NMMA) compete with arginine for binding to the active site of the NOS enzymes and inhibit NO synthesis (Vallance & Leiper, 2002).

1.1.3.1 Endogenous Inhibitors of NOS enzymes

The family of enzymes called the protein arginine methyltransferases (PRMTs) is responsible for the methylation of L-arginine residues within proteins to produce methylarginines. Three main types of methylarginines exist: N^G, N^G -dimethyl-L-arginine (asymmetric dimethylarginine, ADMA), N^G, N^{G'} -dimethyl-L-arginine (symmetric dimethylarginine, SDMA) and N^G -monomethyl-L-arginine (monomethylarginine, L-NMMA). The PRMT1 isoform accounts for the majority of L-NMMA and ADMA production *in vivo* (Tang et al., 2000), whilst PRMTs 5 and 7 methylate both guanidine nitrogen atoms resulting in the production of SDMA (Gary & Clarke, 1998) (Figure 2).

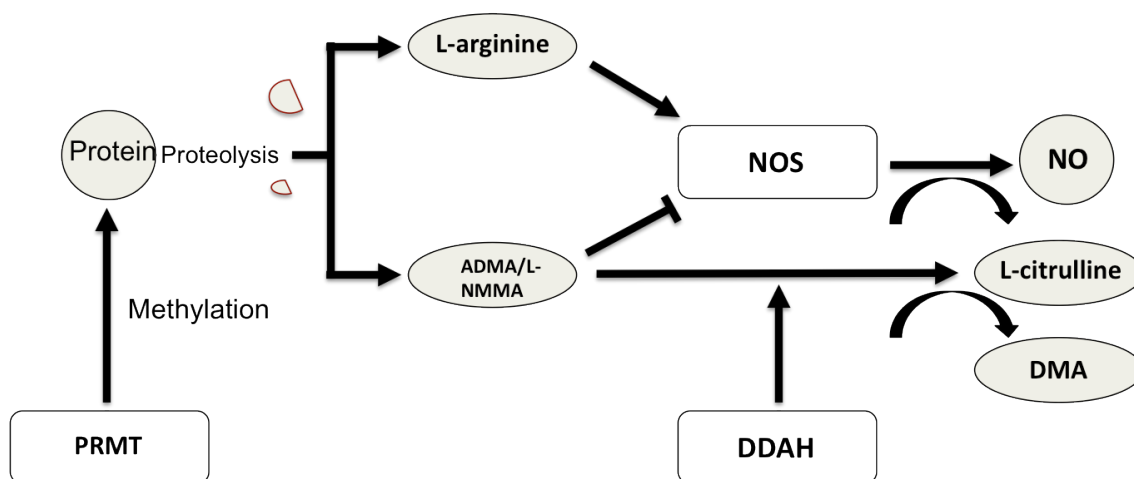


Figure 2. Endogenous regulation of NO production. Protein arginine methyl-transferases (PRMTs) methylate arginine residues that are then released to cytoplasm during proteolysis. Asymmetric dimethylarginine (ADMA) and monomethylated arginine (L-NMMA) are methylarginines that can inhibit NO production. Dimethylarginine dimethylaminohydrolases (DDAH) hydrolyze ADMA and L-NMMA and release NO production by NOS enzymes. ADMA and L-NMMA are metabolized to L-citrulline. This figure was kindly provided by Dr. James Tomlinson.

SDMA is not a NOS enzyme inhibitor but it can play a role in regulation of NO production by antagonizing L-Arginine uptake by the cells (Fleck et al., 2003). Elevated plasma SDMA levels occur in renal failure and closely associate with clinically accepted markers of renal function such as plasma creatinine (Fleck et al., 2003).

L-NMMA was amongst the first NOS inhibitors to be discovered due to its structural similarity to L-arginine. L-NMMA is a NOS enzyme inhibitor that can effectively block NO production from all three NOS isoforms (Vitecek, Lojek, Valacchi, & Kubala, 2012). It can also act as a reaction-based inhibitor for iNOS and nNOS but not for eNOS isoform (Reif & McCreedy, 1995).

Exogenous L-NMMA has been used extensively in experimental models and clinical trials to manipulate NO production. In guinea pigs, treatment with 10 $\mu\text{mol/l}$ of L-NMMA, reduced cardiac output and caused a drop to maximum left ventricular pressure (Casadei & Sears, 2003). Treatment of patients with septic shock with L-NMMA showed decreased cardiac output, increased systemic vascular resistance and improved survival rates (Kilbourn et al., 1990; Kilbourn et al., 1990; Kilbourn, Szabó, & Traber, 1997), although beneficial effects upon outcome were not seen in a subsequent randomized controlled trial (Lopez et al., 2004).

ADMA is another endogenous NOS inhibitor, which like L-NMMA has been shown to be a competitive non-specific inhibitor of all three NOS isoforms (Vitecek et al., 2012). During proteolysis, ADMA is released in the cytoplasm and can act as a NOS inhibitor in the same cell or it can be released into the circulation and be imported into cells elsewhere. Plasma levels of ADMA in healthy humans vary from 0.35-0.70 $\mu\text{mol/l}$ whilst in rodents concentrations up to 1 $\mu\text{mol/l}$ have been reported (Teerlink et al., 2009). Intracellular levels of ADMA have been reported to be up to 10-times higher than plasma levels (Masuda et al., 1999).

ADMA has been proposed as an independent risk factor for renal and cardiovascular diseases (Böger et al., 2009; Böger, 2003a, 2003b; Kielstein & Zoccali, 2005; Leone et al., 1992). In the cardiovascular system, increase in ADMA plasma levels has been associated with prediction of events and mortality (Böger et al., 2009) although in the Framingham Offspring study, ADMA plasma levels were associated with all-cause mortality but were not an effective predictor of cardiovascular events (Feinleib, Kannel, Garrison, McNamara, & Castelli, 1975). Finally, others have reported that increased ADMA in the plasma could predict subsequent myocardial infarctions (MIs) in patients that have survived a first incident (Schnabel et al., 2005).

1.1.3.2 Exogenous Inhibitors of NOS enzymes

A variety of exogenous inhibitors have been characterized based on their mechanism of inhibition and their specificity to the NOS isoforms (Vallance & Leiper, 2002). There are substrate analogues (L-arginine analogues), dimerization inhibitors and cofactor inhibitors.

L-NAME was one of the first synthetic NOS inhibitors to be discovered (Pfeiffer, Leopold, Schmidt, Brunner, & Mayer, 1996). Once L-NAME is transferred into the cells, it is readily hydrolyzed by ubiquitously present esterases to L-N^ω-Nitroarginine (L-NNA) (Vitecek et al., 2012). L-NNA is a potent inhibitor of all three NOS isoforms. L-NNA's binding to iNOS is immediate and can be rapidly reversed by L-arginine whereas it's binding with eNOS and nNOS is slower (Vitecek et al., 2012).

L-NAME has been extensively tested in a number of *in vivo* and *in vitro* experiments along with several clinical trials especially for the treatment of

septic shock. Treatment of endothelial cells with L-NAME showed angiogenesis inhibition, revealing the importance of NO in vessel formation (Papapetropoulos et al., 1997; Ziche et al., 1994). In animal models, L-NAME has been used mainly for the modulation of hemodynamics to treat septic shock (Kilbourn et al., 1997). Treatment of patients with sepsis with L-NAME has been shown to increase systemic vascular resistance and blood pressure but decreases cardiac index (cardiac index is the relation between cardiac output in a minute from the left ventricle to the whole body surface) (Lorente et al., 1993).

Compound 1400W is a slow, tight binding, highly selective inhibitor of iNOS isoform (Garvey et al., 1997). Selectivity of 1400W is 5000-fold higher over eNOS in humans and 1000-fold higher in rats (Garvey et al., 1997). Inhibition of iNOS by 1400W is NADPH dependent, slowly reversible and it does not affect the dimerization of the proteins (Lee et al., 2012). 1400W is a biologically active molecule that has been used for the selective inhibition of iNOS in both *in vitro* and *in vivo* experiments.

In healthy rats, 1400W did not have an effect on blood pressure and resting cerebral flow (Rosengarten et al., 2009). However, in septic rats 1400W managed to stabilize blood pressure but had adverse effects on evoked potential amplitude, the electrical potential measured in the neuronal system as a result of a stimulus (Rosengarten, Wolff, Klatt, & Schermuly, 2009b).

1.1.3.3 Dimethylarginine Dimethylaminohydrolases (DDAH)

The Dimethylarginine dimethylaminohydrolase (DDAH) enzymes hydrolyze ADMA and L-NMMA to L-citrulline and methylamines (Tran et al., 2000). Since ADMA and L-NMMA are NOS enzyme inhibitors, deactivation of these molecules plays an important regulatory role in NO production and signaling.

Two isoforms of the DDAH enzymes have been identified and extensively characterized: DDAH1 and DDAH2. *Ddah1* gene is located on the first chromosome (1p22) while *Ddah2* gene is located in the major histocompatibility complex III (MHC III) region of chromosome 6 (6p21.3) in humans. Phylogenetic analysis of the *Ddah* genes show high genetic conservation among different species (Tran et al., 2003).

Expression pattern analysis of the *Ddah* genes showed specific tissue expression in both embryonic and adult tissues. *Ddah1* expression levels remain unchanged between embryonic and adult samples when *Ddah2* expression is higher in embryonic tissues (Tran et al., 2000). *Ddah1* is expressed in the vasculature, the brain, the liver and the kidneys (Tran et al., 2000). *Ddah2* is predominantly expressed in the liver, the vasculature and the placenta. Both isoforms are also expressed in the heart (Tran et al., 2000). Finally, *Ddah2* is the only isoform that is expressed in macrophages, implicating *Ddah2* in responses of the immune system (Lambden et al., 2015).

Inhibition of DDAH enzymes by pharmacological means or disruption of gene expression has been shown to increase intracellular levels of NOS inhibitors therefore reducing NO production (Leiper et al., 2007). The role of *Ddah* genes along with their distinct expression pattern establishes DDAH enzymes as potential distinctive targets in pharmacological manipulation of NO levels in pathophysiology.

NO synthesis and its associated regulatory pathways has been reported to be modulated during mammalian development (Krumenacker & Murad, 2006). Moreover NO has been shown to play roles in the regulation of proliferation and differentiation of several cell types and tissues in the cardiovascular system (Feng et al., 2002; Nath & Madri, 2006).

1.2 Early Mammalian Developmental Stages

1.2.1 Zygote and Morula Stages

In mammals, early development of the embryo starts at the zygote level and continues in the morula, blastocyst and epiblast stage. The zygote is the first cell of the embryo, forming from the fertilization of the oocyte by the spermatozoon (Gray, 1858). Following sequential divisions called cleavage divisions, the zygote divides and grows to the 16-cell stage to form the morula on E2.5-3.0 days post coitum (dpc) in mice or E3-4 dpc in humans (Larsen et al., 2001) (Figure 3). Up to the morula stage, all cells have not differentiated and remain totipotent. Totipotent stem cells are non-differentiated cells that can give rise to all extraembryonic and embryonic tissues during development

(Mitalipov & Wolf, 2009). Characteristic examples are the cells of the zygote in mammals and the spores in asexual reproduction. Finally, on E3.5-4.5 dpc, the mouse embryo reaches the blastocyst stage. In humans, the blastocyst stage extends between E5-9 dpc (Larsen et al., 2001).

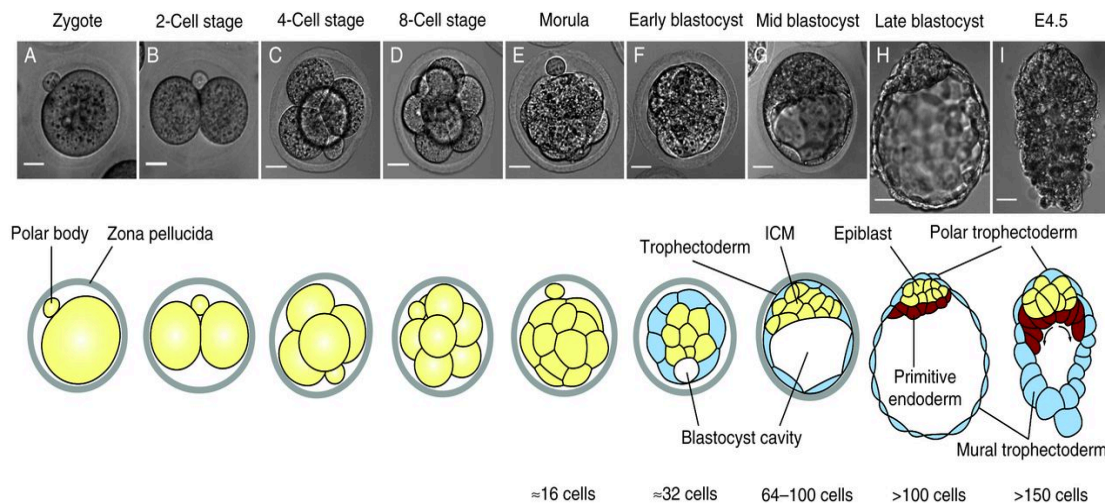


Figure 3. Early mammalian development. Embryonic development starts with the formation of the zygote (A). After sequential divisions and cell compaction the embryo reaches the stage of the morula (E). After the morula level, the embryo undergoes the first cellular differentiation and cavitation in order to form the blastocyst (F, G, H). The blastocyst is consisted of the Inner Cell Mass (ICM) (which gives rise to the embryo) and the trophoblast (giving rise to the placenta). After the formation of the blastocyst, the embryo continues to the development of the epiblast and the attachment of the embryo to the endometrium. (Department of Developmental Biology, Georgia Institute of Technology).

1.2.2 Blastocyst

The blastocyst stage is characterized by two major events: cavitation and cellular differentiation (Larsen et al., 2001). Cavitation is the process during which a cavity is formed in the middle of the blastocyst by the cells of the morula and guides later stages of embryonic development. The blastocyst is structurally divided into two well-defined areas: the Inner Cell Mass and the trophoblast (Martin, 1981) (Figure 4). The trophoblast forms the outer layer of cells of the blastocyst and consists of differentiated cells that in later stages give rise (in combination with the endometrium) to the placenta (Sherk, 2006). The ICM, on the other hand, is a formation of compact cells in the inner part of the blastocyst that give rise to the embryo and one extraembryonic tissue, the amniotic sac (Sherk, 2006). The cells of the ICM are pluripotent (or embryonic)

stem cells and were first isolated from mouse blastocysts in 1981 (Martin, 1981) and from human embryos in 1998 (Thomson, 1998).

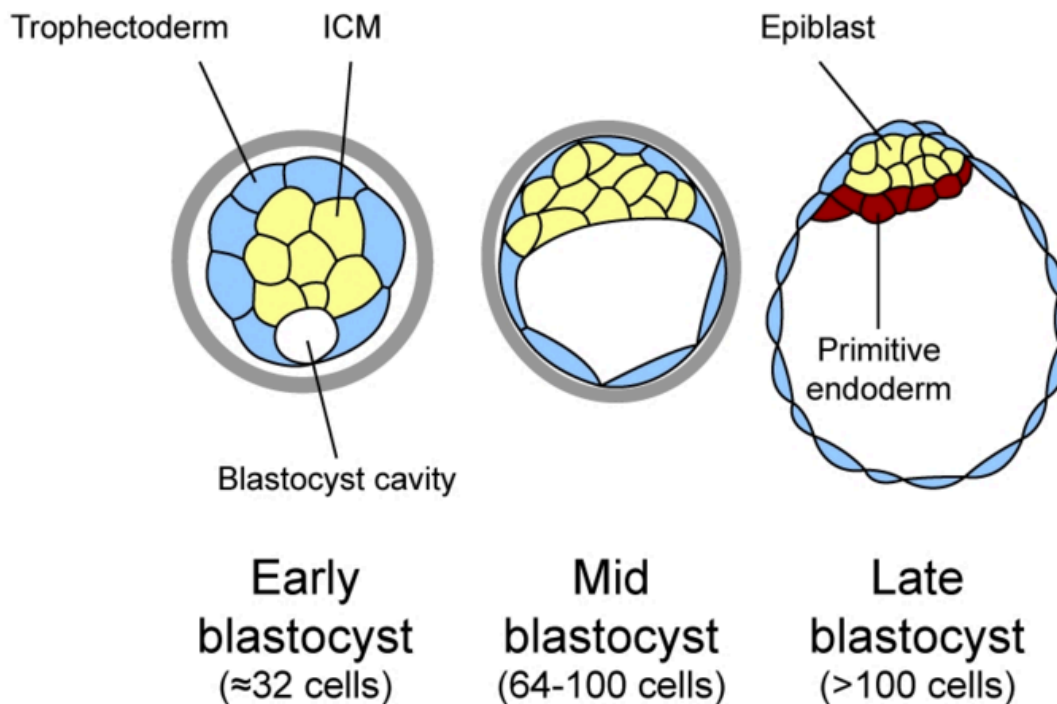


Figure 4. The mouse blastocyst. The ICM cells are portrayed as yellow spheres and the trophoblast is blue. The blastocyst stage is extended from E3.5 to E4.5 in mouse development (Frum & Ralston, 2015).

1.2.3 Isolation and Culture of Mouse Embryonic Stem (ES) cells

Since the first isolation of mouse embryonic stem cells, a variety of stem cell lines have been successfully isolated and cultured *in vitro* (Meissner, Eminli, & Jaenisch, 2009). Mouse blastocysts are removed from female individuals around 76 hours post coitum and are stripped of the zona pellucida (a glycoprotein layer that binds the spermatozoon and facilitates fertilization but prevents the zygote from attaching in the fallopian tubes) as preparation to attach on culture dishes lined with feeder cells (Meissner et al., 2009). Once the blastocysts are attached on the feeder layer, an outgrowth of the ICM is formed and the first colony of pluripotent stem cells is formed. During isolation and subsequently during culture *in vitro* embryonic stem cells are cultured in high-energy medium (15% Fetal Bovine Serum (FBS)) supplemented with Leukemia Inhibition Factor (LIF) to maintain a pluripotent state.

LIF is a cytokine that promotes growth of embryonic stem cells via inhibition of differentiation (Boiani & Schöler, 2005). Decrease in LIF levels are followed by cellular differentiation in pluripotent stem cells. Physiologically, LIF is expressed by the trophectoderm in developing embryos with the LIF receptor to be expressed in the ICM (pluripotent stem cells) (Larsen et al., 2001). Once pluripotent stem cells are isolated from the blastocyst and the trophectoderm has been completely removed, supplementation of LIF in the culture medium is essential for the cells to remain pluripotent (Boiani & Schöler, 2005).

1.2.4 Pluripotency and Master Regulators

Embryonic stem cells are characterized by two unique traits: pluripotency, the ability to differentiate into all three germ layers and renewal, the ability to divide for a long period of time (Martin, 1981).

Pluripotency is a state maintained by a network of transcription factors (LIF signaling cascade, BMP4 and WNT proteins) that bind primarily on promoter regions and result in the induction or repression of genes that implement stem cell pluripotency (Boiani & Schöler, 2005). *Oct4*, *Sox2* and *Nanog* are considered master regulators of pluripotency as shown by the induce pluripotency stem cell experiments carried out in 2006 (Takahashi & Yamanaka, 2006).

Oct4 gene is a transcription factor expressed by pluripotent stem cells during embryogenesis and is quickly downregulated during differentiation (Wu & Schöler, 2014). *Oct4* has been characterized as a master regulator of pluripotency in both mouse and human embryonic stem cells (Boiani & Schöler, 2005). For *Oct4* protein to bind DNA, it is essential for it to dimerize with *Sox2* protein (Guo et al., 2002). *Oct4*-deficient mice develop to the blastocyst stage, but already the cells of the ICM are not pluripotent (Nichols et al., 1998) (Figure 5). *Ex vivo*, cultures of mouse embryos from the stage of morula showed that embryos were able to mature to the blastocyst stage but development was arrested and the structures collapsed (Robertson, 1987). *In vitro*, embryonic stem cell lines with targeting vectors against *Oct4* gene, lost pluripotency upon homologous recombination and silence of the endogenous *Oct4* gene (Mountford et al., 1994). Finally, *Oct4* was one of the initial four factors required

for induction of pluripotency in mouse embryonic fibroblasts (Takahashi & Yamanaka, 2006).

Sox2 gene has been implicated in the regulation of transcription and chromatin architecture (Pevny & Lovell-Badge, 1997). Expression of *Sox2* gene has been confirmed in embryonic and neural stem cells (Pevny & Lovell-Badge, 1997). *In vivo*, *Sox2*-deficient mouse embryos, showed a defective primitive ectoderm due to the involvement of *Sox2* gene in pluripotent stem cells (Avilion et al., 2003). Also, there are strong indications that *Sox2* along with *Oct4* protein are required in early cell fate decisions guiding the first three lineages present at implantation (Avilion et al., 2003). Finally, *Sox2* gene is one of the four transcription factors that are essential for successful derivation of pluripotent stem cells (is cells) as specified by (Takahashi & Yamanaka, 2006).

Nanog is another transcription factor that have been associated with maintaining pluripotency in mouse and human embryonic stem cells (Boiani & Schöler, 2005). *Nanog* gene is expressed in mouse and human embryonic stem cells and is one of the transcription factors that is downregulated upon induction of differentiation (Chambers et al., 2003). *Nanog*^{-/-} mouse embryos show complete absence of primitive ectoderm during development and isolated *Nanog*^{+/-} stem cells show higher chances of spontaneous differentiation (Hatano et al., 2005; Mitsui et al., 2003) (Figure 5). Finally, *Nanog* was not included in the four transcription factors identified as indispensable for induction of pluripotency in mouse embryonic stem cells (Takahashi & Yamanaka, 2006) although it was characterized as a master regulator of pluripotency by previous research results (Chambers et al., 2003; Mitsui et al., 2003).

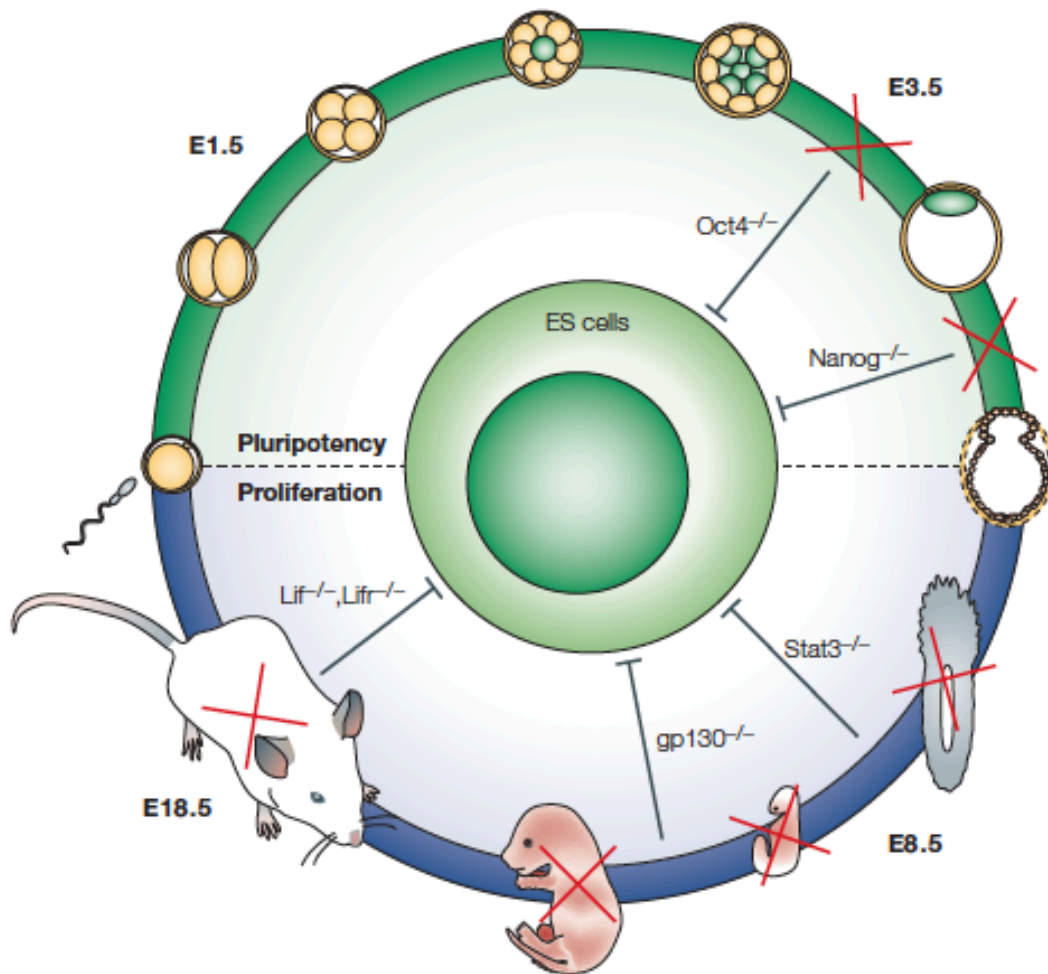


Figure 5. Various pluripotency-associated protein knockout animal models and the arrest stage of embryonic development. *Oct4^{-/-}* is lethal on E3.5 of mouse embryonic development as it mainly affects the pluripotent state of the ICM. *Nanog^{-/-}* embryos pass the blastocyst stage during development but they soon after, arrest their development. Deletion of the LIF or LIF receptor (LIFR) is fatal on E18.5 (Boiani & Schöler, 2005).

1.2.5 Gastrulation and Epithelial-to-Mesenchymal Transition (EMT)

After the formation of the blastocyst and as development further progresses in the mouse embryo, gastrulation is the next developmental hallmark. Gastrulation is the process by which the three embryonic germ layers ectoderm, mesoderm and endoderm are formed from the epiblast (Ferrer-Vaquer et al., 2010). The major role of gastrulation in mammalian development is the rearrangement of cell populations from the epiblast to a multilayered structure that will allow organogenesis to begin. In mouse embryos, this cell allocation starts with the primitive streak formation and occurs via the EMT (Ferrer-Vaquer et al., 2010).

EMT is the first process that leads to gastrulation. During EMT, the cells of the epiblast that are characterized by epithelial characteristics (tight junctions and cellular shape) break their tight formation and start forming the primitive streak (Tam et al., 2001). Formation of the primitive streak establishes bilateral symmetry and beginning of cell migration and germ layer formation (Tam et al., 2001). Once cells migrate through the primitive streak, they acquire either an endodermal or mesodermal identity (Loebel et al., 2003). The cells that migrate first through the primitive streak become endoderm and they guide the formation of the mesoderm in the space defined by the endoderm and the ectoderm layers (Loebel et al., 2003) (Figure 6). Cells that do not migrate through the primitive streak will eventually give rise to the ectoderm (Tam et al., 2001) (Figure 6).

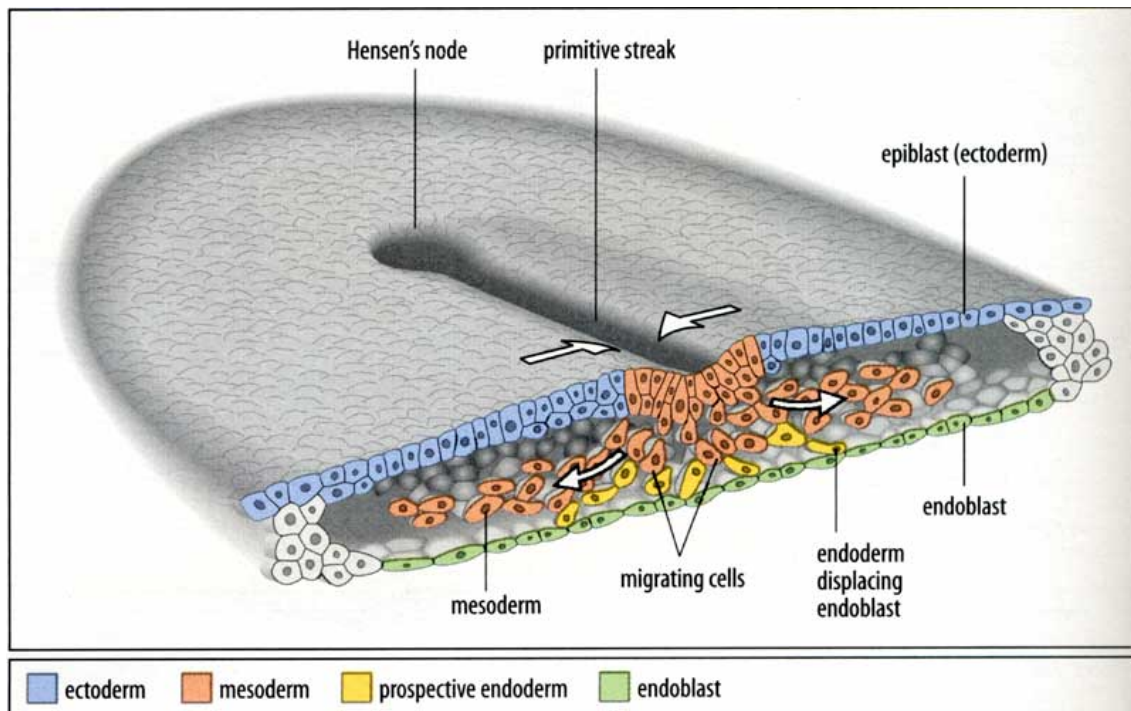


Figure 6. Gastrulation in human embryos. Primitive streak formation is the first process of gastrulation in the mammalian and avian embryos. Cells start migrating through the primitive streak and towards the inner part of the embryo to form the endoderm (yellow) and the mesoderm (red) germ layers. The cells shown in blue do not migrate through the primitive streak and they form the ectoderm (Larsen et al., 2001).

1.2.6 Differentiation of Embryonic Stem (ES) cells *in vitro*

Differentiation of isolated embryonic stem cells to the three germ layers and specific cell types was accomplished via three specific approaches: formation of embryoid bodies (EBs) (Chen, et al., 2011; Ng et al., 2005), coculture with stroma cells (Moore et al., 2004) and differentiation of ES cells in a monolayer on extracellular matrix proteins (Raval et al., 2013).

All three protocols have their advantages and disadvantages when it comes to ES cell differentiation to specific cell types. Culture of ES cells on stroma cells suffer from great variability in specific cell type differentiation and isolation of the differentiated stem cells from the stroma cells can be difficult (Moore et al., 2004). Culture in a monolayer on extracellular matrix proteins can produce large amounts of one specific cell type but it can fail to recapitulate early embryonic differentiation stages (Raval et al., 2013). EB formation has been the 'traditional' protocol for ES differentiation as it provides a 3D structure for stem cells to differentiate, a feature that is needed by a variety of cell types. The 3D structure provided by the embryoid bodies recapitulate closely the physiological structure of the ICM and the developmental stages following each differentiation (Kurosawa, 2007). Also, it is reproducible and with the supplementation of specific signaling factors into the culture medium, differentiation to specific cell types can be enhanced or blocked (Ng et al., 2005).

1.2.6.1 Trophoblast

The trophoblast consists of the outer layer of the blastocyst and in later stages of development gives rise to specific cell types of the embryonic part of the placenta. The trophoblast is the only extraembryonic tissue that can be derived from mouse embryonic stem cells *in vitro* (Hemberger et al., 2003).

Glial cell missing 1 (GCM1) protein is a chorion-specific DNA binding protein encoded by the *Gcm1* gene and is essential for the development of the placenta (Basyuk et al., 1999). In mice, heterozygous *Gcm1* deletion, affected the differentiation and structure of the syncytiotrophoblast, the layer of epithelial cells covering placental villi (Bainbridge et al., 2012) in early developmental

stages and was associated with preeclampsia in later stages of pregnancy (Chen et al., 2004).

1.2.6.2 Ectoderm

Ectoderm is one of the three germ layers and during embryonic development it differentiates to give rise to the nervous system and the epidermis (Keller, 1995).

A wide array of markers have been used to identify embryonic stem cells differentiating to an ectoderm fate (Karsten et al., 2003; Martí et al., 2013) with one of the most well-characterized being *Tuj1* protein (Neuron-specific class III β -tubulin) (Lee et al., 1990). *Tuj1* tubulin has been identified as a neuronal specific type of β -tubulin and that its expression can be located in neuronal progenitor cells (Karsten et al., 2003) and E12 mouse embryonic brains (Menezes & Luskin, 1994).

1.2.6.3 Endoderm

Endoderm is one of the two germ layers (along with mesoderm) that are formed by cells that migrate through the primitive streak during gastrulation of the embryo. In later developmental stages, endoderm develops into the gastrointestinal tube and its associated organs (liver and pancreas), the respiratory tract, endocrine glands and the urinary bladder (Gilbert SF., 2000).

An essential regulator of endoderm formation is *Sox17* gene (Lewis & Tam, 2006). *Sox17* is a transcription factor expressed in early stages of endoderm development such as the formation of visceral and definitive endoderm (Kanai-Azuma et al., 2002). Deletion of *Sox17* gene in mice, effects the development of definitive endoderm and embryos show complete depletion of gut endoderm (Kanai-Azuma et al., 2002). *Sox17*^{-/-} ES cells can form both mesoderm and ectoderm tissues but mid and hindgut endoderm differentiation is completely blocked (Kanai-Azuma et al., 2002).

1.2.6.4 Mesoderm

Mesoderm is the third and last germ layer and is formed between endoderm and ectoderm as the interaction of the two germ layers guides its differentiation. In later stages of development, mesoderm gives rise to the muscles, bones, the heart, the blood vessels and the circulating blood cells (Papaioannou V., 2004).

The *Brachyury* gene is strongly associated with mesoderm differentiation and is one of the transcription factors regulating its establishment (Martin & Kimelman, 2010). *Brachyury* gene deletion was first identified in mouse embryos but its role was not verified until the cloning of the *Drosophila* equivalent *optomotor-blind* gene (Pflugfelder et al., 1992). *Brachyury* expression is required for mesoderm and axial formation (Schulte-Merker & Smith, 1995). Finally, mouse embryos with a gene deletion for *Brachyury* gene, exhibit a truncated body axis towards the caudal end (B. L. Martin & Kimelman, 2010).

1.2.6.5 Hematopoietic Differentiation

Hematopoietic differentiation is the process during embryonic development that gives rise to all blood cell types.

Development of the hematopoietic system is initiated early on during embryonic development and is separated into four different 'waves' of production according to the area of initiation (Orkin & Zon, 2008). The four stages of differentiation and hematopoietic development are: the yolk sac (or primitive) hematopoiesis in parallel with the hemangioblast or hemogenic endothelium, the aorta-gonad mesonephros (AGM) stage, the fetal liver blood production and the bone marrow formation.

The yolk-sac is the first area (blood islands) of hematopoiesis in the developing embryo and differentiation of embryonic stem cells *in vitro* can recapitulate this stage of development accurately (Murry & Keller, 2008). Hematopoietic stem cells produced during yolk-sac hematopoiesis, give rise to early erythropoiesis and later on migrate to the aorta-gonad mesonephros region to give rise to later stages of blood cell types (Galloway & Zon, 2003).

Parallel to the hematopoiesis occurring in the yolk-sac, it has been hypothesized with recent data supporting it, the existence of the hemangio-blast or hemogenic endothelium (Antas et al., 2013). Hemangioblast consists of a common precursor that differentiates to vascular endothelial and blood cells during embryonic development (Choi et al., 1998). A variety of transcription factors and soluble signaling molecules is required for the development of the hemangioblast (Antas et al., 2013). Growth factors like VEGF and transcription factors like *GATA2* have been implicated into the differentiation of embryonic stem cells to the hemangioblast fate (Orkin & Zon, 2008).

The AGM stage is initiated by the formation of the aorta and includes the formation of the mesonephros. The mesonephros has the same function as the adult kidney even though none of the mature kidney structure arises from it. This stage is detectable between E10.5-E11 in mice (Orkin & Zon, 2008).

Finally, following the AGM stage, hematopoietic stem cells migrate and colonize the fetal liver and eventually they migrate to form the bone marrow (Galloway & Zon, 2003). Adult hematopoiesis gives rise to all circulating blood cells and is restricted to the bone marrow. Organs like the thymus or the spleen play a role in maturation of specific cell types of the immune system (Murry & Keller, 2008).

A selection of transcription, surface or functional marker genes were used to identify different stages and cell types during *in vitro* differentiation of embryonic mouse stem cells. *Aml1* (or *Runx1*) and CD34 were used to identify stem cells with a hematopoietic fate and CD31 (or *Pecam-1*) and smooth muscle actin (SMA) were used as specific markers for endothelial and smooth muscle cells.

Runx1 gene is a transcription factor required for the establishment of hematopoiesis during development (Lorsbach et al., 2004). In *Runx1*^{-/-} mice, definitive hematopoietic progenitors are absent and only primitive erythrocytes were obtained from *Runx1*^{-/-} ES cell differentiation to hematopoietic cell lines (Swiers, De Bruijn, & Speck, 2010). Deletion of *Runx1* gene blocked hematopoietic differentiation emerging from the endothelial cells lining major vessels such as the aorta and more specifically the endothelial-hematopoietic transition indicating a specific differentiation role of the gene during the hemangioblast stage of embryonic development (Swiers et al., 2010). Finally, *Runx1* gene has been shown to regulate major developmental genes such as

Scl, *Lmo2*, and *Gata2*, genes of both hematopoietic and cardiac differentiation pathways (Antas et al., 2013).

CD34 is a surface antigen that has been widely associated with bone marrow cells and their differentiation (Healy et al., 1995). Even though its exact role in bone marrow differentiation has not been yet verified, it has traditionally been used for the identification of hematopoietic cells for clinical treatments (Goodell, 1999). CD34 protein expression does not affect the differentiation and development of the hematopoietic stem cells but increased CD34 protein expression enhances the adhesion ability of the hematopoietic stem cells to the stroma cells therefore implicating CD34 in compartmentalization of the bone marrow (Healy et al., 1995).

CD31 protein belongs to the cell adhesion molecule family and is specific to endothelial cells (Choi et al., 1998). During embryonic development, endothelial and hematopoietic precursors emerge together with endothelial cells displaying higher expression of cell adhesion molecules such as CD31 (Choi et al., 1998). Even though deletion of CD31 gene does not affect endothelial development and vessel formation, CD31 remains an important surface molecule with involvement in immunological responses in adult mouse individuals (Ma et al., 2012).

Finally, expression of α -smooth muscle actin (*Acta2*) was used to assess the differentiation of embryonic stem cells to smooth muscle cells. *Acta2* gene is expressed in mature smooth muscle cells making over 70% of a smooth muscle cells actin (Owens, 1995). Also, *Acta2* gene is expressed in a variety of cell types rendering it a good marker for smooth muscle cells but not a specific one. Other smooth muscle specific markers include smooth muscle calponin, smoothelin and smooth muscle myosin heavy chain (Rensen, Doevendans, & van Eys, 2007).

1.2.6.6 Early Cardiac Development and Differentiation

The heart is the first organ to form during embryonic development and the cardiac precursor cells are among the first cells to be allocated and begin to differentiate (Harvey, 2002).

Early cardiac differentiation during embryonic development is characterized by two major events: the first migration of cardiac precursors

during gastrulation and the second migration that is responsible for the heart tube formation. These events are known as first and second cardiac fields.

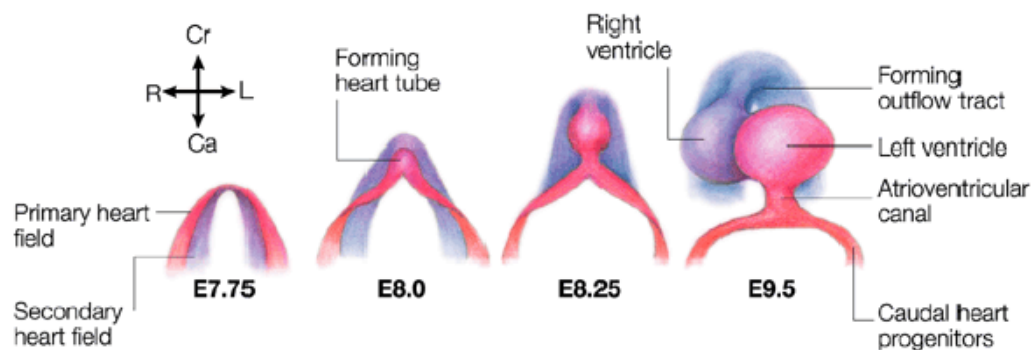


Figure 7. Early cardiac differentiation and first stages of heart tube formation in mice. Initially, cells that migrate through the primitive streak form the cardiogenic region on the cranial end of mesoderm. After cardiogenic region is defined, the first (FHF) and second (SHF) heart fields are formed. Later, the two fields fuse and form the heart tube (Lin et al., 2012). On E9.5, the left and right ventricle form along with their respective flow tracts. The compass indicates the body axes. Ca, caudal; Cr, cranial; L, left; R, right. Figure adapted from (R. Kelly, Brown, & Buckingham, 2001).

The first cardiac field is established during gastrulation and formation of mesoderm and consists of establishment and allocation of cardiac precursors (Evans et al., 2010). Amongst the first cells that migrate through the primitive streak are cardiac precursors. Once these cells migrate through the primitive streak, they are allocated close to the cranial end of mesoderm, called cardiac mesoderm, and give rise to the first cardiac field (Evans et al., 2010). The formation of the first cardiac field is orchestrated by a variety of signaling molecules produced by the cardiac precursors themselves or by neighboring tissues that drive the differentiation of cardiac precursor cells (Figure 7). Bone Morphogenic Proteins (BMPs) and Fibroblast Growth Factor (FGF) are the most characterized signaling molecules for cardiac differentiation produced by cells of endoderm fate neighboring the cardiac fields (Harvey, 2002). Alongside with external signaling, a variety of transcription factors act within the nuclei of cardiac precursors such as *Gata4* and *Nkx2.5* genes and initiate cardiomyocyte differentiation (Devine et al., 2014).

Gata4 is a transcription factor that plays a central role in cardiac formation and cardiomyocyte differentiation during early embryonic development (McCulley & Black, 2012). Expression of *Gata4* gene is retained from embryonic development throughout adulthood (Molkentin et al., 1997). *Gata4* upregulates the expression of cell cycle regulators therefore controlling

the proliferation of cardiomyocytes (Rojas et al., 2008) and in combination with transcription factors such as *Nkx2.5* can regulate the expression pattern of a plethora of genes involved in cardiac development (McCulley & Black, 2012). In mice, deletion of the *Gata4* gene, is lethal on day E7 due to severe developmental abnormalities associated with heart tube formation (Molkentin et al., 1997).

Nkx2.5 is one of the most important transcription factors in cardiac development since it is implicated in a wide variety of regulatory processes affecting cardiomyocyte formation. Its expression is regulated by GATA and SMAD proteins and is also retained during adulthood (McCulley & Black, 2012). *NKX2.5* protein promotes later stages of cardiac differentiation and chamber identity mainly by driving the expression of other cardiac differentiation associated genes (Targoff, Schell, & Yelon, 2009). Finally, *Nkx2.5*^{-/-} mouse embryos arrest their development on E9 due to abnormal heart formation and growth retardation due to failure of loop formation by the cardiac tube (Lyons et al., 1995).

Even though the majority of cells involved in cardiac formation derive from the first heart field, a significant amount of cells contributing to smooth muscle cells, endothelial cells and cardiomyocytes originate in the second heart field (Epstein, 2010). The second heart field is formed ventral to the first heart field and its major contribution is towards the outflow tracks of the heart (Dyer & Kirby, 2010). Its contribution is vital for the correct alignment of the aorta and the pulmonary artery by elongation of the outflow tracks of the heart (Dyer & Kirby, 2010). Finally, malformation of the second heart field has been associated with a variety of cardiomyopathies in adults (Epstein, 2010).

After the formation of the two heart fields and their fusion to create the heart tube, formation of the heart continues with the creation of the four chambers and their inflow and outflow tracks (Moorman et al., 2003).

1.3 Heart Formation and Function

1.3.1 Late Cardiac Development

The anatomical formation of the heart as it is presented in adult individuals starts right after the establishment of the two heart fields and the

formation of the heart tube (Christoffels, Burch, & Moorman, 2004). Late heart development is characterized by two major events: the loop formation by the heart tube that gives rise to the two ventricles and the formation and elongation of the atrioventricular canal that gives rise to both atria (Moorman et al., 2003) (Figure 8).

The first major event towards the formation of the four cardiac chambers is the looping of the elongated heart tube. First, the elongated tube starts looping towards the right side of the developing body, a motion that creates a bulge towards the left side (Harvey, 2002). The bulge created on the left is going to give rise to the left ventricle, the first of the four chambers to be formed. The right ventricle is formed adjacent to the left ventricle and starts developing in parallel with the left ventricle (Harvey, 2002). The master regulator, *Nkx2.5*, orchestrates the cardiac looping as well as the formation of the ventricles. Its expression is maintained during development and is restricted to ventricular cardiomyocytes in later developmental stages (McCulley & Black, 2012).

Once the cardiac loop has been formed and the ventricles have commenced their development, the atria start to form at the end of the atrioventricular canal towards the inflow tract (Stennard, 2005) (Figure 8). The atria are formed after the elongation of the atrioventricular canal, which further divides into the right and left atria (Moorman et al., 2003). Finally, the forming atria move in place and fuse with the developing ventricles forming the cardiac valves (Stennard, 2005). Complete development of the atria and especially the left atrium requires the formation of the lungs since the formation of the pulmonary vein drives the formation of the atria (Moorman et al., 2003).

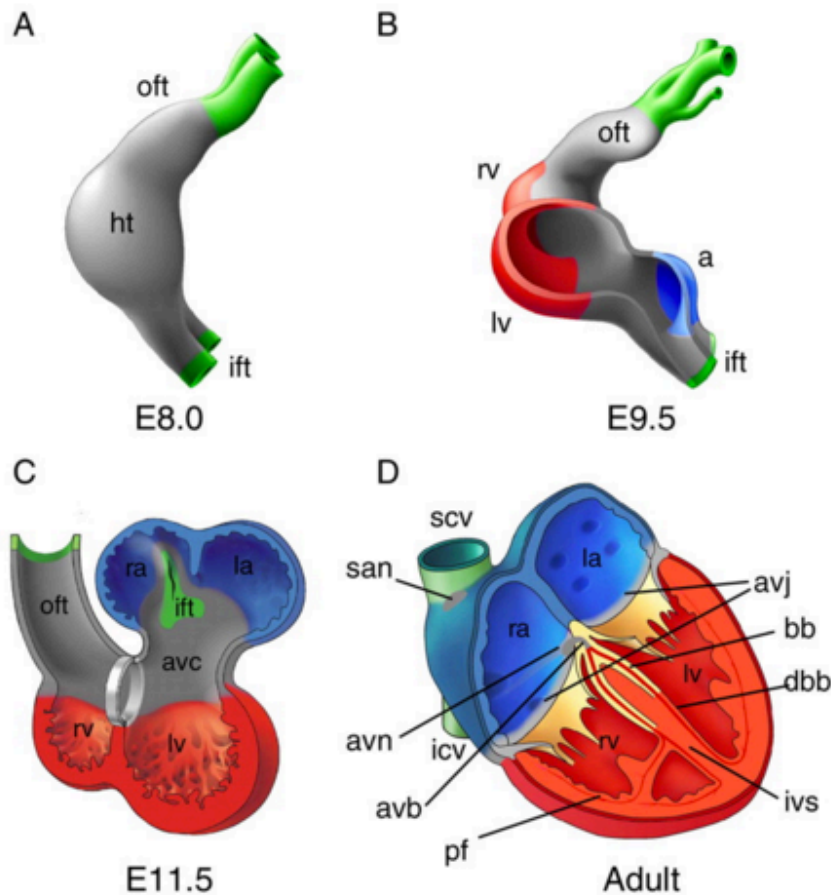


Figure 8. Heart tube formation, looping and chamber formation of the mouse heart during embryonic development. **A.** Heart tube formation by fusion of the first and second heart field on E8. ht: heart tube, ift: inflow track, oft: outflow track **B.** Looping of the heart tube creating the precursors of the two ventricles and the atria on E9.5. lv: left ventricle, rv: right ventricle, a: atria. **C.** Further development of the heart by formation of the two atria via the extension of the atrioventricular canal on E11.5. avc: atrioventricular canal, ra: right atrium, la: left atrium. **D.** The fully developed adult mouse heart. Figure was modified from (Christoffels et al., 2004) and acquired by (Stennard, 2005).

1.3.2 Anatomy of the Adult Heart

The mammalian heart is located in the middle of the thorax between the lungs and is surrounded by the pericardium, which contains pericardial fluid to reduce friction between the heart and surrounding tissue. The mammalian heart consists of four chambers: two atria and two ventricles. This particular anatomy makes it the most efficient layout when compared to less evolved species.

The cardiac anatomy can be divided by the two separate blood flows that occur during cardiac cycle: the pulmonary flow (deoxygenated blood going through the lungs) from the right side of the heart and the body flow (oxygenated blood delivered to body tissues) from the left side of the heart

(Figure 9). The right side consists of the right atrium, the right ventricle, the pulmonary and tricuspid valve and the pulmonary artery (Gray, 1858). The left side contains the left atrium, the left ventricle, the mitral and aortic valve and the aorta (Gray, 1858). Finally, both sides of the heart and their respected circulations are divided by the atrioventricular septum.

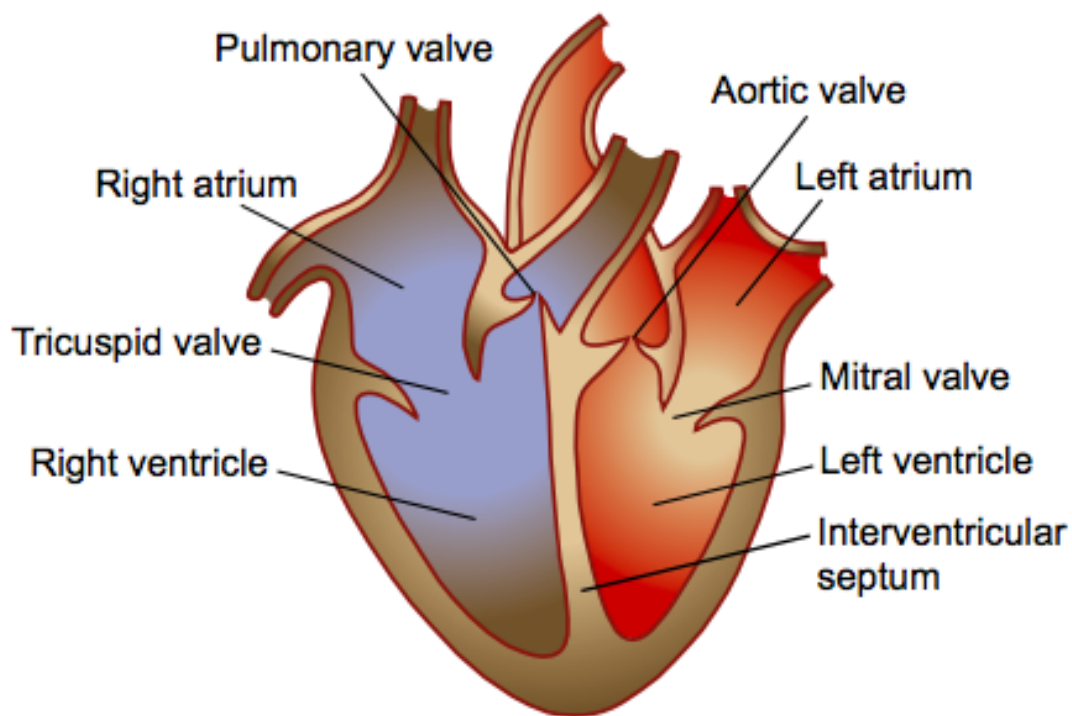


Figure 9. Anatomy of the mammalian heart. The right side of the heart (blue) consists of the right atrium, the right ventricle and the two valves, the pulmonary and tricuspid valve. The left side contains the left atrium, the left ventricle, the mitral valve and the aortic valve. (Shah et al., 2009).

The heart is connected to the circulatory system through six major vessels, two arteries and four veins. The blood coming from the body enters the right atrium through the superior and inferior vena cava and exits the right ventricle with direction to the lungs by the pulmonary artery (Shah et al., 2009). Two pulmonary veins return the oxygenated blood to the left atrium and the blood then exits the left ventricle towards the head and the body circulation through the aorta (Shah et al., 2009).

Cardiac muscle is characterized by high requirements of oxygen and energy in order to maintain blood circulation. The heart receives blood through the coronary circulation. Two main arteries, the left and right coronary artery,

originating from the beginning of the aorta immediately after the aortic valve, supply the cardiac muscle with oxygenated blood (Gray, 1858). The left coronary artery that supplies the left ventricle with blood immediately divides into the left circumflex and the left anterior descending artery (LAD). Deoxygenated blood is drained by the coronary sinus, the anterior cardiac veins and the lesser cardiac veins back into the right atrium (Shah et al., 2009).

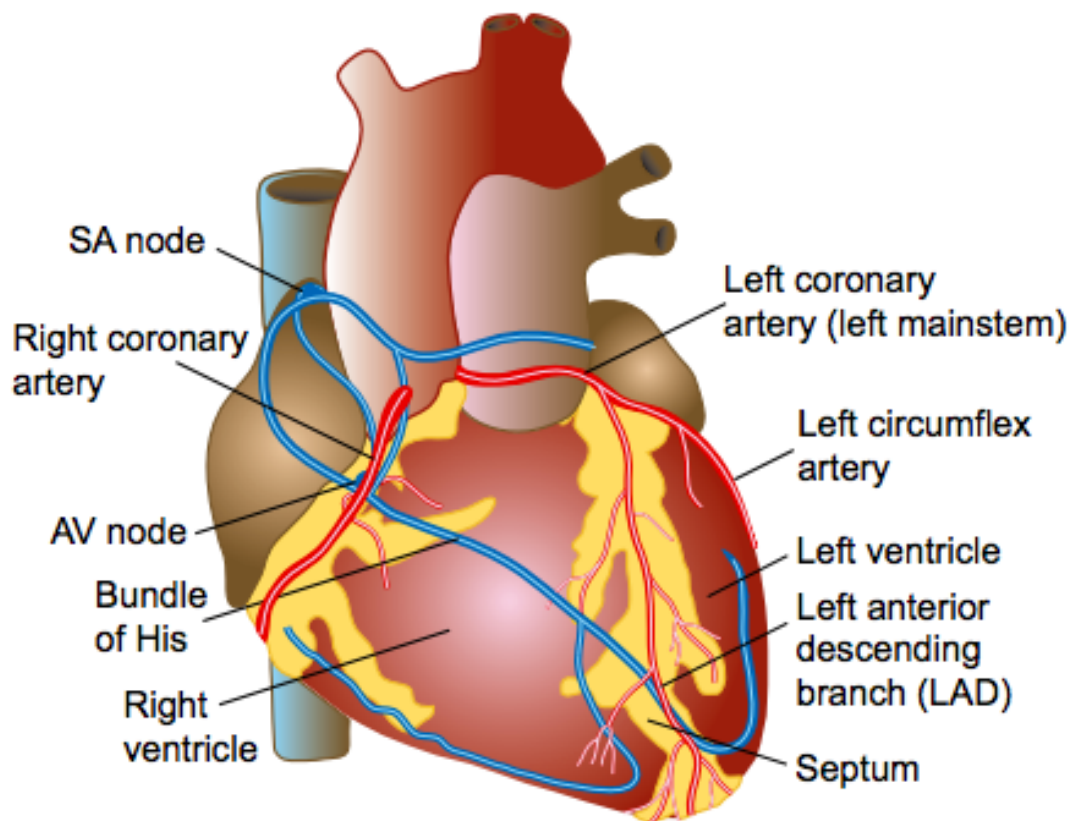


Figure 10. Circulatory and conduction system of the mammalian heart. Coronary arteries (red) supply cardiac muscle with oxygen and nutrients. The conduction system (blue) of the heart orchestrates the cardiac cycle. (Shah et al., 2009).

Finally, essential for orchestrating the cardiac cycle is the conduction system of the heart. A highly specialized network of neurons is responsible for coordinating the contraction of all parts of the heart involved in the cardiac cycle in a timely manner (Shah et al., 2009). The Sinoatrial node (SA) is responsible for initiation of the cardiac cycle. The stimulus then travels through the intermodal tracts to the atrioventricular node (AV) and through the His bundle to the Purkinje fibers to complete the cardiac cycle (Figure 9).

1.3.3 Cardiac Cycle

A cardiac cycle consists of contraction and relaxation of both atria and ventricles. The purpose of the cardiac cycle is maintaining the blood flow through the body.

The onset of the cardiac cycle is the depolarization of the pacemaker cells (specialized cardiomyocytes), which reside on the SA node and the sequential contraction of the right and left atria (Shah et al., 2009). Heart rate, which is the number of contractions per minute, is controlled by the pacemaker cells and is also regulated by nerve fibers of the sympathetic and parasympathetic system. This event is portrayed as the P wave on an electrocardiogram (Figure 11). Atrial contraction results in increased pressure into the atria causing the atrioventricular valves to open allowing blood flow into the ventricles.

The impulse generated by the SA node, travels and reaches the AV node. There the impulse is delayed giving the opportunity to the atria to complete their contraction before the initiation of ventricular contraction (Guyton & Hall, 2011). Leaving the AV node, the electric impulse travels through the ventricular bundles and the Purkinje fibers to the rest of the ventricular myocardium causing it to contract (Guyton & Hall, 2011). At this point, the pressure rises into the ventricles causing the opening of the aortic and pulmonary valve and blood being ejected from the ventricles. Both atrioventricular valves close due to high ventricular pressure blocking blood ejection towards the atria. Contraction of the ventricles appears at an electrocardiogram as the QRS complex (Figure 11). During ventricular contraction, the atria start to fill with blood, preparing for the initiation of the next cardiac cycle.

After completion of the QRS complex, the ventricles relax and repolarize. Repolarization of the ventricles is shown as the T wave on an electrocardiogram (Figure 11) (Shah et al., 2009). At the end of ventricular repolarization, the heart is ready to commence another cardiac cycle.

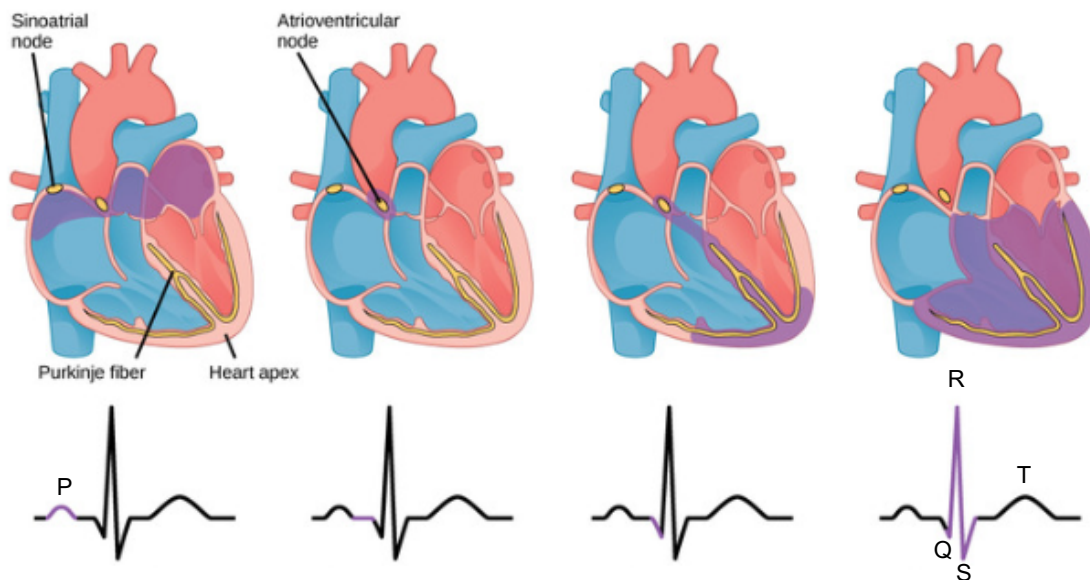


Figure 11. Electrocardiogram trace of a cardiac cycle related to major events of the cardiac muscle. P wave is the first event of cardiac cycle and signifies the contraction of the atria. QRS complex follows P wave and is caused by the activation of the ventricles. Finally, repolarization of the ventricles is depicted as the T wave on an electrocardiogram trace. (American Heart Association).

1.3.4 Contraction Mechanism of Cardiomyocytes in a Molecular Level

The cytosol of cardiomyocytes contains a large number of myofibrils, which are the basic unit of the contraction device (ter Keurs, 2012). Myofibrils consist of two major units: the thick and thin filaments. The thick filament contains mainly cardiac myosin chains (heavy and light) and titin proteins whereas the thin filament is comprised of cardiac actin, tropomyosin and the three troponins: C, T and I (Moss, Razumova, & Fitzsimons, 2004) (Figure 12).

Excitation contraction coupling is the process that leads from electrical excitation of the myocyte, through intracellular Ca^{2+} influx, to the contraction of the heart (Bers, 2002). In different experimental models, there have been quite a few differences recorded in between experimental models such as mice, rats and human stem cell derived cardiomyocytes and fetal ventricular myocytes (Hintz et al., 2002; Zhu, Santana, & Laflamme, 2009). Isolated C57/BL6 adult mouse cardiomyocytes exhibit higher peak shortening, shortened duration of contraction and relaxation, elevated velocity of relengthening associated with different Ca^{2+} homeostasis when compared to isolated adult rat cardiomyocytes (Hintz et al., 2002). Finally, in human cardiomyocytes there is a tight control of EC coupling from earlier stages of development, a characteristic that

distinguishes human cardiomyocytes from other model species such as rat and mouse (Zhu et al., 2009). Overall, it is important to take caution when experimental results are transferred between model organisms and humans.

During excitation contraction coupling, NO has been shown to play a major role through implication in various pathways. It has been shown to play a role through interaction with calcium-handling proteins (Ca^{2+} is a cofactor for the production of NO by the NOS enzymes) (L. Xu, Eu, Meissner, & Stamler, 1998), contractile myofilaments (Layland, Li, & Shah, 2004) and respiratory complexes (Clementi, Brown, Feelisch, & Moncada, 1998). Finally, experiments in knockout animals has shown that $\text{eNOS}^{-/-}$ mice exhibit mildly lower basal contractility and $\text{nNOS}^{-/-}$ mice exhibit the opposite phenotype, slightly elevated basal contractility (Hare, 2003; Khan et al., 2003).

Initiation of contraction is based on one main event: the release of Ca^{2+} into the cytosol from the endoplasmatic reticulum (ER) and the intracellular matrix (Fearnley, Roderick, & Bootman, 2011). Ca^{2+} release and reabsorbance into the ER is tightly orchestrated between heart beats by a variety of proteins and channels (Fearnley et al., 2011). Some of the major proteins involved in the process are Na^{+} and Ca^{2+} channels, $\text{Na}^{+}/\text{Ca}^{2+}$ exchange proteins and $\text{Na}^{+}/\text{K}^{+}$ pumps (mainly involved in cardiac action potential).

Contraction of cardiomyocytes in a molecular level is best characterized by the sliding filament model (Huxley & Niedergerke, 1954). According to this model, during cell rest state, thin filaments are not bound by the thick filaments due to available sites being obstructed by the structure of tropomyosin (Shah et al., 2009). During neuronal excitation and increase of intracellular $[\text{Ca}^{2+}]$, Ca^{2+} binds troponin C (of the troponin-tropomyosin complex) and causes a structural reconfiguration, which exposes the binding sites of cardiac actin to the myosin chains (ter Keurs, 2012). This reconfiguration involves troponins T and I and the tropomyosins. Upon exposure of the actin sites to the thick filament, myosin forms cross bridges and converts ATP into force (Huxley & Niedergerke, 1954) (Figure 12). As a result, both filaments start to slide and the sarcomere shortens (ter Keurs, 2012). Once contraction has been achieved, Ca^{2+} pumps reabsorb Ca^{2+} into the ER or into the intracellular space causing the troponin-tropomyosin complex to reconfigure its structure and the filaments to slide back into their relaxed state (Kobayashi, Jin, & de Tombe, 2013).

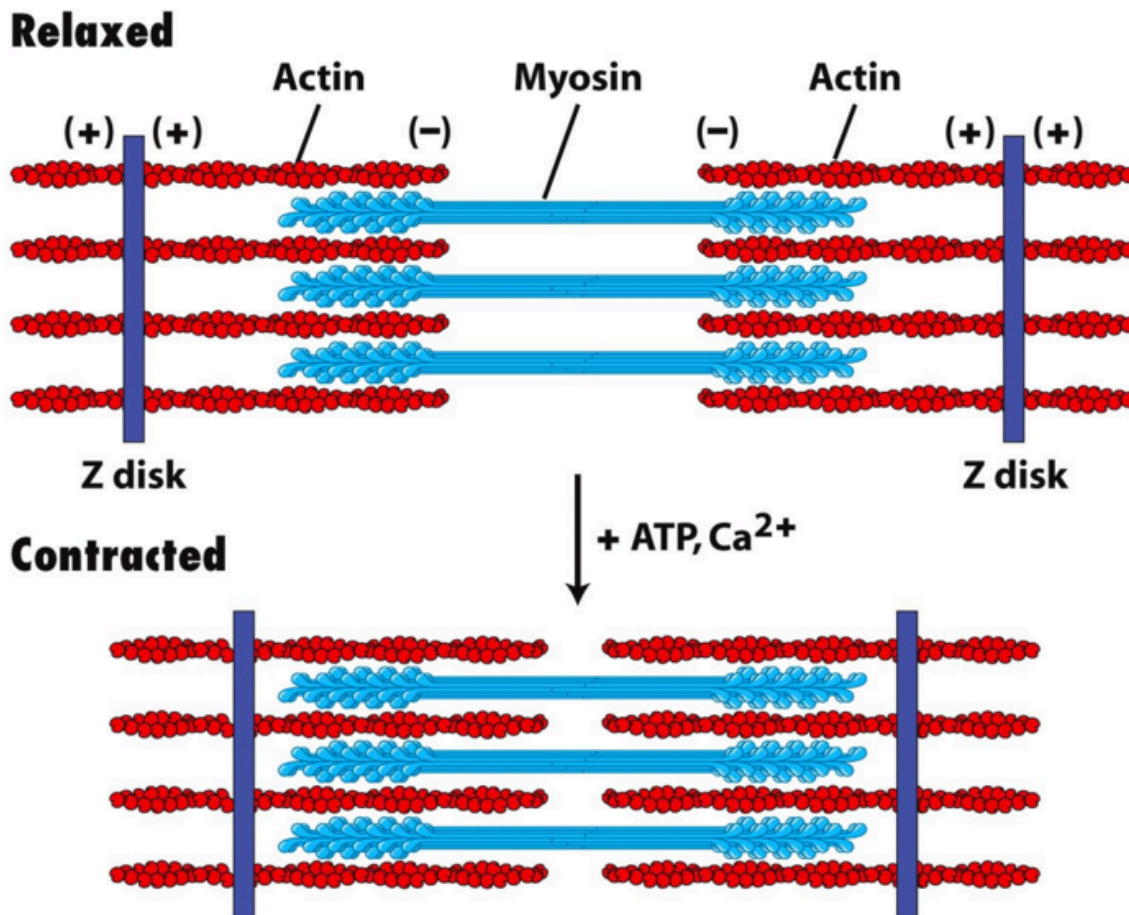


Figure 12. Schematic of the myosin-actin sliding model in relaxation and contraction. During relaxation, Ca^{2+} is retracted from the cytoplasm and therefore actin and myosin are in relaxed state. During contraction, $[\text{Ca}^{2+}]$ rises and reconfiguration of the troponins drive myosin to bind on actin. Due to this binding, the myofilaments decrease in length and the muscle contracts. Figure adapted from the book, Molecular Cell Biology, Sixth Edition (2008).

1.4 The Cardiovascular System in Disease

1.4.1 Major Cardiovascular Associated Disease Categories

Cardiovascular disease (CVD) is a general term to describe a range of pathophysiological instances that are associated with the heart or blood vessels (Bhatnagar et al., 2015).

Disease occurs due to inflammation and decreased blood flow to the heart, the brain or specific parts or areas of the body causing significant irreversible damage to the area affected. Decreased blood flow results from two main processes that often co-exist: atherosclerosis, the steady build-up of fatty

deposits within arterial walls that harden and narrow vessels, or more acutely, thrombosis; the partial or complete blockage of an artery by a blood clot.

Depending on the region affected we can further categorize cardiovascular diseases into four main clinically relevant categories: Coronary Artery disease (CAD), Stroke, Aortic disease and Peripheral Arterial disease.

Stroke occurs when blood supply is cut off in an area of the brain due to a blood clot (ischemic) or a weakened vessel rupturing (hemorrhagic). In the United Kingdom, stroke is the third most common cause of death after heart disease and cancer, being the cause of death for over 110,000 people every year (S. Lee, Shafe, & Cowie, 2011).

Aortic disease affects the normal function of the aorta. The aorta is the largest vessel in the human body and its function is to supply blood from the heart to the body. The most common type of aortic disease is the aortic aneurysm formation whereby the aorta swells outwards causing disrupted organ perfusion and in the case of acute rupture, carries a high mortality (Aboyans et al., 2014).

Another type of cardiovascular disease is peripheral Arterial disease that affects blood supply in the upper or lower limbs of the body. The most common part of the body that is affected by peripheral Arterial disease are the legs, causing pain on exertion (claudication), skin ulcers and even ischemic loss of toes and feet (Gornik & Beckman, 2005).

1.4.2 Coronary Heart Disease

Coronary Artery disease is the most common type of disease of cardiovascular system. Reduced blood flow to the myocardium can lead to reduced oxygenation and nutrient deprivation leading to cellular death and cardiac contraction imbalance. A number of physiological and pathophysiological factors have been identified that can reduce the blood flow to the coronary arteries: decreased aortic diastolic pressure, increased intraventricular pressure and myocardial contraction, aortic valve stenosis, vasoconstriction, increased right atrial pressure and coronary artery stenosis (Archacki & Wang, 2004).

Coronary artery stenosis is the decrease in coronary blood flow in the coronary arteries due to a stenosis in the vessels. Reduced blood flow has been attributed to a variety of factors such as fixed coronary stenosis and vasoconstriction with acute plaque change (vessel rupture, hemorrhaging) and coronary artery thrombosis being the most common causes of stenosis (Uren et al., 1994).

1.4.2.1 Thrombosis

Thrombosis is the formation of a blood clot in a blood vessel that can block blood flow through the circulation system. Blood clots can form either physiologically as a response to an injured vessel acting against blood loss or pathophysiological due to a disturbance in the physiological mechanisms that control blood coagulation (Freedman, 2005). Endothelial cells due to their position, located in between blood flow and the walls of the vessels, are involved in initiation of blood clot formation (Wu & Thiagarajan, 1996). Damaged endothelium and subendothelium cell types that are exposed to blood flow due to damage initiate platelet aggregation and adhesion along with fibrin accumulation (Stoll, Kleinschnitz, & Nieswandt, 2008). Once blood clots are formed they can either reduce blood flow in the area of formation or can detach from the vessel wall and travel through the circulation as an embolus. Depending on the vessel that the embolus blocks it can lead to a stroke, a peripheral artery obstruction or a myocardial infarction.

1.4.2.2 Atherosclerosis

Atherosclerosis gives rise to coronary artery disease through a slow progression of lesion formation and luminal narrowing of the vessels and more specifically the arteries. Coronary artery disease induced by atherosclerosis is the leading cause of death and morbidity in the world and especially in countries that have adapted the Western lifestyle (Mackay & Mensah, 2004).

Atherogenesis, the formation of an atherotic plaque, is usually attributed to a variety of risk factors contributing to different stages of the condition. Atherosclerosis is a complex disease that is usually triggered by a combination of underlying conditions. 'A combination of imbalanced lipid metabolism and a

maladaptive immune response entailing a chronic inflammation of the arterial wall' are the major conditions that initiate atherosclerosis (Weber & Noels, 2011). Clinical manifestation of atherosclerosis is consisted of four stages (Hopkins, 2013) (Figure 13):

1. Initiation of endothelial activation and inflammation.
2. Promotion of intimal lipoprotein deposition, retention, modification and foam cell formation.
3. Progression of complex plaques by plaque growth, enlargement of the necrotic core, fibrosis, thrombosis, and remodeling.
4. Precipitation of acute events.

Initiation of atherogenesis is caused by activation of the endothelium due to reduced or disturbed flow in the vessel (Hopkins, 2013) (Figure 13). Endothelial cell activation is driven by lipoprotein accumulation and is further progressed by a reduced availability of NO along with other risk factors (Aikawa et al., 2002). Activation leads to vascular smooth muscle cell accumulation (Allahverdian, Chehroudi, McManus, Abraham, & Francis, 2014). First stages of atherogenesis essentially follow the first steps of an immune response (Abbas, Lichtman, & Pillai, 2012). Initially, adhesion molecules are produced by the activated endothelium such as selectins and vascular cell adhesion molecule-1 (VCAM-1). Release of these molecules, attract circulating cells of the immune system and more specifically monocytes (Audran et al., 1996).

Promotion of atherosclerosis is characterized by further lipoprotein accumulation along with monocytes and monocyte-derived macrophages (Hansson & Hermansson, 2011; Moore & Tabas, 2011; Woollard & Geissmann, 2010) (Figure 13). Critical for this stage are the circulating levels of low density lipoprotein (LDL) cholesterol and high density lipoprotein (HDL) cholesterol (Badimon & Vilahur, 2012; Berliner et al., 1995; Tomkin & Owens, 2012). A hallmark in this level of atherogenesis is the formation of foam cells (Valledor, Lloberas, & Celada, 2015; Yu et al., 2013).

Foam cells are lipid-containing macrophages that are generated by the uptake of low density lipoproteins and are present in atherosclerotic plaque (Yu et al., 2013). Foam cells are important in all stages of atherogenesis from fatty-streak formation to fully established atherosclerotic plaques (Valledor, Lloberas,

& Celada, 2015). Foam cells and accumulating lipoproteins start forming and expanding the atherosclerotic plaque.

During expansion, more activated macrophages reach the area and along with activated endothelial cells secrete cytokines and proinflammatory signaling molecules (Libby, 2009). These molecules increase fibrosis and in combination with the accumulation of apoptotic cells and cholesterol create a necrotic core that becomes fragile and is prone to rupture (Weber & Noels, 2011).

At the final stage and once the atherosclerotic plaque becomes unstable, thrombi consisted mainly of fibrin and platelets can be released from the initial plaque and be transferred through circulation and block blood flow (Hopkins, 2013) (Figure 13). This process is called embolism and could be life threatening related to the importance of the vessel that is blocked. A coronary artery occlusion by either a blood clot or an embolus originating from an atherosclerotic plaque is termed a myocardial infarction and can cause damage to the heart muscle (Libby, 2009).

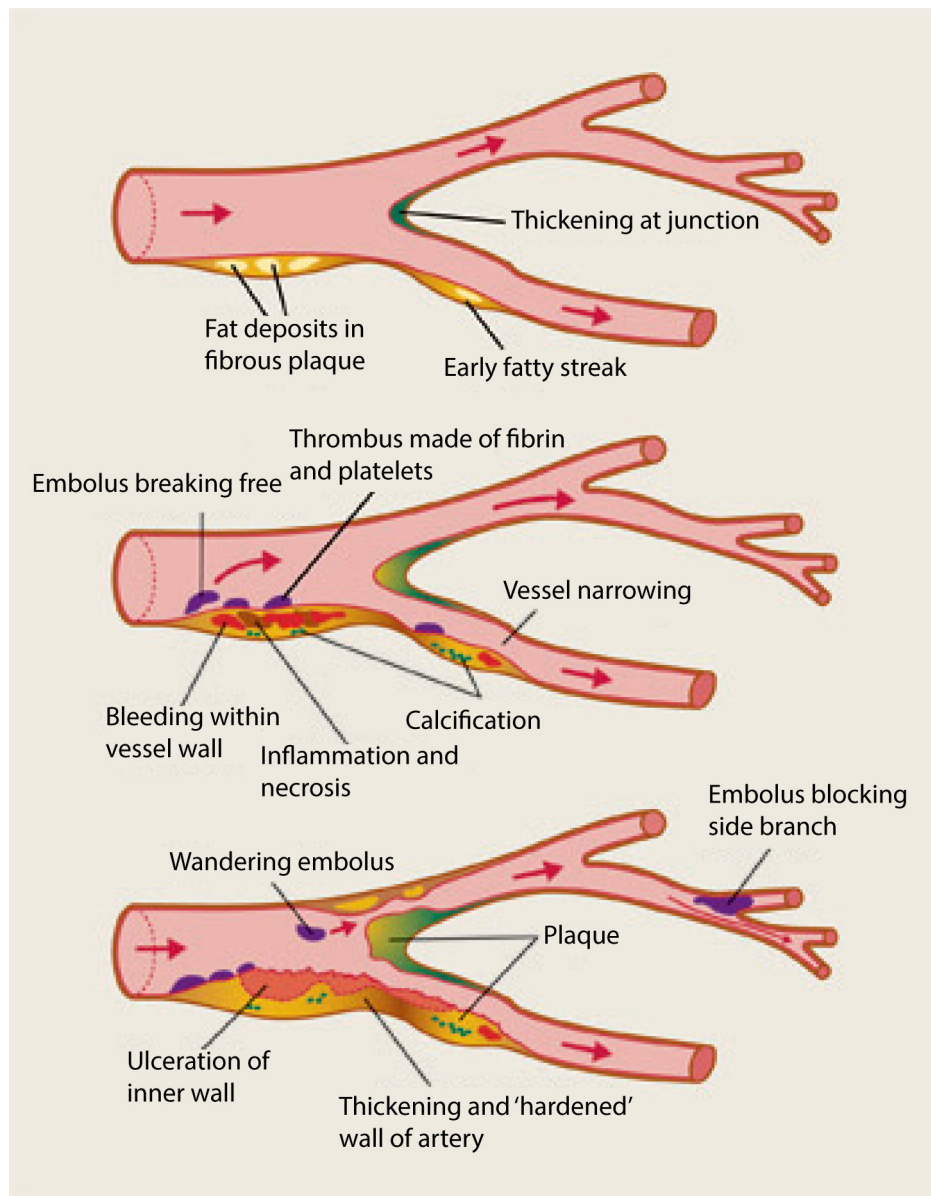


Figure 13. Stages of atherosclerotic plaque and embolus formation. Thrombosis is the formation of a blood clot that can lead to decreased blood flow to a vessel. Atherosclerotic plaque formation is the process of lipid deposition on the walls of the vessels leading to reduced blood flow and stiffness of the vessel. These two conditions, can affect blood flow individually or in combination. Both conditions, can lead to vessel blockage leading to ischemia or anoxia to an area of the body. If the vessels blocked are the coronary arteries, the condition is called myocardial infarction and can have a severe effect on the health of the individual as it could lead to death. Figure adapted from the European Federation of Pharmaceutical Industries and Associations.

1.4.3 Myocardial Infarction (MI)

1.4.3.1 Definition and Clinical Classification of MI

Myocardial infarction is defined as myocardial cell death due to prolonged ischemia (Jennings & Reimer, 1983) (Figure 14). Cell death is caused by an imbalance between the oxygen supply and demand from the myocardium (Thygesen et al., 2007). It is clinically characterized by electrocardiographic (ECG) findings, elevated values of biochemical markers, imaging and pathology (Thygesen et al., 2012). MI is usually caused by an underlying condition such as the occlusion of one of the coronary arteries by an embolus, an atherosclerotic plaque or a spasm of the arterial vessel (Jennings & Reimer, 1983) (Figure 14).

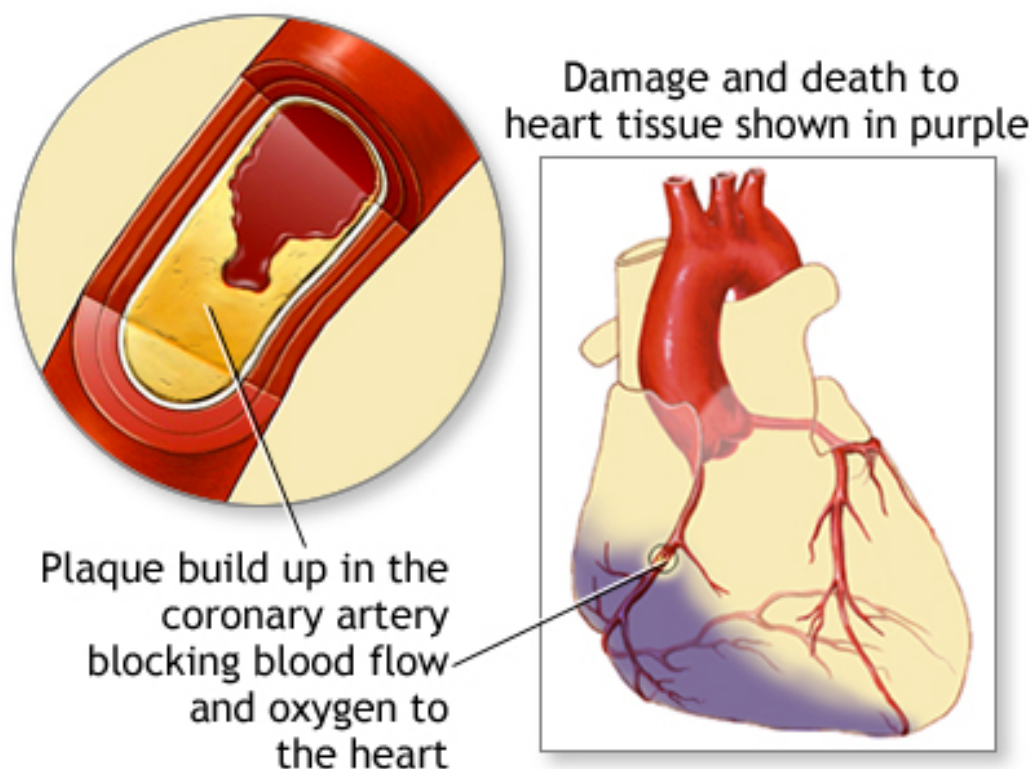


Figure 14. A myocardial infarction caused by a complete obstruction of the blood flow in the coronary artery by an atherosclerotic plaque. As a consequence of blood flow obstruction, the affected area becomes hypoxic and if the problem is not resolved the tissue becomes necrotic (purple area). Figure adapted from the American Accreditation Healthcare Consortium.

MI can be classified into two categories depending on symptoms manifestation: Acute and Prior or 'Silent' myocardial infarction (Thygesen et al., 2007). The term acute MI is used when there is evidence of myocardial necrosis

in a clinical assessment. Specific criteria such as abnormalities in the ECG and elevated biomarkers should be met before an MI incident is characterized as acute. A prior or 'Silent' MI is an incident that has no symptom manifestation but can be detected by specialized clinical procedures such as blood analysis of troponin T levels.

A second clinical classification system has been developed based on the underlying condition causing the incident (Thygesen et al., 2007) (Tele 1).

Tele 1. Clinical classification of MI. (Thygesen et al., 2007)

Type of MI	Underlying Condition
Type 1	Spontaneous myocardial infarction related to ischemia due to a primary coronary event such as plaque erosion and/or rupture, fissuring or dissection
Type 2	Myocardial infarction secondary to ischemia due to either increased oxygen demand or decreased supply (coronary artery spasm, embolism, arrhythmias etc.)
Type 3	Sudden unexpected cardiac death often with symptoms suggestive of myocardial ischemia accompanied by presumably new ST elevation etc.
Type 4a	Myocardial infarction associated with percutaneous coronary interventions (PCI)
Type 4b	Myocardial infarction associated with stent thrombosis as documented by angiography or at autopsy
Type 5	Myocardial infarction associated with coronary artery bypass grafting (CABG)

1.4.3.2 Epidemiology

Cardiovascular disease (CVD) is an umbrella term for conditions of the heart and the circulation system. CVD remains one of the leading causes of mortality worldwide and especially societies that have been culturally adapted

to the Western lifestyle (Roger, 2007). In the United Kingdom (UK), CVD is responsible for 27% of all deaths every year even though this number has declined by 50% since 1961 (Townsend et al., 2012) (Figure15). For the first time in 2012, CVD went from being the main cause of death to the second cause of death in the UK after cancer (Bhatnagar et al., 2015). Each year the economic burden in the UK is estimated to be £15 billion with healthcare costs alone to be up to £11 billion (CEBR, 2014).

Coronary Heart Disease (CHD) according to British Heart Foundation (BHF) and World Health Organization (WHO) is the single biggest killer in the UK and worldwide (Townsend et al., 2012; WHO, 2012). CHD is responsible for 70000 deaths in the UK each year (BHF, 2012) with around 2.3 million people currently living in the UK with a coronary heart disease (BHF, 2013). The annual cost of coronary heart disease for the National Health System (NHS) is calculated to be £1.8 billion (Townsend et al., 2012).

Myocardial infarction is the major cause of death for people that suffer from a coronary disease (Bhatnagar et al., 2015). Even though MI is the major cause of death from a coronary disease around 7 out of 10 people survive a heart attack living an impaired life (Smolina et al., 2012).

The same prevalence of cardiovascular disease and more specifically myocardial infarction is documented in the United States of America (USA) (Mozaffarian et al., 2015).

Myocardial infarction is affecting a large number of individuals every year. Since the development of new technologies for the diagnosis of the underlying conditions leading to a MI and the advancement in procedures that minimize the effects after an MI, the number of individuals surviving an incident has significantly risen. The quality of life of those individuals is impaired and the economic strain on the NHS is increasing. There is an important need in minimizing the effects of MI immediately after the incident or later on by regenerating the lost tissue in order to improve the quality of life of the surviving individuals.

UK PREMATURE CVD DEATH RATES (UNDER-75s)
by LOCAL AUTHORITY 2010/12

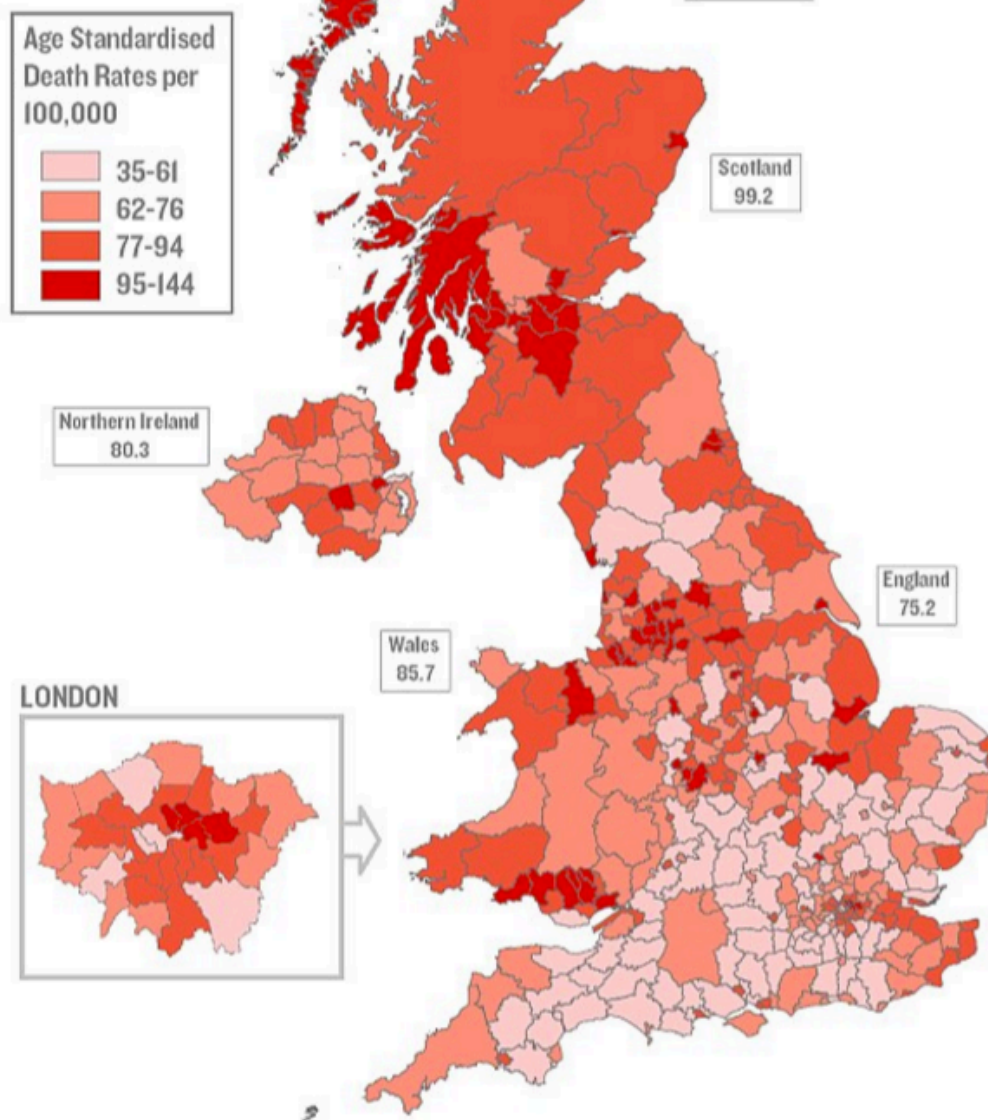


Figure 15. Annual death rates of cardiovascular disease in the UK by local authority. (BHF, 2012)

1.4.3.3. Risk Factors

A variety of risk factors have been linked with increased predisposition to a cardiovascular disease leading to a myocardial infarction. Risk factors can be divided into two major categories: risk factors associated with underlying conditions and risk factors associated with lifestyle.

Myocardial infarction has been attributed to underlying conditions such as diabetes, high blood pressure (chronic condition) and high cholesterol levels (BHF, 2015; Diabetes, 2015) or in some cases with clinical procedures such as coronary artery bypass and stent associated thrombosis (BHF, 2012; Thygesen et al., 2007; Thygesen et al., 2012).

Lifestyle habits especially in Western societies have also been identified as myocardial infarction risk factors. Smoking, high alcohol consumption, physical inactivity that can lead to obesity and poor diet have been linked to an increase in MI incidence (BHF, 2015).

1.4.3.4 Symptoms and Clinical Diagnosis

Symptoms of a MI vary between different individuals and sex of the individual can play a major role on the type and number of symptoms reported (Mallinson, 2010). Men traditionally report a higher number of symptoms than women (Canto, 2007). Common symptoms of a MI are chest pain that usually radiates to the left arm, shortness of breath, sweating and dizziness (Mallinson, 2010). In women, most common symptoms include dyspnea, weakness and fatigue (Canto, 2007). Other symptoms include nausea, vomiting and palpitations (Mallinson, 2010). A quarter of all MI incidents are termed as prior or 'silent' and manifest no symptoms (Valensi, Lorgis, & Cottin, 2011).

Clinical diagnosis of MI is consisted of biomarker evaluation and ECG analysis along with patient history (Thygesen et al., 2007). Biomarkers associated with an MI and more specifically with necrosis of the myocardium are different proteins released to the circulation by damaged cardiomyocytes. Biomarkers with high clinical sensitivity and tissue specificity are cardiac troponins (T and I) (Mythili & Malathi, 2015). Even though troponin levels in circulation are indicative of a MI incident, they do not reflect the underlying pathophysiological mechanism. Blood samples are drawn during first assessment of the patient and 6-9 hours later for the measurement of troponin. Comparison of troponin levels in the two samples is important for patients health evaluation and distinguish between other conditions that are characterized by increased troponin levels (Thygesen et al., 2012).

ECG analysis is also used to assess patients health and verify a MI incident (Thygesen et al., 2012). Through the ECG analysis the date of the

event along with the artery affected and the amount of myocardium at risk can be estimated (Thygesen et al., 2012). Acute or evolving changes in the ECG affect the PR segment, the QRS complex and the ST segment or T waves (Szczepański & Saeed, 2014) (Figure 16).

Non-invasive imaging techniques are used in cases of a known or suspected MI incident to diagnose and characterize the infarction (Thygesen et al., 2012). Such techniques include: echocardiography, magnetic resonance imaging (MRI), X-Ray computed tomography (CT) and radionuclide imaging.

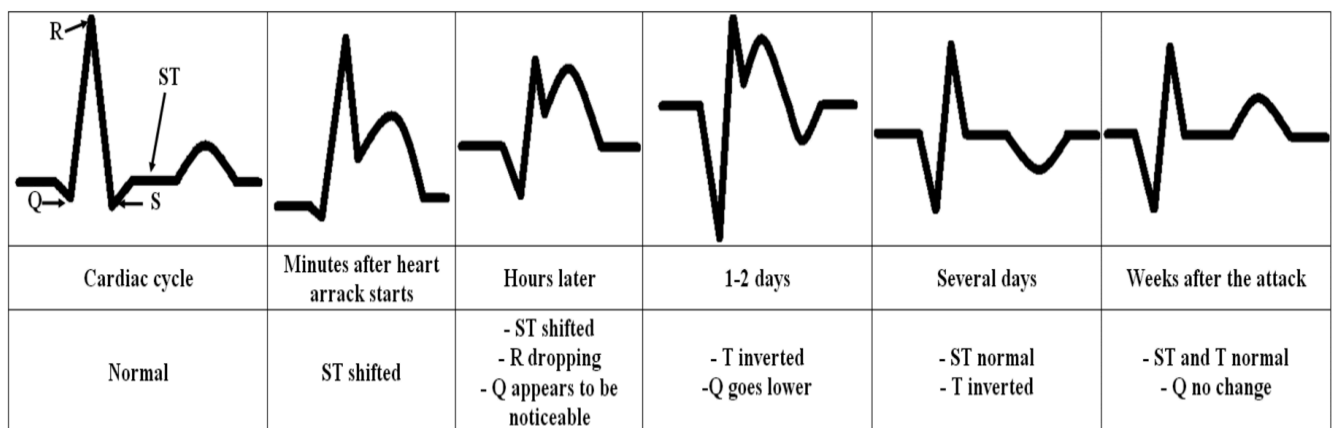


Figure 16. Evolution of ECG recording after an MI incident. All changes to the normal cardiac cycle ECG can be considered as disturbances. (Szczepański & Saeed, 2014)

1.4.4 Pathophysiology

1.4.4.1 Ischemic Injury

Myocardial ischemia triggered by an infarction is the major condition that leads to myocardial cell injury and death. Injury of the myocardium is induced immediately after the loss of oxygen when cellular death is a response to prolonged ischemia (Reimer & Ideker, 1987). The myocardium responds immediately to the ischemic insult through the activation of ischemia associated pathways (L. M. Buja, 2005a).

The heart is characterized by high metabolic activity requiring high energy levels at all times. Energy in the myocardium is produced by the mitochondria in the form of ATP through the mitochondrial oxidative phosphorylation (Hoeks, Hesselink, & Schrauwen, 2012). With loss of oxygen during ischemia, aerobic ATP production by the mitochondria rapidly stops and is substituted by anaerobic glycolysis. Activation of anaerobic glycolysis leads to

intracellular accumulation of hydrogen ions and lactate resulting in acidosis and inhibition of all residual energy metabolism (L. M. Buja, 2005b). Energy depletion inactivates active transport of ions such as K^+ , Na^+ , Ca^{2+} initiating a high ion imbalance in the cytoplasm. Intracellular levels of Na^+ increase leading to cell swelling due to water influx (L. M. Buja, 1998). Intracellular $[Ca^{2+}]$ also increases leading to Ca^{2+} -induced proteases activation affecting cell contractility (Buja, Hagler, & Willerson, 1988).

Due to ionic imbalance and water influx, structural elements of the cardiomyocytes begin to be affected. Mitochondria start swelling causing irreversible damage to the inner and outer membrane, releasing pro-apoptotic factors to the cytoplasm (Majno & Joris, 1995). A major role in mitochondria dysregulation and damage is played by the regulation of mitochondrial permeability transition pore (MPTP) by the ERK/AKT pathway (Ong et al., 2015; Rasola & Bernardi, 2011).

Progression from reversible to irreversible myocardial cell injury is characterized by changes to the myocardial interstitium and microvasculature (Buja, 2005). Prolonged increase in $[Ca^{2+}]$ activates phospholipases and lipid degradation affecting the cytoskeleton along with cellular integrity (Buja, 1991). Disruption of the cellular membrane is one of the final stages of ischemic damage and leads to derangements in intracellular electrolytes and ATP exhaustion (Buja, 2005).

1.4.4.2 Reperfusion Injury

In patients that have survived a myocardial infarction incident is essential to keep the infarcted area size at minimum so as to limit the effect of the incident on the patient's everyday life.

Reperfusion of the myocardial tissue after a MI incident has been shown to be one of the most successful approaches on limiting the necrotic area (S. R. Maxwell & Lip, 1997). The effect of reperfusion on the area is strongly related to the time of intervention (Buja, 2005). Even though, reperfusion is one of the most successful approaches in limiting a MI effect on the myocardium, it is characterized by a number of deleterious effects that can be termed as reperfusion injury. Reperfusion of the tissue activates the immune system

initiating an inflammatory response and can accelerate the progression of injured cardiomyocytes to death (Park & Lucchesi, 1998).

Reperfusion injury is mainly mediated through production of oxygen radicals, accumulation of intracellular Ca^{2+} and neutrophil accumulation and activation (Park & Lucchesi, 1998). During an ischemia/reperfusion injury, oxygen radicals are produced, overwhelming antioxidant defenses of injured cardiomyocyte and endothelial cells (Dhalla et al., 2000). Increased production of oxygen radicals leads to Ca^{2+} loading due to inactivation of sarcoplasmic reticulum ATPases and inhibition of Na^+/K^+ ATPases (Zweier & Talukder, 2006). Lipid degradation is also initiated by oxygen radicals leading to cellular membrane degradation and further destruction of cellular integrity (Park & Lucchesi, 1998). Finally, release of oxygen radicals result in the chemotaxis of neutrophils, which further enhance the inflammatory reaction but also limit blood circulation in the microvasculature (S. R. Maxwell & Lip, 1997).

Reperfusion remains the most effective process in limiting infarct size but it has also been associated with irreversible damage of cardiomyocytes that have been heavily injured during ischemia. Further research into the complex mechanisms that regulate reperfusion will help us understand and minimize reperfusion injury.

1.4.4.3 *Ex vivo* experimental model of Ischemia/Reperfusion Injury: Langendorff apparatus

In 1895, Oscar Langendorff was a pioneer in his field as he managed to develop the first *ex vivo* system for the perfusion of the mammalian heart (Langendorff, 1895). With his system, Oscar Langendorff established the retrograde mammalian heart perfusion which since then has been an invaluable system in our understanding of the mammalian heart physiology. Especially, since the emergence of genetic manipulation of the mammalian genome, Langendorff has helped us shed light in the role of specific genes in physiological and pathophysiological processes of the heart. Finally, the opportunity that the Langendorff apparatus offers for the investigation of gene targets in an ischemia/reperfusion injury have been crucial for the understanding of the mechanisms involved in myocardial infarction (Headrick et al., 2001; Liao, Podesser & Lim, 2012; Reichelt et al., 2009).

Retrograde mammalian heart perfusion is the fundamental principle of the Langendorff system. During perfusion, the aorta is cannulated and oxygenated perfusion buffer (Krebs solution) is pumped through the coronary arteries supplying the myocardium with oxygen and nutrients (Bell, Mocanu, & Yellon, 2011). The perfusate then is directed through the coronary veins and is released in the right atria. Retrograde perfusion shows that coronary circulation (with constant perfusion pressure) is the only essential factor for the myocardium to retain all its physiological characteristics when it is isolated from the other physiological systems of the mammalian body.

The Langendorff apparatus can record a variety of physiological parameters that are essential in the understanding of the heart physiology. Some of the most important parameters that can be measured are the aortic pressure, the coronary flow, the left ventricular pressure, an ECG and the cardiac pace (Liao et al., 2012). Measuring those parameters during an ischemia/reperfusion model, can provide an insight into the physiological pathways involved in the damage or protection of the myocardium against that type of injury.

Finally, the Langendorff system has been widely used for the isolation of adult Ca^{2+} tolerant mouse cardiomyocytes (Li et al., 2014). A digestion buffer containing collagenase is pumped through the coronary circulation using retrograde heart perfusion in order to soften the extracellular matrix and release cardiomyocytes that are then cultured for up to 72h *in vitro*. Isolation of adult Ca^{2+} tolerant mouse cardiomyocytes has been a useful tool for the understanding of contraction in the cellular level and hypoxic/normoxic responses of cardiomyocytes during an ischemia/reperfusion injury.

1.4.5 Treatment and Therapeutic Approaches

Cardiovascular disease is still one of the primary causes of death in the UK despite its decline in recent years (BHF, 2012). Despite CVD mortality rate, there is no available cure for the effects of myocardial infarction, the number one cause of death in patients suffering of a CVD (BHF, 2015). Treatment of MI patients is limited to treating any acute symptoms and protect the patient from a second incident (reinfarction) (S. Maxwell, 1999). Pharmacological treatment of the underlying conditions that led to a MI is available but the injury inflicted by

the MI incident is not yet reversible (Doppler et al., 2013). A variety of clinical trials are currently trying to tackle the irreversible loss of the myocardium by cardiac regeneration with the use of stem cells to provide MI patients with a cure (Doppler et al., 2013).

Emergency management of a MI incident includes analgesia, oxygen therapy and nitrate supplementation (improve blood flow and can relieve acute myocardial pain) (Maxwell, 1999) alongside interventions to re-establish coronary artery flow. Aspirin supplementation and thrombolytic agents have been shown to achieve recanalization (restore of blood flow) in 80% of cases (Baigent et al., 1998) and is one of the primary procedures used in medical centers. More recently, primary angiogram followed by intervention using angioplasty or stenting is becoming the mainstay of acute MI management. (Waxman, Ishibashi, & Muller, 2006).

Since the isolation of human embryonic stem cells and the continuous advancement in cardiac differentiation technology, regeneration of the myocardium after a myocardial infarction incident has been the ultimate target of the field. A variety of different approaches have been developed in the regeneration of the myocardium. Three major approaches have emerged in the lab that give promise for future clinical trials: cell based therapies, tissue engineering and reprogramming of scar fibroblasts (Doppler et al., 2013).

Cell based therapies involve isolation of specific cell types with differentiation capacity (pluripotent, multipotent, oligopotent) such as embryonic pluripotent stem cells (Puliafico, Penn, & Silver, 2013), bone marrow cells (Orlic et al., 2001), adipose-tissue derived regenerative cells (Fraser et al., 2006; Zuk et al., 2001) and cardiopoietic stem cells (Teng, Zhao, & Huang, 2012) (Table 2). Differentiated cells are then injected as fully differentiated cardiomyocytes or cardiac progenitor cells or are mobilized through activating agents to the necrotic area of the myocardium (Doppler et al., 2013).

In the tissue engineering approach, embryonic stem cells or cardiac progenitors cells are differentiated to cardiomyocytes in combination with various scaffolds and are then implanted into the necrotic area (Vunjak-Novakovic et al., 2011).

Since the discovery of reprogramming of embryonic fibroblasts to iPS cells in 2006 (Takahashi & Yamanaka, 2006), a new approach evolved in the field of regenerative medicine. This approach includes reprogramming of

fibroblasts belonging to the patient to iPS cells, differentiation of iPS cells to terminally differentiated cardiomyocytes or cardiac progenitor cells and autologous transplantation to the necrotic area (Li et al., 2013; Nelson et al., 2009; Singla et al., 2011). Another reprogramming approach, includes the direct reprogramming of fibroblasts or bipotential myocardial/smooth muscle cells to cardiac progenitor cells or cardiomyocytes prior to injection to the myocardium (Islas et al., 2012; Moretti et al., 2006; Wu et al., 2006). Reprogrammed cells can either be injected to the necrotic area or the reprogramming agents can be infused via injection to transdifferentiate resident fibroblasts (Doppler et al., 2013).

Table 2. List of clinical trials using adult stem cells. Information derived from (Doppler et al., 2013).

Cell type used	Name of clinical trial	Year
Bone Marrow Cells	BOOST	2009
	REPAIR-AMI	2010
	TOPCARE-AMI	2011
Adipocyte Progenitor Cells	APOLLO	2012
Cardiosphere Derived cells	CADUCEUS	2012
	SCIPIO	2012

1.5 The role of NO in pluripotency and cardiac development and disease

1.5.1 NO regulation in cardiac development, function and disease

Since the discovery of NO, increasing research interest has focused on its role in regulating many physiological functions. NO has been proven to act as a powerful agent through a number of different pathways in physiological processes ranging from pluripotency of embryonic stem cells to cardiac function and failure (Beltran-Povea et al., 2015; Massion et al., 2003; Massion et al., 2005; Schulz, Kelm, & Heusch, 2004).

Analysis of the mechanisms through which NO acts can be separated into two main categories depending of the involvement of cGMP as a secondary

signaling molecule (Krumenacker et al., 2006; Mujoo et al., 2008; Mujoo, Krumenacker, & Murad, 2011). cGMP-dependent NO signaling relies on the effect of NO in activation of sGC and the production of cGMP by conversion of GTP (Krumenacker et al., 2006). cGMP initiates various cascades by interacting with downstream targets such as cGMP-dependent protein kinases, cGMP-dependent phosphodiesterases and cyclic nucleotide channels (Krumenacker et al., 2006). cGMP-independent pathways mainly involve direct interaction of NO with a targets such as metal complexes, oxygen, superoxide and cytochrome C oxidase (CcO) (Brown & Cooper, 1994). Interaction of NO with those molecules is highly favorable in high NO concentrations (Brown & Cooper, 1994). Protein nitrosylation and nitration is another signaling pathway in which NO is actively involved and can have a variety of effects (Anand & Stamler, 2012; Lima et al., 2010). The effects of both signaling categories can vary depending on the amount of NO produced (Vallance & Leiper, 2002).

Endogenous regulation of NO production during cardiac development, function and disease at the level of inhibition by methylarginines has not been thoroughly tested and there is no clear evidence of their role. Also, since methylarginines are produced by protein degradation, an active process in remodeling of cells in tissues, regulation of NO synthesis by methylarginines may be particularly significant during development. Finally, limited are the evidence for the role of DDAH genes during development of the heart.

1.5.2 NO regulation in pluripotency

Implication of NO regulation in pluripotency is mainly dependent on the amount of NO produced and revolves around induction of apoptosis, survival and regulation of differentiation via down-regulation of pluripotency genes or repression of differentiation-inducing genes (Beltran-Povea et al., 2015; Mora-Castilla et al., 2010).

Supplementation of low amounts of diethylenetriamine NO adduct (DETA-NO), an NO donor, prevents the loss of expression of self-renewal genes and blocks differentiation of both mouse and human ES cells (Tejedo et al., 2010). In addition, NO supplementation increases the expression of Nanog, Oct4 and Sox2 genes and represses the expression of differentiation markers such as Brachyury, Gata4 and Gata6 (Tejedo et al., 2010). Finally, constitutive

overexpression of eNOS gene maintained the expression of pluripotency markers even under LIF withdrawal conditions, protected them from apoptosis and promoted cell survival (Tejedo et al., 2010).

1.5.3 NO regulation in stem cell differentiation

NO-cGMP signaling pathway has been shown to be involved in differentiation of embryonic pluripotent stem cells and other progenitor cells to cardiovascular system related tissues (Aicher et al., 2003; Bøyum et al., 2004; Guthrie et al., 2005; Kanno et al., 2004; Michurina et al., 2004; Shami & Weinberg, 1996).

In vitro experiments using NO supplementation via NO donor treatment have shown potential roles for NO in cell fate determination during differentiation (Bøyum et al., 2004; Guthrie et al., 2005; Shami & Weinberg, 1996). NO supplementation during bone marrow progenitor cell differentiation favored myeloid rather than erythroid fate during development (Shami & Weinberg, 1996). Also, NO production inhibited colony-forming ability of Sca1 or Thy1⁺ cells (Bøyum et al., 2004). Finally, hematopoietic stem cells isolated from eNOS^{-/-} mice exhibited impaired mobilization as bone marrow progenitor cells and reduced endothelial progenitor cell differentiation from an hematopoietic fate (Aicher et al., 2003; Guthrie et al., 2005; Michurina et al., 2004). Adult eNOS^{-/-} mice suffered from reduced revascularization after an ischemic injury (Guthrie et al., 2005).

During cardiomyogenesis, eNOS and iNOS genes but not nNOS has been shown to be expressed from E8.5 and possibly play a role in development of the myocardium in rat embryos (Bloch et al., 1999). *In vitro* experiments of embryonic stem cell differentiation with the supplementation of NOS inhibitors (1mM L-NAME or 10mM L-NA) revealed reduced differentiation to cardiomyocytes (Bloch et al., 1999; Kanno et al., 2004). Supplementation of 200µM S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP), an NO-donor (SNAP) showed increased cardiomyocyte differentiation (Kanno et al., 2004). Finally, overexpression of iNOS in embryonic stem cells increased the amount of cardiomyocytes formed during differentiation (Kanno et al., 2004).

NO regulation seems to play an important role in the development of the cardiovascular system and the cell types associated with it. Also, reports show

a potential role of NO in development of cortical or enteric neurons in insects (Haase & Bicker, 2003; Polleux, Morrow, & Ghosh, 2000).

1.5.4 NO regulation in cardiac development and function

Regulation of NO is as well implicated in later developmental stages of the cardiovascular system and more specifically during development and adult function of the heart (Bloch et al., 1999; Y. Liu & Feng, 2012; Nath & Madri, 2006; Zhao, Lu, & Feng, 2002).

During development, iNOS and eNOS genes are highly expressed until E9.5 in rat embryos hearts (Bloch et al., 1999). By E14.5, iNOS expression decreases and at E18.5 iNOS and eNOS expression remains but is detectable only at low levels (Bloch et al., 1999). In embryonic stem cell derived cardiomyocytes that resemble physiologically neonatal cardiomyocytes, iNOS and eNOS genes were highly expressed in early stages but were downregulated in later stages of maturation (Bloch et al., 1999). Adult cardiomyocytes express high levels of eNOS and nNOS genes in the basal state but not iNOS (Massion et al., 2005). The iNOS gene is expressed in cardiomyocytes only after an inflammatory stimulus or hypoxia (Massion et al., 2005).

Studies with NOS knock out mice revealed a role of NOS genes and more specifically eNOS in cardiac development. eNOS^{-/-} mice were viable but demonstrated increased incidents of bicuspid valve and atrioventricular septal defects (Feng et al., 2002). Disruption of eNOS gene expression had an effect on normal development of coronary arteries, affecting their diameter, density and volume. eNOS expression levels were directly linked to survival, cardiac fractional shortening and pulmonary congestion (Y. Liu & Feng, 2012). Finally, high eNOS expression decreased caspase 3 activity and myocardial and endocardial cushion apoptosis during development (Nath & Madri, 2006).

During fetal development, DDAH isoforms show a different expression pattern, implying a different role of each isoform in the developmental process (Tran et al., 2000). DDAH2 shows expression in a variety of tissues during development including the placenta (Tran et al., 2000). DDAH1 on the other hand, is expressed in fetal brain, kidney, lung and liver but shows weak expression in the developing heart (Tran et al., 2000). The role of DDAH

isoforms in embryonic development has not been extensively studied in the past. Disruption of *Ddah1* expression has been reported to be lethal, a phenotype, which was not reproduced in later DDAH1^{-/-} animal models (Leiper et al., 2007). DDAH1^{-/-} and DDAH2^{-/-} mice showed no developmental defects but show increased blood pressure and lower survival rate after a septic insult (Breckenridge et al., 2010; Lambden et al., 2015).

In the adult myocardium, the NO signaling pathway is involved in regulation of basal physiological parameters. The role of NO is dose-dependent demonstrating positive effects in low and negative effects on high concentrations (Massion et al., 2003). Treatment of isolated rat cardiomyocytes with an NO donor increased muscular contraction force but only by 15% (Kojda et al., 1996). Single disruption of NOS gene expression in animal models showed no effect on basal contractile function and heart rate (Massion et al., 2005). It is suggested though that nNOS might play a role in calcium reuptake into the SR (Sears et al., 2003).

1.5.5 NO regulation during an ischemia/reperfusion injury of the myocardium

During ischemia, cellular levels of O₂ drop, creating a hypoxic environment inside the cell and more specifically to the mitochondria. Low O₂ creates a hypoxic environment inside the mitochondria, the major site of ATP production through glycolysis for which O₂ is absolutely essential. During hypoxia, the mitochondrial respiratory chain is dysregulated and ROS production is increased leading to cellular damage and apoptosis (Buja, 2005). NO signaling plays a significant role in regulation of ROS production and protection of the cell from hypoxia-induced apoptosis (Charlotte Farah & Reboul, 2015a; Schulz et al., 2004; Vegh, Szekeres, & Parratt, 1992). Two mechanisms of signaling have been identified for NO in cardioprotection during an ischemia-reperfusion injury. In the first one, NO activates protein kinase G (PKG) through sGC activation and cGMP production. In the second one, NO modifies a variety of proteins through S-nitrosylation, leading to cardioprotection during an ischemia-reperfusion injury (Charlotte Farah & Reboul, 2015a). Involvement of NO in cardioprotection is influenced by three specific factors:

localization, concentration and time of production (Charlotte Farah & Reboul, 2015a).

The main target of NO signaling during an ischemia-reperfusion injury is ROS production in the mitochondria (Schulz et al., 2004). Three main pathways of cardioprotection have been identified involving cytoplasmic signaling molecules along with mitochondrial specific target proteins (Heusch, Boengler, & Schulz, 2008). The first pathway is activated by adenosine, bradykinin and opioids and involves activation of Akt and eNOS proteins by phosphorylation (Heusch et al., 2008). NO production then activates sGC and through a cascade of proteins ROS is produced priming MPTP for opening (Heusch et al., 2008). This pathway is activated during ischemic preconditioning. The second pathway is activated through growth factor binding on G-protein-coupled receptors and is called reperfusion injury salvage kinase system (RISK). RISK system involves the parallel activation of PI3K/Akt and the extracellular regulated kinase system leading to GSK3 β activation and inhibition of MPTP opening (Heusch et al., 2008). In the third pathway, NO increases the expression of tumor necrosis factor α (TNF α) followed by activation of janus-activated kinase (JAK) and signal transducer and activator of transcription (STAT) pathway with further involvement of the nucleus and the mitochondria (Heusch et al., 2008; Thielmann et al., 2002).

S-Nitrosylation of a variety of proteins is the second mechanism of NO signaling that can have a cardioprotective role. S-Nitrosylation of ND3 subunit of mitochondrial complex I can lead to a decrease in ROS production during reperfusion (Chouchani et al., 2013). Also, S-nitrosylation of G protein-coupled receptor kinase (GRK) can inhibit its translocation to the mitochondria therefore preventing the activation of the apoptosis pathway (Huang et al., 2013). Finally, S-nitrosylation might have a negative effect on cardiac tissue survival during an ischemia/reperfusion injury. Hypernitrosylation of ryanodine receptor (RyR2) through TNF- α /caspase 8 pathway could lead to an increase in Ca²⁺ leak from the SR contributing to cell death (Fauconnier et al., 2011).

Since NO regulation during an ischemia/reperfusion injury has mainly a cardioprotective role depending on the stage of synthesis and release, proteins such as DDAH1 and 2 that can regulate NO production through inactivation of endogenous NO inhibitors could be ideal pharmacological targets since they could limit the negative effects of NO on the myocardium (Massion et al., 2003).

So far, little is known about the expression of these genes in the different cell types of the heart and their role during an ischemia/reperfusion insult. DDAH2 seems to be localized in the endothelium and the endocardium of the healthy rat heart following a pattern similar to that of eNOS (Gray et al., 2010). DDAH1 was below detectable levels in the myocardium (Gray et al., 2010). A rat coronary artery ligation model revealed DDAH1 expression in cardiomyocytes bordering the infarct region and DDAH2 expression in surviving cardiomyocytes and in blood vessels in the infarct zone (Gray et al., 2010).

2. Hypothesis

In this study, we wanted to investigate the effect of NO signaling pathway on cardiac development, function and disease. In order to achieve this, we tested the following hypotheses:

1. NO synthesis inhibition via ADMA can regulate embryonic stem cell differentiation.
2. Global *Ddah2* deletion can affect cardiac tissue survival during an ischemia/reperfusion injury in an NO dependent or independent manner.

Since little is known for the role of NO during pluripotency and differentiation, we aim to study the effect of NOS inhibition via ADMA in pluripotency and document the regulation of NO signaling during differentiation of embryonic stem cells to the different germ layers. Finally, we are going to further test the effect of NO synthesis inhibition via ADMA during differentiation of embryonic stem cells to the three germ layers with a higher focus on mesoderm and endoderm.

In the adult heart, we are going to investigate the effect of various NOS inhibitors, both endogenous and synthetic, and the effect of NO synthesis inhibition during an ischemia/reperfusion injury on tissue survival using an *ex vivo* Langendorff model. Finally, using the ischemia/reperfusion *ex vivo* model, we are going to investigate the effect of *Ddah2* deletion on tissue survival at the organ and cellular level.

3. Materials and Methods

3.1 *In vitro* Methods

3.1.1 Production and culture of irradiated Mouse Embryonic Fibroblasts (MEFs) for feeder cells

Production of feeder cells is crucial for the successful culture of E14 embryonic stem cells *in vitro*. Isolated MEFs from 13.5 days post coitum CD1 embryos were acquired from Stem Cell Technologies. The cells were thawed and cultured in Dulbecco's Modified Eagle Medium (DMEM) with high glucose concentration (4500 mg/L) (Sigma-Aldrich) without L-glutamine and supplemented with 10% (v/v) FBS (Sigma-Aldrich), 2mM L-glutamine (Gibco), and 1% (v/v) penicillin/streptomycin (Gibco). The cells were cultured and expanded up to passage number 4 in 140 mm dishes (Thermo Scientific) and maintained in an incubator at 37°C with 5% CO₂. After expansion, all cells were collected using 0.05% trypsin/EDTA and were mitotically inactivated by irradiation of 1 Gy per minute for 30 minutes in a commercial cell irradiator (CIS Bio International). Following irradiation, the cells were counted using a hemocytometer (WEBER) and 6.000.000 cells were aliquoted per freezing vial. The cells were stored in liquid nitrogen in freezing medium containing 10% DMSO (Sigma-Aldrich). 24 hours prior to E14 embryonic stem cells passaging, one vial of feeder cells was thawed and cultured in a 6-well plate (Thermo Scientific) coated with 0.2% (w/v) gelatin (Sigma-Aldrich) with cell density of 1.000.000 cells per well. Viability and quality of the cells was assessed using a light microscope. Culture and preparation of feeders was done according to this protocol (Meissner et al., 2009)

3.1.2 *In vitro* Culture of E14 Embryonic Stem Cells

E14 embryonic stem cells were kept pluripotent *in vitro* by the addition of Leukemia Inhibition Factor (LIF) to the culture medium and co-culture with MEFs as feeder cells. Stem cell culture medium contains Knock Out DMEM

(Gibco), 15% (v/v) Heat Inactivated FBS (Sigma-Aldrich), 2mM L-glutamine (Gibco), 1% (v/v) penicillin/streptomycin (Gibco), 1% (v/v) Non-Essential Amino acids (Gibco), 2000 units of LIF (Millipore) per ml of medium and 0.8% (v/v) β -mercaptoethanol (Sigma-Aldrich). The E14 embryonic stem cells were cultured in 6-well plates (Thermo Scientific) treated with 0.2% (v/v) gelatin and 1.000.000 mitotically inactivated MEFs per well and maintained at 37°C with 5% CO₂. The cells were checked daily for signs of differentiation under a light microscope and passaged every 3 days. Frozen stocks were kept in liquid nitrogen in freezing medium containing 10% (v/v) DMSO (Sigma-Aldrich). The Culture of E14 embryonic stem cells was done according the following protocol. (Meissner et al., 2009)

3.1.3 E14 Embryonic Stem Cell Differentiation to Mesoderm

The E14 embryonic stem cells were differentiated to mesoderm in vitro by the embryoid body formation method. This was done by centrifugation in a low attachment round bottom 96-well plate (Kurosawa, 2007).

The E14 stem cell cultures were collected using 0.05% trypsin/EDTA (Gibco) and placed in 0.2% gelatin (Sigma-Aldrich) coated 6-well plates (Thermo Scientific) for 2 hours at 37°C with 5% CO₂. This enabled the separation of the E14 embryonic stem cells from the feeder cells. After 2 hours, the supernatant of the cultures was collected and the cells resuspended in embryoid body (EB) formation media which contained: High Glucose (4500 mg/L) DMEM (Sigma-Aldrich), 20% (v/v) FBS (Sigma-Aldrich), 2mM L-glutamine (Gibco), 1% (v/v) penicillin/streptomycin (Gibco), 1% Non-Essential Amino acids (Gibco) and 0.8% β -mercaptoethanol (Sigma-Aldrich) (Crespo, Sobrado, Gomez, Cervera, & McCreath, 2010; Rathjen & Rathjen, 2001). For all EBs/ Asymmetric Dimethylarginine (ADMA) treatment studies, ADMA (Sigma-Aldrich) was added to the culture medium to achieve final concentrations of 100 μ M. Embryonic stem cells were counted using an hemocytometer and their concentration was adjusted to 1000 cells per 100 μ l (Bauwens et al., 2008; Ng et al., 2005). 100 μ l of cells were placed in each well of a low attachment round bottom 96-well plate (Thermo Scientific) and centrifuged at 1000 rpm per minute for 5 minutes at room temperature to form the EBs. After centrifugation, the cells were cultured for 48h at 37°C with 5% CO₂. After the first 48h, EBs were

transferred into 6-well plates (Thermo Scientific), coated with 0.2% gelatin (Sigma-Aldrich) at a density of 16 EBs per well and allowed to differentiate for up to 18 days. The cells were checked daily under an optical microscope and medium was replaced every 48 hours.

3.1.4 Adult Mouse Cardiomyocyte Isolation and Culture

Adult male mice of 10-12 weeks old were used for the isolation of calcium (Ca^{2+}) tolerant adult mouse cardiomyocytes. All mice were anaesthetized using 40 mg/ml Ketamine (Vetalar™V, Zoetis) and 3.2 mg/ml xylazine (Rompun, Bayer). 500U/ml of Heparin (Fannin) was administered to the mice prior to the administration of anaesthesia and regular pedal reflexes checked to establish and confirm desired level of surgical anaesthesia.

Once surgical anesthesia of the animals was confirmed, the chest area and rib cage were opened to expose the beating heart. The exposed heart, along with the lungs and the thymus were immediately excised and were placed in PBS at room temperature. The contracting heart was then left in PBS for 1 minute in order to expel blood contained in the atria and ventricles. The lungs, thymus and the pericardium were removed and the aorta cut right below the brachiocephalic artery to enable the aorta to be cannulated.

After cannulation, the heart was connected to the Langendorff apparatus (Harvard Apparatus) and perfused with a high concentration calcium Perfusion Buffer (Table 3) at 1ml/minute at 37°C for 5 minutes to expel the remaining blood from the coronary arteries. Perfusion buffer was changed to Digestion buffer after 5 minutes. Digestion buffer contained Perfusion buffer supplemented with 0.2mg/ml of Liberase TM (Roche), 5.5mM 2.5% Trypsin (Gibco) and 12.5µM CaCl_2 . The heart was then perfused with the digestion buffer for approximately 6 minutes. After 4 minutes of digestion, the heart was gently squeezed with a pair of forceps (F.S.T) and the perfusate checked for individual contracting cells under an optic microscope. Once individual cells are visible, the ventricles were removed into Stopping buffer 1, which contained Perfusion buffer supplemented with 10% FBS (Sigma-Aldrich) and 12.5µM CaCl_2 . The ventricles were then dissociated using a pair of forceps. Complete dissociation of the tissue was achieved using a plastic pipette Pasteur (Sarstedt). After complete disassociation of the tissue, the supernatant

containing the cardiomyocytes was passed through a 100 mm cell strainer (Falcon) into a 50ml Falcon tube. The cells were left for 20 minutes to sediment. Following sedimentation, the supernatant was removed and resuspended in 10ml Stopping Buffer 2, which contained Perfusion buffer supplemented with 5% FBS and 12.5 μ M CaCl₂. The resuspended cells were left to sediment for 10 minutes in the stopping buffer 2. After the 10 minutes, the supernatant was removed and discarded and the cells resuspended in T1 solution, which contained Stopping buffer 2 supplemented with 62 μ M CaCl₂. The cells were then subsequently resuspended and left to sediment in solutions T2-T5 (112 μ M-1mM CaCl₂) in order to reintroduce the cells to physiological concentrations of calcium (Ca²⁺). After the final Ca²⁺ reintroduction, the cells were resuspended into Culture media consisting of M199 medium (Sigma-Aldrich) supplemented with 2mM L-carnitine (Sigma-Aldrich), 5mM creatine (Sigma-Aldrich) and 5mM taurine (Sigma-Aldrich). The cells were then plated in 30mm dishes (Thermo Scientific) coated with 1 μ g/ml of laminin (Sigma-Aldrich) in order to adhere the cells to the plate. The cells were then placed and maintained in an incubator at 37°C with 5% CO₂ for an hour. After the hour, dead and unattached cells were removed by the removal of the culture medium. Culture media was then supplemented with 25 μ M of blebbistatin (Sigma-Aldrich) for overnight culture in the incubator at 37°C with 5% CO₂.

Table 3. Reagents contained in the Perfusion Buffer of adult mouse cardiomyocytes

Perfusion Buffer

Reagents (Sigma-Aldrich)	Concentration
Sodium Chloride (NaCl)	113mM
Pottasium Chloride (KCl)	7.46mM
Pottasium Phosphate Monobasic (KH ₂ PO ₄)	0.6mM
Sodium Phosphate Dibasic (Na ₂ HPO ₄)	0.6mM
Magnesium Sulfate Heptahydrate (MgSO ₄ -7H ₂ O)	1.2mM
Sodium Bicarbonate (NaHCO ₃)	12mM
Pottasium Bicarbonate (KHCO ₃)	10mM
Phenol Red	0.032mM
HEPES Sodium Salt	0.922mM
Taurine	30mM
2,3-Butanedione Monoxime (BDM)	10mM
Glucose	5.5mM
pH was adjust using 1M HCl to 7.4	

3.1.5 Hypoxia-Reoxygenation Injury Model in Adult Mouse Cardiomyocytes

Adult mouse cardiomyocytes were successfully isolated from DDAH2 WT and DDAH2 global KO 10-12 weeks old male mice. After overnight culture of the cells in the presence of 25μM blebbistatin, the cells were washed once with Culture Media to remove the blebbistatin and placed back in the incubator for 30 minutes to equilibrate at 37°C with 5% CO₂ prior to the hypoxia treatment. After the 30 minutes, the cardiomyocytes were placed into a hypoxia chamber (Coy Laboratory Products Inc.). The media was removed and was replaced with Hypoxia buffer (Table 4) which had been placed into the hypoxia chamber overnight to equilibrate its oxygen O₂ concentration to 5%. The cells were then incubated in the hypoxic chamber for 3 hours at 37°C with 5% O₂, 5% CO₂ and 90% N₂. At the end of hypoxia treatment, Hypoxia media was substituted by

Normoxia media (Table 5) and the cultures were incubated for 1 hour at 37°C with 5% CO₂. Samples for mRNA, Mass-Spectrometry and Protein analysis were collected before hypoxia, after 3 hours of hypoxia and after an hour of normoxia as described in chapters 2.2.1 and 2.2.7.

Table 4. Reagents contained in Hypoxia Buffer (Schäfer et al., 2000)

Hypoxia Buffer

Reagents (Sigma-Aldrich)	Concentration
Sodium Chloride (NaCl)	127.8mM
Pottasium Chloride (KCl)	14.8mM
Pottasium Phosphate Monobasic (KH ₂ PO ₄)	1.2mM
Magnesium Sulfate (MgSO ₄)	1.2mM
Sodium Bicarbonate (NaHCO ₃)	2.2mM
Calcium Chloride (CaCl ₂)	1mM
Sodium Lactate	10mM
pH=7.4	

Table 5. Reagents contained in Normoxia Buffer (Schäfer et al., 2000)

Normoxia Buffer

Reagents (Sigma-Aldrich)	Concentration
Sodium Chloride (NaCl)	118mM
Potassium Chloride (KCl)	14.8mM
Pottasium Phosphate Monobasic (KH ₂ PO ₄)	1.2mM
Magnesium Sulfate	1.2mM
Sodium Bicarbonate (NaHCO ₃)	22mM
Calcium Chloride (CaCl ₂)	1mM
Glucose	10mM
pH=6.4	

3.1.6 Ischemia/Reperfusion Model in the Isolated Adult Mouse Heart

In this model, DDAH2 WT, DDAH2 global KO and C57/BL6 8-10 week old male mice were used. 30 minutes prior to commencing the experiment, the

mice were injected with 125U/ml of heparin (Fannin). All animals were anesthetized with 40 mg/ml Ketamine (Vetalar™V, Zoetis) and 3.2 mg/ml xylazine (Rompun, Bayer). Once complete anesthesia was achieved, the chest and the rib cage were cut open to expose the beating heart. The heart along with the lungs and thymus were removed and placed in ice cold PBS in order to cease the contractions of the heart. The aorta was cannulated, ligated and attached to the Langendorff apparatus probe after the removal of the lungs and the thymus. Perfusion of the heart with Perfusion buffer was started immediately at 5 ml/min at 37°C. The heart was perfused for 10 minutes to restart the contractions and also to equilibrate the heart to the new conditions. After the initial stabilization period the experiment began and the heart's physiology (Aortic Pressure, Heart Rate, Coronary Flow and Electrophysiology) was monitored. After 15 minutes, perfusion of the heart was completely stopped and the 30 minutes of Ischemia commenced. The heart was reperfused for 45 minutes after the 30 minutes of ischemia. The hearts were submerged throughout the equilibration and experimental time in Perfusion buffer contained in an organ bath (Radnoti). mRNA, Protein and Mass spectrometry samples were collected after Perfusion, after Ischemia and at the end of Reperfusion.

Finally, using the Ischemia/Reperfusion Model on the Langendorff apparatus, the effect of methylarginines and Nitric Oxide Synthase Inhibitors were measured. During these experimental studies, the Perfusion buffers were supplemented with physiologically concentrations as shown in table 6.

Table 6. Reagents contained in Perfusion Buffer for physiological analysis of adult mouse hearts using a Langendorff apparatus.

Perfusion buffer	
Reagents (Sigma-Aldrich)	Concentration
Sodium Chloride (NaCl)	119mM
Potassium Chloride (KCl)	4.7mM
Sodium Bicarbonate (NaHCO ₃)	22mM
Magnesium Chloride (MgCl ₂)	1.2mM
Pottasium Phosphate Monobasic (KH ₂ PO ₄)	1.2mM
Calcium Chloride (CaCl ₂)	2.5mM
EDTA	0.5mM
Sodium Pyruvate	2mM

pH was equilibrated to 7.4 by using 95% O₂/ 5% CO₂ (BOC)

SDMA	10µM
ADMA	10µM
L-NMMA	10µM
L-NAME	25µM
1400W	5µM

3.2 Molecular Biology Techniques

3.2.1 RNA Extraction from Cells and Tissue Samples

All treatment media were removed and the cells washed twice with Phosphate Buffered Saline (PBS) (Sigma-Aldrich) to remove all traces of the treatments and culture medium. 800 µl of Qiazol® Lysis Buffer (Qiagen) were added per 6-well plate and the cells detached from the plate using a cell-scraper (Thermo Scientific). 160 µl of chloroform (Sigma-Aldrich) were added per sample and the samples were manually mixed. The samples were then centrifuged at 16000g for 15 minutes at 4°C in order to separate the phases. The upper phase was collected and transferred into new sample tubes and equal volumes of 70% ethanol (Fisher Chemical) were added. RNA was isolated from the samples using a column based isolation kit (RNeasy mini kit, Qiagen) following the company's protocol.

Mouse tissues were isolated and snap frozen in liquid nitrogen immediately after removal. The samples were stored in -80°C until further process. The samples were homogenized using a steel ball bearing grinder (5 minutes at 30Hz; TissueLyser II, Qiagen). The tissue lysates were then centrifuged at 8000g for 15 minutes at room temperature to remove cell debris. RNA was isolated from the tissue samples using the column based isolation kit (RNeasy mini kit, Qiagen) following the company's protocol.

All isolated RNA samples were stored at -80°C until further analysis.

3.2.2 RNA quantification and cDNA synthesis

RNA concentration for each sample was quantified by using a Nanodrop™ Spectrophotometer (Thermo Scientific). Briefly, 2 µl of each sample were loaded on to the machine to form a column. Based on the light absorption of each sample on different wavelengths the RNA concentration and quality were measured.

After measuring the concentration of each RNA sample, 1µg of total RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (BioRad). A reaction mix was created for each sample as outlined in table below (Table 7).

Table 7. Reverse Transcription reaction reagents and cDNA synthesis protocol

Reaction Mix

RNA (1µg) + Water	15 µl
Reverse transcriptase	1 µl
5x iScript Reaction Mix	4 µl
Total Reaction Volume	20 µl

cDNA synthesis protocol

Reverse Transcription	30 min at 42°C
Reverse Transcriptase inactivation	5 min at 85°C

3.2.3 Real-Time Polymerase Chain Reaction (RT-PCR)

All samples were analyzed using the iTaq Fast SYBR Green Supermix (BioRad) fluorescent dye and the ROX reference dye (Invitrogen). Each sample was run and analyzed in duplicate and the average C_T values used in the analysis. Each reaction was set up as shown in Table 8.

A fluorescence threshold (C_T) was set manually at the base of the exponential phase of the curve for each reaction plate. A standard curve was created on each plate and for each set of primers in order to measure the mRNA concentration of each gene. All standard curves and sample C_T values were plotted on a logarithmic scale and the relative sequence transcription

calculated (Nolan, Hands, & Bustin, 2006). All sample values were normalized to a housekeeper gene in order to correct for the amount and quality of the cDNA of each reaction. A selection of housekeeping genes was analyzed in order to determine the most stably expressed gene throughout the treatments. GAPDH was found to be the most stably expressed housekeeper gene according to our analysis. Finally, negative controls were also run on each plate with water instead of cDNA.

Table 8. cDNA synthesis protocol and reagents

Reaction Mix	
cDNA (5ng)	1 μl
Primers (10μM)	0.5 μl (per primer)
iTaq Fast SYBR Green Supermix (2x)	10 μl
Water	8 μl
PCR cycling protocol	
Initial step	95°C 20 seconds
Thermal cycling (x40 cycles)	
Denaturation	95°C 1 second
Annealing	60°C 20 seconds

3.2.4 Protein Extraction from Cells and Tissue Samples

For protein extraction from cell, all media was aspirated from each well and the cells washed twice with PBS. After the wash, the cells were collected using 0.05% Trypsin and centrifuged at 1.000 rpm for 5 minutes at room temperature. The supernatant was aspirated and the cells were lysed in Radioimmunoprecipitation Assay (RIPA) buffer (Thermo Scientific) composed of the following: Tris 50mM, NaCl 150mM, SDS 0.1%, sodium deoxycholate 0.5%, Triton X 100, 1mM PMSF and supplemented with Complete Protease Inhibitors (Roche). The samples were pipetted up and down to facilitate cell lysis and centrifuged at 14.000 rpm for 15 minutes at 4°C. The supernatant was

transferred into a new eppendorf tube and stored at -80°C until further processing.

RIPA buffer added to samples containing adult mouse cardiomyocytes were additionally supplemented with the following phosphatase inhibitors: NaF 5mM, BgP 20mM and NaPPi 5mM.

At the end of all *in vivo* or *ex vivo* experiments, the tissue samples were immediately snap frozen in liquid nitrogen (-180°C) and stored at -80°C until further processing. The samples were later thawed on ice and RIPA buffer supplemented with Complete Protease Inhibitors added. The tissue samples were then lysed using the TissueLyser II (Qiagen) at 30 Hz for 5 minutes. Following lysis, the homogenized tissues were then centrifuged at 12.000 g for 15 minutes at 4°C and the supernatant stored at -80°C for future analysis.

3.2.5 Protein Quantification

Protein quantification was performed using the Pierce® BCA Protein Assay Kit (Thermo Scientific). Pierce BCA Protein Assay is a detergent-compatible formulation based on bicinchonic acid (BCA) for colorimetric detection and quantitation of total protein. The absorbance shift was measured by spectrophotometry (Fluostar Omega, BMG Labtech). This method is based on the reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline solution.

10 µl of each sample were loaded in each well of a 96-well plate (Thermo Scientific) and mixed with 200 µl of the Working Reagent. The working reagent consisted of two reagents mixed at a 1:50 ratio. Each plate also contained known concentrations of controls to generate a standard curve. The standard controls used were 0, 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg/ml Bovine Serum Albumin (BSA). The plate was then incubated at 37°C for 30 minutes. After incubation, the plate was placed in a spectrophotometer and the light absorbance measured at 562nm. The protein concentrations of the samples were then calculated using the standard curve.

3.2.6 SDS-Page Gel Analysis

Proteins were separated according to their size by SDS-PAGE gel analysis (Burnette, 1981). For proteins smaller than 100 kDa, 12%

polyacrylamide gels were used for the analysis and 7.5% polyacrylamide gels were used for larger proteins. A 4% stacking gel was placed on top of every gel. Gels were made according to the following recipe (Table 9).

Table 9. SDS-PAGE gel reagents (depending on acrylamide %)

Reagents	Resolving Gels		Stacking Gel
	2x12% gels	2x7.5% gels	2x4% gels
dH ₂ O	6.95 ml	8.75 ml	6.32 ml
40% Acrylamide			
/Bis 37.5:1	4.8 ml	3 ml	1 ml
0.5M Tris-HCl			
pH=6.8	-	-	2.52 ml
1.5M Tris-HCl			
pH=8.8	4 ml	4 ml	-
10% SDS	160 µl	160 µl	100 µl
TEMED	16 µl	16 µl	20 µl
10% APS	160 µl	160 µl	100 µl

All protein samples were prepared as following:

Table 10. Reagents contained in protein samples analyzed by SDS-PAGE gel

Protein samples	
	Quantity (µl)
Protein (100 µg)	1-16 µl
H ₂ O	0-16 µl
5x Laemli Buffer	4 µl
10% w/v SDS	
10mM β-mercaptoethanol	
20% v/v Glycerol	
0.2M Tris-HCl, pH=6.8	
0.05% Bromophenolblue	
Final Volume:	20 µl

10-20 μ l of sample was loaded per well depending on the type of protein being analyzed (Table 10). Precision Plus Protein™ All Blue Prestained Protein Standards marker (BioRad) was used to identify the size of the proteins. The SDS-PAGE gels were then run at constant voltage (150V) for up to 90 minutes at room temperature. Right after, the gels were equilibrated in transfer buffer containing 20% methanol for 30 minutes. After equilibration, the gels were set up to transfer the proteins onto a PVDF membrane using a BioRad Mini-Transblot system. The transfer was set up on ice for 2 hours at constant current of 260 mA.

3.2.7 Western Blotting

After transfer, the membrane was washed once in PBS, then submerged in 100% methanol (Fisher Scientific) and left to air-dry at room temperature for 30 minutes. After the 30 minutes, the primary antibody (Table 11) was added at an appropriate dilution in 5% milk (OXOID) or 5% BSA (Sigma-Aldrich) in PBS-T and the membrane was incubated overnight at 4°C. The next day, the membrane was washed 3 times with 100 ml PBS-T for 10 minutes each time. Following washing, the membrane was incubated with the secondary antibody (Table 11) at an appropriate dilution in 5% milk PBS-T or 5% BSA PBS-T at room temperature for 2 hours. After incubation, the membrane was again washed 3 times with PBS-T as specified above. Two detection technologies were used for the visualization of the proteins of interest: chemiluminescence or fluorescence detection. For the chemiluminescence technique, the 20x LumiGlo® Reagent and 20x Peroxide kit (Cell Signaling Technologies) was used whilst for the fluorescence detection, the Odyssey CLx Imager System (LI-COR Biosciences) was used.

Table 11. Primary and secondary antibodies

Primary antibodies

Protein	Company	Species	Catalogue #
eNOS (140kDa)	Cell Signaling	Rabbit	9572S
iNOS (130kDa)	Cell Signaling	Rabbit	2982S
DDAH1 (32kDa)	-	Goat	-
DDAH2 (29kDa)	-	Goat	-
AKT (60kDa)	Cell Signaling	Rabbit	9272S
Troponin T (38kDa)	Abcam	Mouse	ab10214
α -Tubulin (55kDa)	Abcam	Mouse	ab7291
Oct4 (39kDa)	Abcam	Rabbit	ab27985-100
Sox2 (40kDa)	Abcam	Goat	ab59776
Nkx2.5 (35kDa)	Abcam	Rabbit	ab35842
SMA (42kDa)	Abcam	Rabbit	ab5694

Secondary antibodies

Species	Company	Catalogue #
Goat anti-Mouse	LiCOR	926-68070
Goat anti-Rabbit	LiCOR	926-32211
Rabbit anti-Goat	Life Technologies	A21084
Goat anti-Rabbit	DAKO	P0448
Goat anti-Mouse	DAKO	P0447
Rabbit anti-Goat	DAKO	P0449

3.2.8 Liquid Chromatography-mass Spectrometry / Mass Spectrometry (LC-MS/MS)

Liquid chromatography-mass spectrometry (LC-MS/MS) is a technique traditionally used for the detection of substances even in pico-molar concentrations within complex samples. The combination of the two techniques allows higher specificity in the analysis.

All samples were pumped using high pressure down a chromatography column containing silica. Binding of the substances on the column is based on hydrophobic and ionic interactions. Washing the column using elution buffers with a pH or a salt gradient, all compounds are separated.

Compounds were separated by washing the column with elution buffers with pH or a salt gradient. The eluted substances were then passed through a spectrometer. The samples are vaporized before ionization with an electron beam. The substances are then separated based on their mass-to-charge ratio (m/z) by passing through an electro-magnetic field. After the first separation, all ions are fragmented as they pass through a chamber containing an inert gas like nitrogen. Finally, the ions accelerate in a second m/z field just before detection. Samples containing pure forms of the molecules in question are used to set the selectivity of the filters making the technique highly specific.

All biological samples were prepared by methanol extraction. Specific volume of each sample was mixed with pure methanol in a ratio of 1:5. D7-ADMA (Cambridge Isotope Laboratories) was added as an internal standard before precipitation in order to measure extraction efficiency and ion suppression at detection. The samples were centrifuged at 12000 g for 15 minutes at 4°C. The supernatant was transferred into a new eppendorf tube and the samples were placed in a heat block (Star Lab) at 95°C until complete evaporation. The samples were then resuspended in 50 µl of 0.1% Formic Acid (Fisher Scientific). The Agilent 6400 Series Triple Quadrupole LC-MS/MS System was used for the analysis. Methylarginines (Calbiochem) and aminoacids (Calbiochem) were used for the optimization of the detection system.

All data were collected using the Agilent MassHunter Data Acquisition Software (Agilent Technologies) and were analyzed with the MassHunter Qualitative Analysis Software (Agilent Technologies).

3.2.9 Nitric Oxide (NO) Analysis

The majority of NO produced is derived to nitrates in the presence of myoglobin (Bourassa, Ives, Marqueling, Shimanovich, & Groves, 2001; Garry, Kanatous, & Mammen, 2003). Nitric oxide can be re-derived from nitrites and nitrates by reduction in boiling solution of vanadium chloride (Sigma-Aldrich).

NO is released and measured using the chemi-luminescent reaction with ozone, which emits light in the red spectrum.

Proteins were removed from the samples using methanol precipitation method. The samples were diluted 1:5 into pure methanol and centrifuged at 12000 g for 15 minutes at 4°C. The supernatants were used for the analysis. 40 µl of each sample were loaded on the Sievers NOA 280i (GE Analytical Instruments) and analyzed in duplicates. Known concentrations of sodium nitrate (NaNO₃) (Sigma-Aldrich) were used as standards and to generate standard curves. The standard curves were used for the quantification of NO in the samples. The concentrations were also normalized to the total amount of protein contained in each sample. All results were expressed as µM/µg of total protein.

3.3 *In vivo* Methods

3.3.1 Breeding and Generation of Animal Lines

All *in vivo* experiments were carried out under a Home Office Project and Personal License (PPL 70/7372, PIL 70/25030) and in accordance with the 3R principles of replacement, refinement and reduction. Experiments were designed to reduce the number of animals used and to reduce any pain, discomfort, or stress to the animals involved in the studies. All individuals were maintained on a 12-hour day/night cycle and had continuous access to food and water *ad libitum*. The animals were maintained in social groups with appropriate environmental enrichment. Animals were age and sex matched in experiments and littermate controls were used. Mr. Matthew Delahaye carried out all daily work for the maintenance of breeding colonies.

Global DDAH1 knockout mice were maintained on a mixed C57BL/6 background. The cre lox-P system was used for the deletion of the first exon of the gene. Cre protein is a DNA recombinase, which catalyzes the recombination of a DNA region enclosed by two loxP sites. The alpha-actin promoter was used to drive the expression of the cre. All breeding pairs were set up as follows: heterozygous Cre⁺ X heterozygous Cre⁻.

A high throughput gene trapping approach based on a retroviral vector was used for the creation of the global DDAH2 knockout mice. Heterozygous

individuals were obtained from Texas Institute for Genetic Medicine and were bred in a mixed B6/129 background.

3.3.2 Genotyping End Point PCR

Tail or ear sections were collected from the mice at the age of 4 weeks and used for genotyping. DNA extraction was done using proteinase K digestion buffer containing: 10mM Tris base, 1.5mM MgCl₂, 50mM KCl, 0.01% gelatin, 0.45% Igepal Ca-630 and 0.45% Tween 20 supplemented with proteinase K. All samples were digested at 53°C overnight and then further heated for 10 minutes at 100°C for the deactivation of the proteinase K enzyme.

An End Point PCR was used to amplify specific target regions of DNA. For all reactions, the ReadyMix Taq PCR Reaction Mix (Sigma) was used containing Taq polymerase, dNTPs and a red loading dye. All reactions were set up as shown in table (Table 12) and the thermocycler was programmed as shown in the same table.

Table 12. Genotyping PCR reaction reagents and protocol

Reagents	Quantity
DNA	1 µl
RedTaq PCR Master Mix (2x)	6 µl
Primers (10mM)	0.5 µl each
Water	4 µl

Thermocycler Protocol

2 minutes 94°C

x40 cycles:

20 seconds 94°C

40 seconds 60°C

1 minutes 72°C

5 minutes 72°C

3.4 Data analysis

Statistical analysis was performed using the Prism software package (GraphPad Inc., UK). All data are presented as means $\pm$ Standard Error of the Mean (SEM). For comparisons between two groups for a single variable, an unpaired two-tailed t-test (parametric data) was used. Differences between multiple groups were assessed by one-way analysis of variance (ANOVA) followed by Bonferroni's post-test for multiple comparisons. Two-way ANOVA was used where comparisons were being made between groups subject to two experimental variables, followed by Bonferroni's post-test for multiple comparisons. P values of <0.05 were considered statistically significant.

Chapter 4

4. NO signaling in maintenance of pluripotency and differentiation of mouse embryonic stem cells

4.1 The effect of methylarginines in maintaining pluripotency in mouse embryonic stem cells

4.1.1 Introduction

Embryonic stem cells possess two unique characteristics, the ability to replicate and to differentiate into all cell populations of the embryo (Boiani & Schöler, 2005). Pluripotency is a unique cell state and it requires a variety of genes and factors for its maintenance (Martí et al., 2013b). Nitric oxide has been implicated in maintenance of pluripotency (Beltran-Povea et al., 2015; Mora-Castilla et al., 2010). Low levels of NO *in vitro*, blocks embryonic stem cell differentiation by preventing the loss of self-renewal genes (Beltran-Povea et al., 2015). On the other hand, high concentrations of NO donors can promote differentiation of mouse stem cells by down-regulating the pluripotency markers *Nanog* and *Oct4* (Mora-Castilla et al., 2010).

Since NO levels could play a role in maintaining pluripotency, we hypothesized that ADMA, a NOS inhibitor, could affect the maintenance of pluripotency by inhibiting NO production.

In order to identify if mouse embryonic stem cells upon treatment with 100µM ADMA have started differentiating to the three germ layers or extraembryonic tissues, we selected specific factors for each germ layer and we monitored their expression pattern.

Gcm1 gene was selected as a trophoblast specific marker due to its strict tissue-specific and prolonged (spanning from early to late developmental stages) expression pattern (Basyuk et al., 1999).

Tuj1 was selected as the ectoderm specific marker for our experiments due to its early expression in neuronal progenitor cells and the continuous expression in mature neuronal cell types (Menezes & Luskin, 1994).

Sox17 gene expression is essential for the formation of endoderm (Kubo, 2004), a trait that makes it a suitable marker for the identification of cells with commitment to this germ layer.

Finally, *Brachyury* has been used as a mesoderm marker (B. L. Martin & Kimelman, 2010) as it is expressed early on during mesoderm differentiation and its expression is strictly associated with the establishment of this specific germ layer.

4.1.2 Experimental Design

E14 mouse embryonic stem cells were used in this experiment to identify the potential role of ADMA and NO inhibition in maintenance of pluripotency. E14 embryonic stem cells were maintained pluripotent *in vitro* by LIF supplementation in the media and culture on a feeder layer. 100µM of ADMA was supplemented to the media of treated cells. Control and 100µM ADMA treated cells were cultured in the same conditions for 4 days. RNA and protein samples were collected on Day 0, Day 2 and Day 4 of culture and were used to analyze pluripotency and differentiation markers as well as NO regulation-associated genes.

4.1.3 Results

4.1.3.1 AP staining

Expression of alkaline phosphatase (a pluripotency marker) was used to confirm pluripotency on day 4 of culture. Control and ADMA treated samples were stained for the activity of alkaline phosphatase on Day 4 to confirm that cells are pluripotent. Both control and ADMA treated cells were positively stained showing that ADMA treatment did not have an effect on pluripotency by Day 4 (Figure 17).

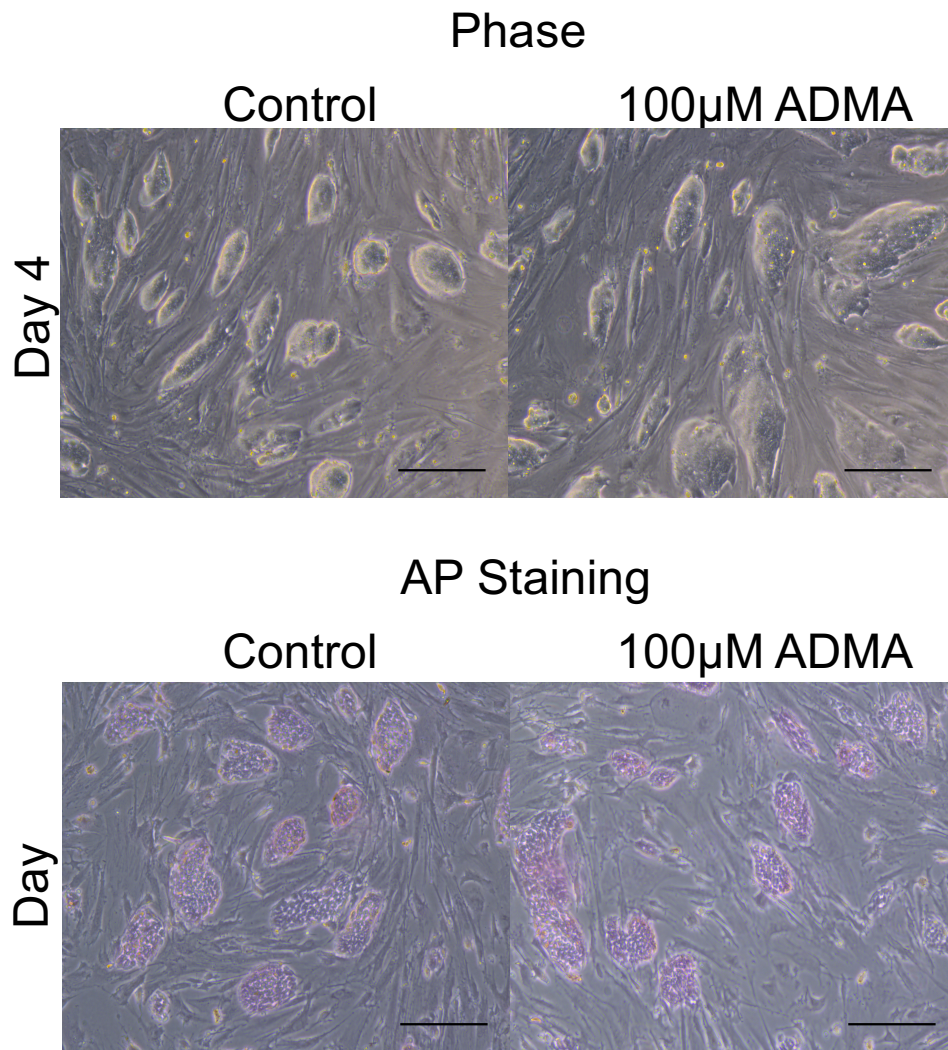


Figure 17. AP staining on Day 4 of Control and 100µM ADMA treated cells. No difference was detected in both sample types. Both sample types were positively stained for the presence of active alkaline phosphatase showing that pluripotency was not affected. n=3

4.1.3.2 RNA and protein analysis of pluripotency markers

RNA and protein samples of control and ADMA treated cells were collected on Day 0, Day 2 and Day 4 and were analyzed for the expression of pluripotency-associated markers *Oct4* and *Sox2*. Both markers showed no change in their gene or protein expression level in Control or ADMA treated cells (Figure 18). No changes in the expression pattern of these markers shows that treatment of cells with the NOS inhibitor, ADMA, had no significant effect on the maintenance of pluripotency state of embryonic stem cells.

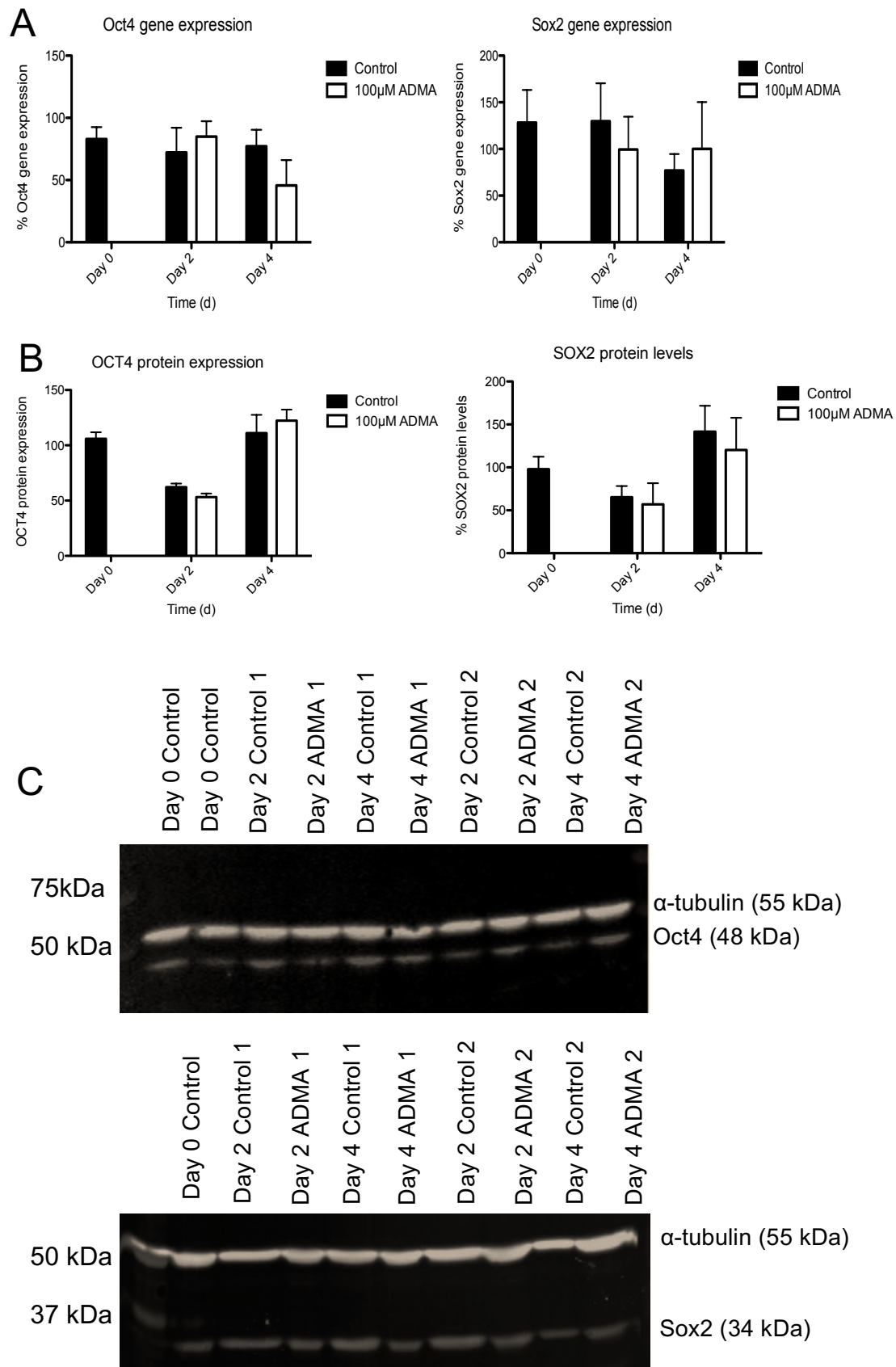


Figure 18. mRNA and protein analysis of pluripotency associated genes *Oct4* and *Sox2* of control and 100µM ADMA treated samples on Day 0, Day 2 and Day 4. **A.** mRNA levels analysis of *Oct4* and *Sox2* genes by qPCR *Oct4* (Day 0 Control (82.946 ± 9.678), Day 2 Control (72.187 ± 19.815) vs 100µM ADMA (84.992 ± 12.358), Day 4 Control (77.127 ± 13.249) vs 100µM ADMA (45.681 ± 20.440)) *Sox2* (Day 0 Control (128.272 ± 35.015), Day 2 Control (129.669 ± 40.811) vs 100µM ADMA (99.417 ± 35.155), Day 4 Control (76.950 ± 17.643) vs 100µM ADMA (100.166 ± 50.091)). $n=4$ Two-way ANOVA ns. **B-C.** Protein analysis of *Oct4* and *Sox2*

during treatment in control and ADMA treated samples OCT4 (Day 0 Control (105.877 ± 5.877), Day 2 Control (62.187 ± 3.196) vs 100 μ M ADMA (53.135 ± 3.296), Day 4 Control (111.056 ± 16.481) vs 100 μ M ADMA (122.340 ± 9.927)) SOX2 (Day 0 Control (97.886 ± 14.575), Day 2 Control (65.295 ± 12.907) vs 100 μ M ADMA (56.887 ± 24.646), Day 4 Control (141.656 ± 30.028) vs 100 μ M ADMA (120.198 ± 37.515)). α -Tubulin was used as a loading control. n=4 Two-way ANOVA ns.

4.1.3.3 DDAH gene expression analysis by qPCR

Expression analysis of DDAH genes by qPCR showed no change with ADMA treatment. Both *Ddah1* and *Ddah2* genes did not show any difference in their gene expression levels (Figure 19).

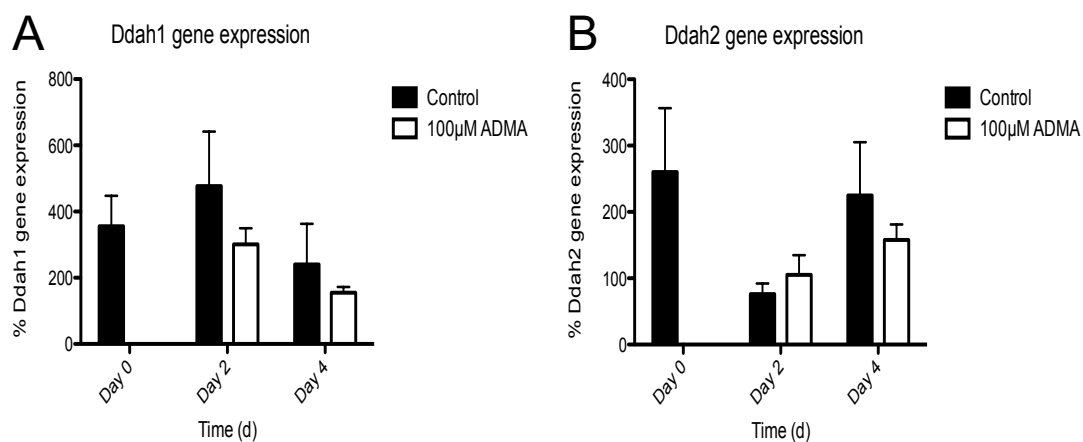


Figure 19. DDAH gene expression pattern during treatment of control and ADMA treated pluripotent stem cells. **A.** Expression analysis of *Ddah1* gene (Day 0 Control (355.513 ± 92.010), Day 2 Control (477.075 ± 164.911) vs 100 μ M ADMA (301.173 ± 48.813), Day 4 Control (240.648 ± 122.474) vs 100 μ M ADMA (154.844 ± 17.586)). **B.** mRNA levels of *Ddah2* gene (Day 0 Control (260.324 ± 96.063), Day 2 Control (76.326 ± 15.857) vs 100 μ M ADMA (105.234 ± 29.623), Day 4 Control (224.750 ± 80.296) vs 100 μ M ADMA (157.826 ± 23.453)). n=4 Two-way ANOVA ns.

4.1.3.4 Germ layer marker pattern determination by qPCR

Markers associated with early stages of differentiation to the three germ layers, were assessed by qPCR. There was no significant difference in the expression levels of these markers when pluripotent stem cells were treated with 100 μ M ADMA (Figure 20). Mesoderm differentiation was assessed by Brachyury analysis, endoderm differentiation by *Sox17* gene expression and *Tuj1* actin was used for analyzing differentiation towards the ectoderm. Analysis of these markers showed that pluripotency was maintained and there was no differentiation towards a specific germ layer upon treatment with 100 μ M ADMA.

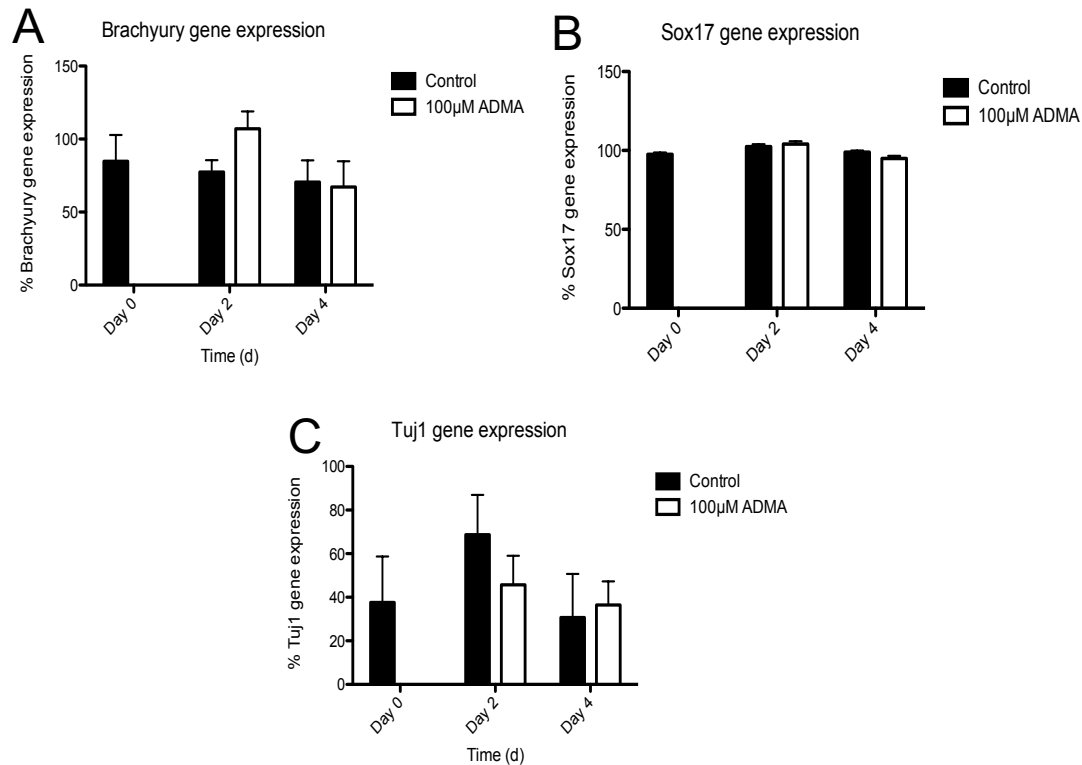


Figure 20. qPCR analysis of mRNA levels of early germ layer markers of control and ADMA treated pluripotent stem cells. No significant difference in expression of those markers during ADMA treatment was identified. **A.** Mesoderm differentiation was assessed by analysis of Brachyury gene, an early mesoderm differentiation marker (Day 0 Control (84.813±18.016), Day 2 Control (77.381±8.225) vs 100µM ADMA (107.004±11.914), Day 4 Control (70.620±14.798) vs 100µM ADMA (67.160±17.614)). **B.** Sox17 expression pattern (endoderm marker) (Day 0 Control (97.625±1.028), Day 2 Control (102.416±1.484) vs 100µM ADMA (104.040±1.765), Day 4 Control (98.882±1.099) vs 100µM ADMA (94.936±1.581)). **C.** *Tuj1* actin (Ectoderm marker) mRNA level analysis by qPCR (Day 0 Control (37.595±21.092), Day 2 Control (68.724±18.222) vs 100µM ADMA (45.688±13.385), Day 4 Control (30.746±19.939) vs 100µM ADMA (36.460±10.831)). n=4 Two-way ANOVA ns.

4.2 Expression pattern analysis of pluripotency and (NO) regulation-associated genes during differentiation of embryonic stem cells

4.2.1 Introduction

Expression pattern of genes associated with NO regulation has been thoroughly studied in adult physiology and pathophysiology (Tran, Leiper, & Vallance, 2003; Tran, Fox, Vallance, & Leiper, 2000; Vallance & Leiper, 2002) but little is known about the expression of these genes during embryonic development (Breckenridge et al., 2010) and early differentiation of stem cells (Wuchen Wang, Lee, & Lee, 2015). In order to determine the effect of ADMA-

mediated NOS inhibition on early differentiation, embryonic stem cells were differentiated for 10 days under normal conditions and expression of genes associated with NO regulation was assessed by qPCR and western blot in order to monitor the expression pattern of those genes during the first days of differentiation. Manipulation of NO production during differentiation could lead embryonic stem cells in acquiring a different cellular fate.

4.2.2 Experimental Design

E14 embryonic stem cells were cultured in the presence of feeder cells and LIF in order to remain pluripotent (Boiani & Schöler, 2005). Pluripotent stem cells were forced to aggregate and were left to differentiate for 10 days. Samples were taken during differentiation to analyze the expression pattern of pluripotent and NO regulation-associated genes.

4.2.3 Results

4.2.3.1 Expression pattern analysis of mRNA and protein levels of pluripotency genes during differentiation

The expression pattern of two pluripotency markers, *Oct4* and *Sox2*, was analyzed at the level of mRNA and protein in order to show successful transition of the embryonic stem cells from a pluripotent to a differentiation state. qPCR analysis of the two genes showed significant downregulation of both markers by day 6 of differentiation when compared to pluripotent cells on Day 0 (Figure 21). Low levels of *Oct4* and *Sox2* were also detected on Day 10 of differentiation showing adequate differentiation of the embryonic stem cells (Figure 21). Protein analysis of OCT4 protein at the same time points, verified the loss of pluripotency during differentiation (Figure 21).

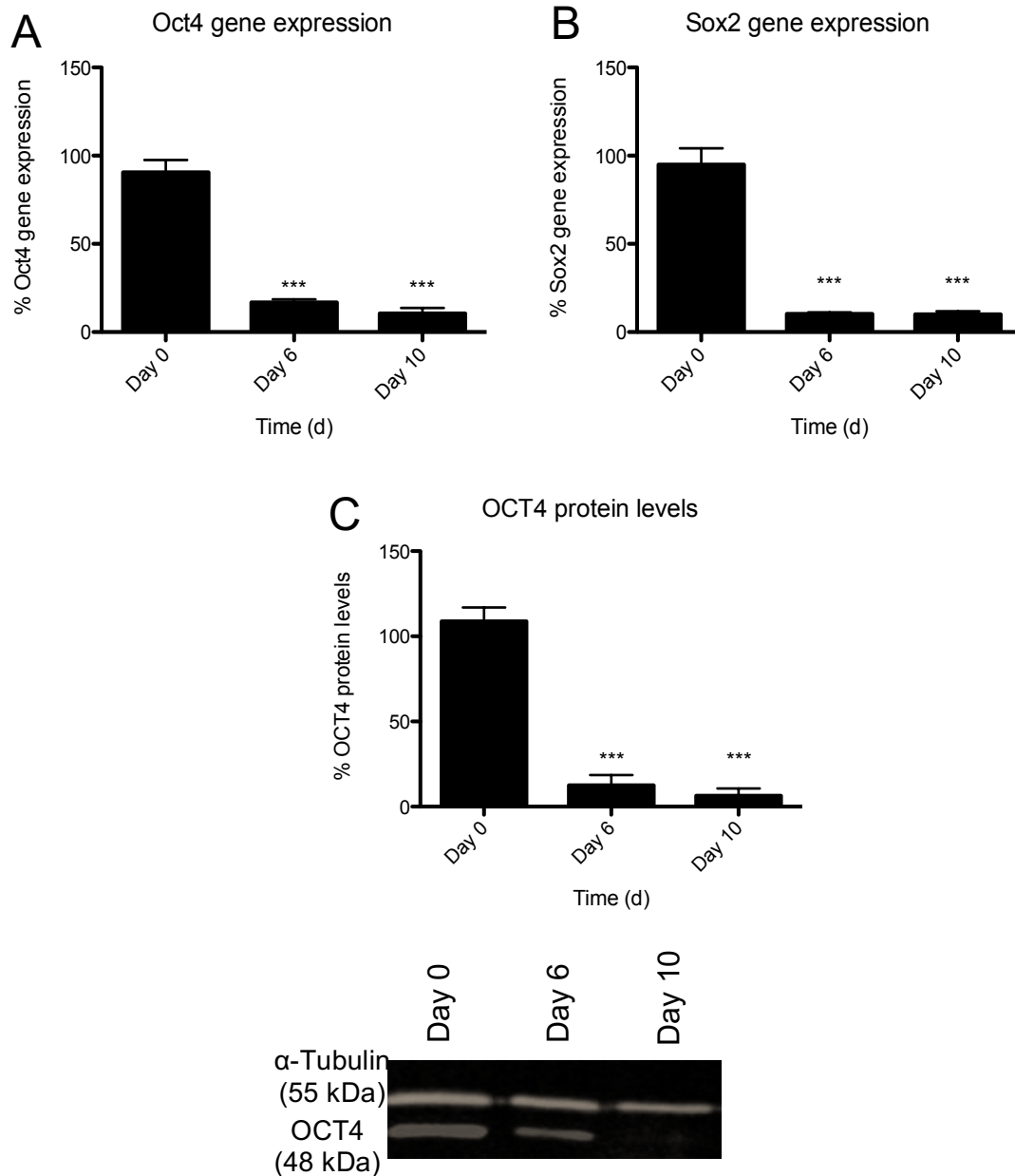


Figure 21. Pluripotency marker expression in mRNA and protein levels. **A-B.** mRNA expression of *Oct4* and *Sox2* pluripotency markers during differentiation *Oct4* (Day 0 (90.54±6.969), Day 6 (16.78±1.783), Day 10 (10.55±3.080)), *Sox2* (Day 0 (94.94±9.249), Day 6 (10.35±0.8814), Day 10 (10.01±1.800)). Expression of both pluripotency markers was significantly downregulated during 10 days of differentiation showing successful differentiation of embryonic stem cells. **C.** OCT4 protein levels during 10 days of differentiation (Day 0 (108.8±8.186), Day 6 (12.57±6.010), Day 10 (6.367±4.250)). OCT4 protein levels were significantly decreased during differentiation further supporting successful differentiation of pluripotent stem cells. n=6 One-way ANOVA *** p< 0.001.

4.2.3.2 mRNA and protein level analysis of NO regulatory genes during differentiation

Expression pattern of NO regulatory genes were assessed by qPCR and western blot analysis in order to monitor the expression levels of these genes during differentiation. *Ddah1* expression assessed by mRNA levels was

significantly down-regulated during differentiation while *Ddah2* expression was steadily and significantly up-regulated throughout differentiation (Figure 22). Protein level analysis of the same genes was consistent with mRNA expression and further supported our findings (Figure 23). All three NOS isoforms were analyzed during differentiation and showed different expression patterns. *eNOS* levels on day 10 showed a slight trend in expression compared to pluripotent stem cell expression levels but without reaching significance (Figure 22). Conversely *nNOS* was significantly down-regulated by Day 6 and maintained low expression levels throughout differentiation (Figure 22). Finally, *iNOS* expression was steadily upregulated during the process of differentiation but reached significant levels only on Day 10 compared to Day 0 (Figure 22).

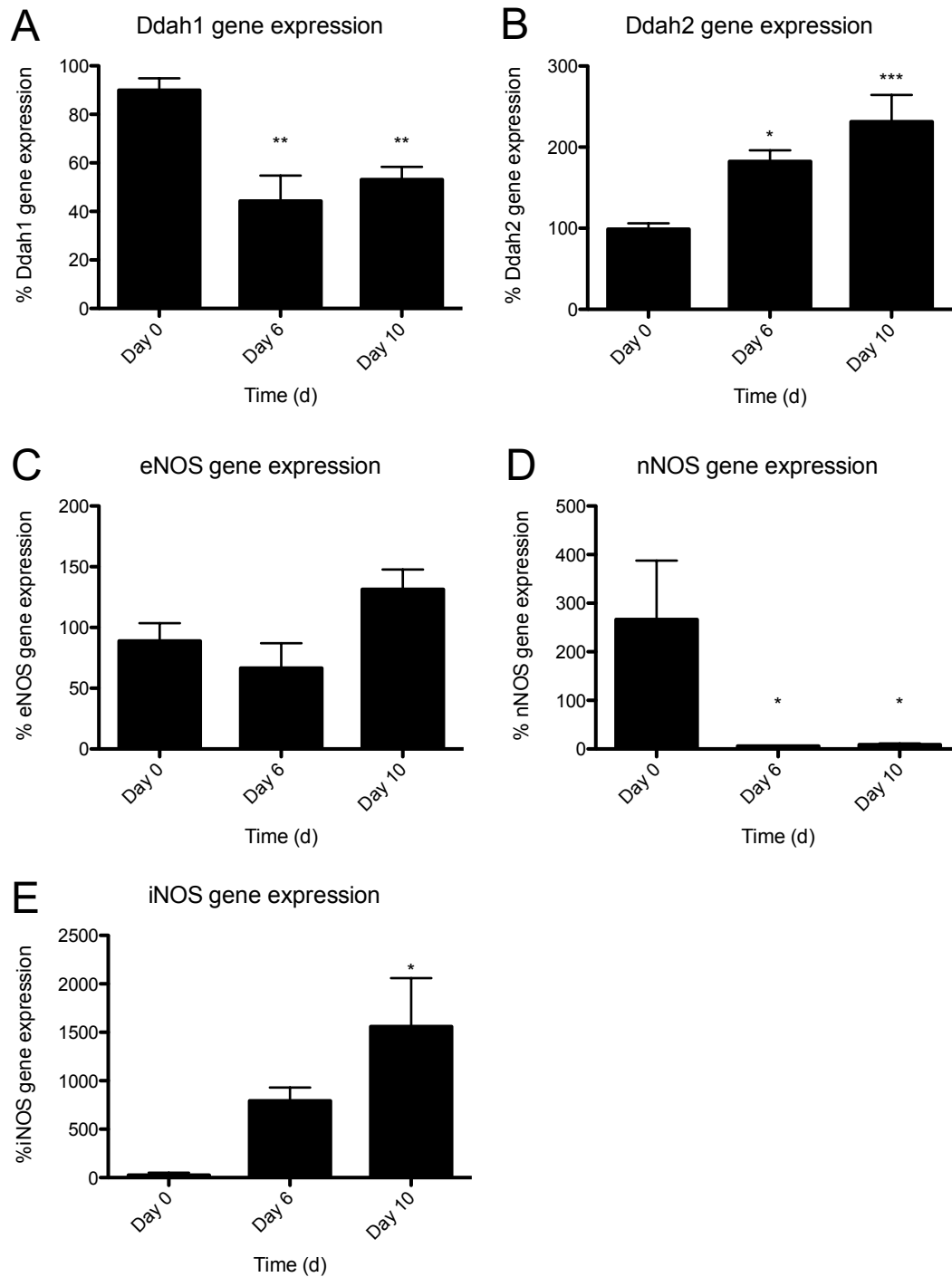


Figure 22. Nitric oxide regulatory genes expression during differentiation of embryonic stem cells. **A-B.** mRNA levels of *Ddah1* and *Ddah2* genes, *Ddah1* (Day 0 (89.99±4.896), Day 6 (44.26±10.56), Day 10 (53.09±5.308)), *Ddah2* (Day 0 (98.89±7.307), Day 6 (182.3±13.81), Day 10 (231.3±32.99)). *Ddah1* expression was significantly downregulated in comparison to *Ddah2* which was steadily upregulated during differentiation. **C-E** *eNOS* showed no significant changes and *nNOS* showed significant downregulation during differentiation *eNOS* (Day 0 (88.84±14.70), Day 6 (66.54±20.49), Day 10 (131.5±16.30)), *nNOS* (Day 0 (266.6±121), Day 6 (6.011±0.9731), Day 10 (8.876±2.622)). *iNOS* was steadily upregulated during differentiation *iNOS* (Day 0 (25±25), Day 6 (792±137.9), Day 10 (1559±500.2)). n=6 One-way ANOVA * p<0.05, ** p<0.01, *** p<0.001.

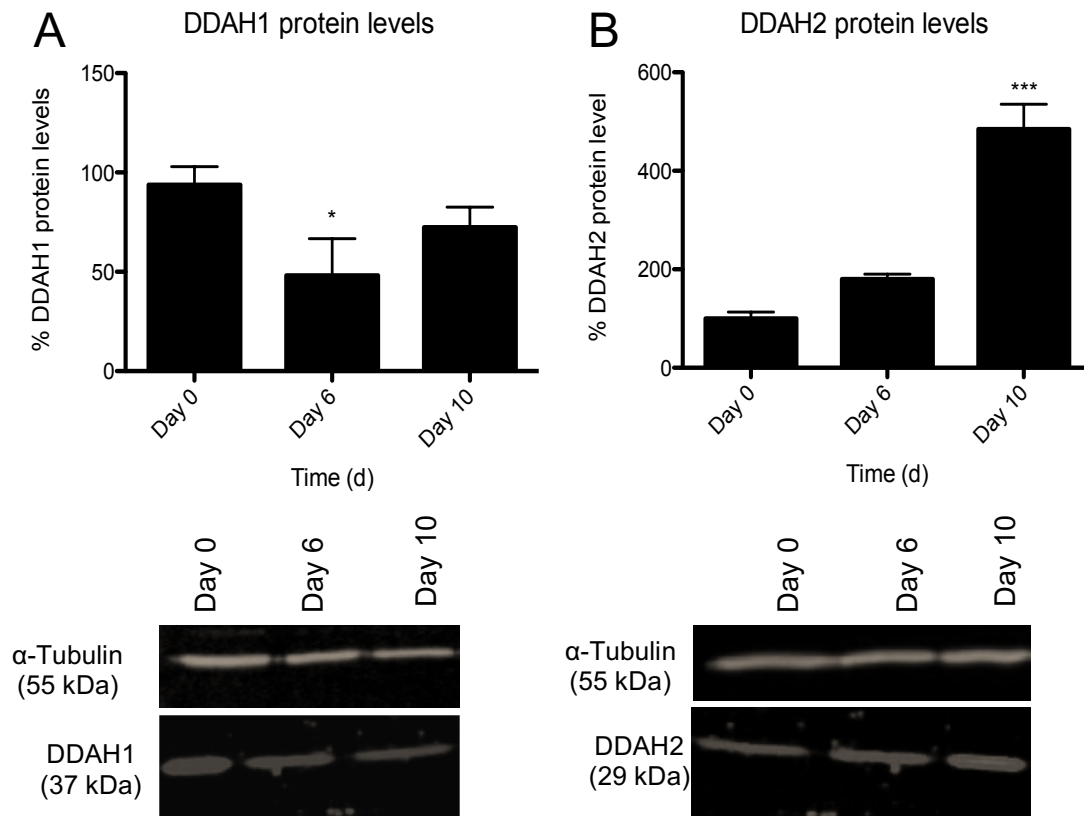


Figure 23. Protein levels during differentiation for *Ddah1* and *Ddah2* genes. **A.** DDAH1 protein levels initial showed a significant downregulation on Day 6 of differentiation, which was followed by a small upregulation by Day 10 (Day 0 (93.77±9.222), Day 6 (48.18±18.46), Day 10 (72.49±10.12)). **B.** DDAH2 protein levels were significantly and steadily upregulated during differentiation (Day 0 (100±13.15), Day 6 (180.4±10.04), Day 10 (484.8±50.53)). n=6 One-way ANOVA * p<0.05, *** p<0.001.

4.3 The Effect of ADMA in Spontaneous Differentiation of Mouse Embryonic Stem Cells.

4.3.1 Introduction

A variety of roles have been attributed to NO, during embryonic development and more specifically during differentiation of embryonic stem cells to a variety of cell types (Beltran-Povea et al., 2015; Kanno et al., 2004; Mujoo et al., 2008). Reduced NO levels during differentiation have been shown to decrease cardiac differentiation while supplementation of embryonic stem cells, during differentiation, with NO donors has the opposite effect (Kanno et al., 2004). ADMA, as a competitive endogenous NOS inhibitor, can affect the levels of NO during differentiation and change the fate of the differentiating embryonic stem cells.

In order to identify the different cellular fates that the embryonic stem cells acquired during differentiation in control and ADMA treated samples, we

used a variety of cell specific markers. Those markers have been excessively used in the past and have been shown to be linked to a particular cell type.

Its selective expression pattern and the importance of *Runx1* gene in hematopoietic differentiation during embryonic development render *Runx1* the perfect candidate for a hematopoietic specific marker. Also, CD31 gene has been traditionally used as an endothelial specific marker and due to its ability to distinguish hematopoietic from endothelial cells, it was selected as an endothelial specific marker.

4.3.2 Experimental Design

E14 embryonic stem cells were used in this experiment. Pluripotent stem cells were forced to aggregate into embryoid bodies and then cultured for up to 10 days in order to differentiate. Control embryoid bodies were supplemented with normal differentiation medium containing 20% FBS. ADMA treated embryoid bodies were cultured in normal differentiation medium containing 100 μ M ADMA. Samples for mRNA, protein, mass spectrometry and nitric oxide concentration analysis were collected on Day 0 (day of embryoid body formation), Day 6 and Day 10. Additionally, during the first two days of culture, the size of the embryoid bodies was analyzed in order to identify a potential role of NO in differentiating stem cell proliferation rate. Finally, the number of yolk-like sac formations were analyzed in control and ADMA treated cells on Day 6 of differentiation.

4.3.3 Results

4.3.3.1 Embryoid Body Size

Embryoid body size (area) analysis during the first two days of differentiation showed no significant difference between control and ADMA treated cells (Figure 24).

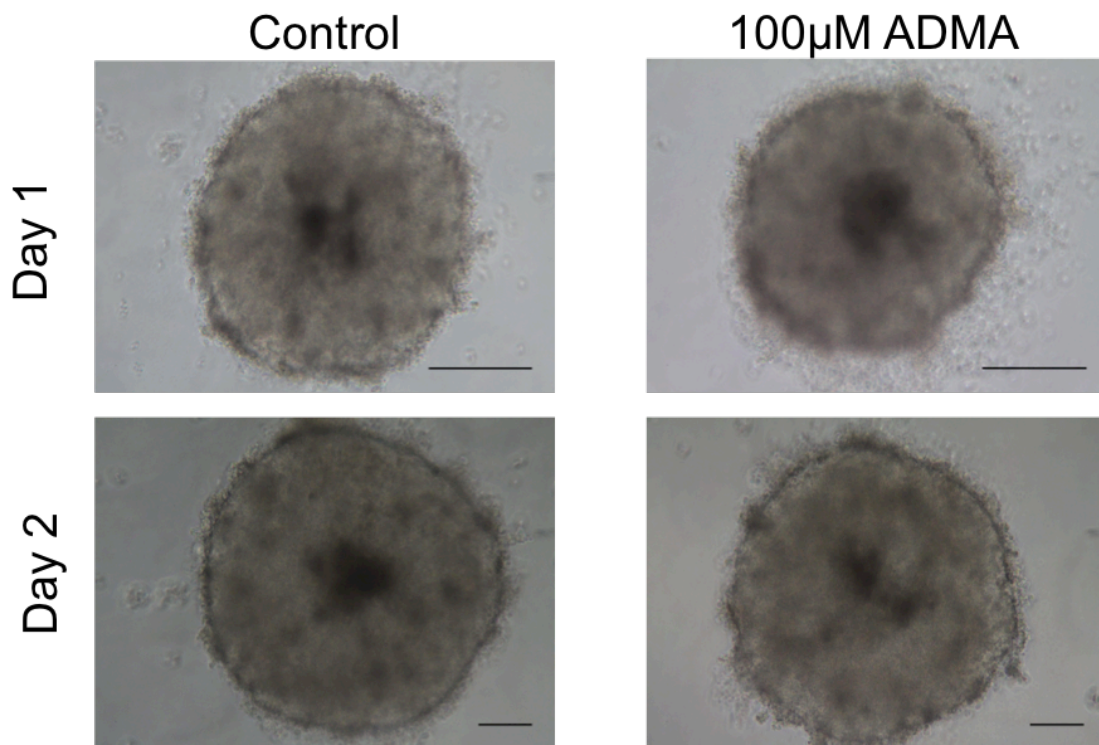
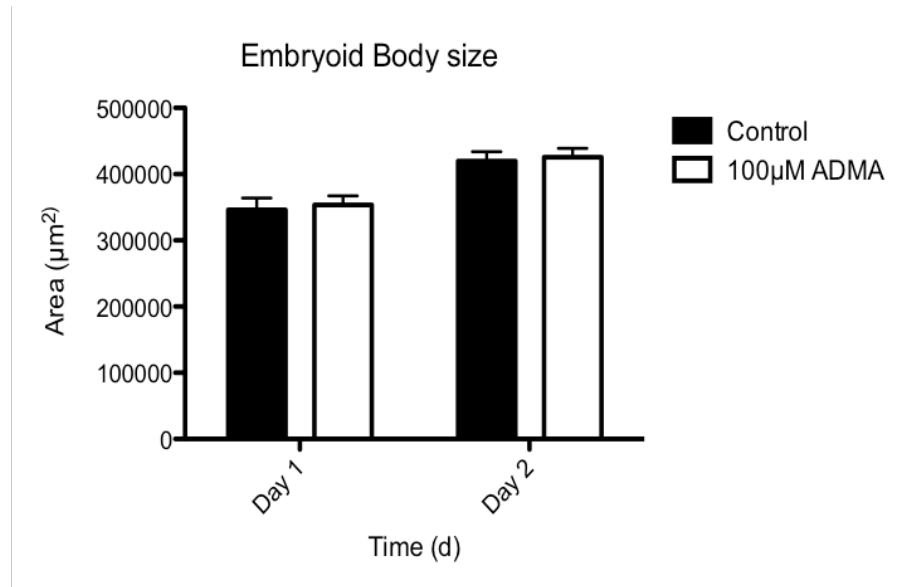


Figure 24. Embryoid body size analysis during the first 2 days of culture. Comparison between Control and 100µM ADMA treated EBs showed no difference in embryoid body size (Control Day 1 (345869.5±18109.38) vs 100µM ADMA Day 1 (353650.3±13808.810), Control Day 2 (419751±14043.83) vs 100µM ADMA Day 2 (425427.7±13676.74)). n=4 experiments, 96 embryoid bodies were analyzed per experiment. Two-way ANOVA ns.

4.3.3.2 Embryoid Sac Formation

On Day 4 of differentiation, yolk-like sacs associated with early hematopoiesis were formed on embryoid bodies (EBs) (Poon, Clermont, Firpo, & Akhurst, 2006). Analysis of the number of yolk-like sacs per embryoid body showed a significant increase of the number and size of sacs in ADMA treated cells ($p < 0.001$). Mean values (Control, mean (1.435±0.1638)- 100µM ADMA,

mean (2.904 ± 0.1421)), showed an almost two-fold increase in the formation of sacs in ADMA treated samples compared to controls (Figure 25).

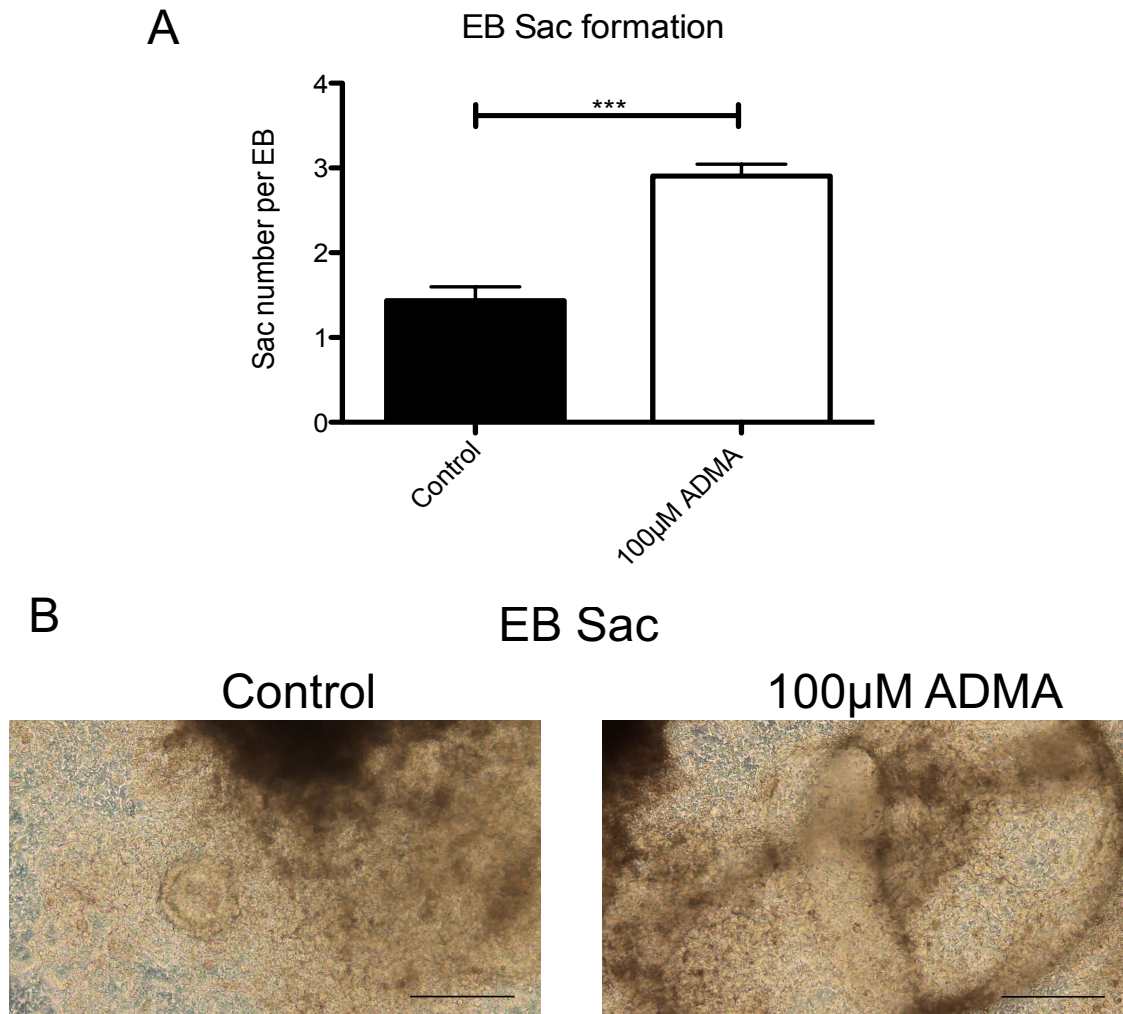


Figure 25. Sac formation in embryoid bodies during differentiation of control and 100µM ADMA treated cells. **A.** Quantification of sac formation normalized to EB numbers (Control (1.435 ± 0.1638) vs 100µM ADMA (2.904 ± 0.1421)). **B.** Control and 100µM ADMA treated embryoid bodies on Day 6 of differentiation. In control samples, small sac formations are visible when in 100µM ADMA treated EBs larger and higher in number sacs is forming. $n=4$ experiments, 96 embryoid bodies were analyzed per experiment. t-test *** $p < 0.001$.

4.3.3.3 Nitric Oxide Concentration

Nitric oxide concentration was measured in control and ADMA treated samples during differentiation. Treatment with 100µM ADMA significantly reduced ($p < 0.05$) the intracellular concentration of NO on Day 6 of differentiation (Figure 26) but it did not significantly reduce NO levels by Day 10.

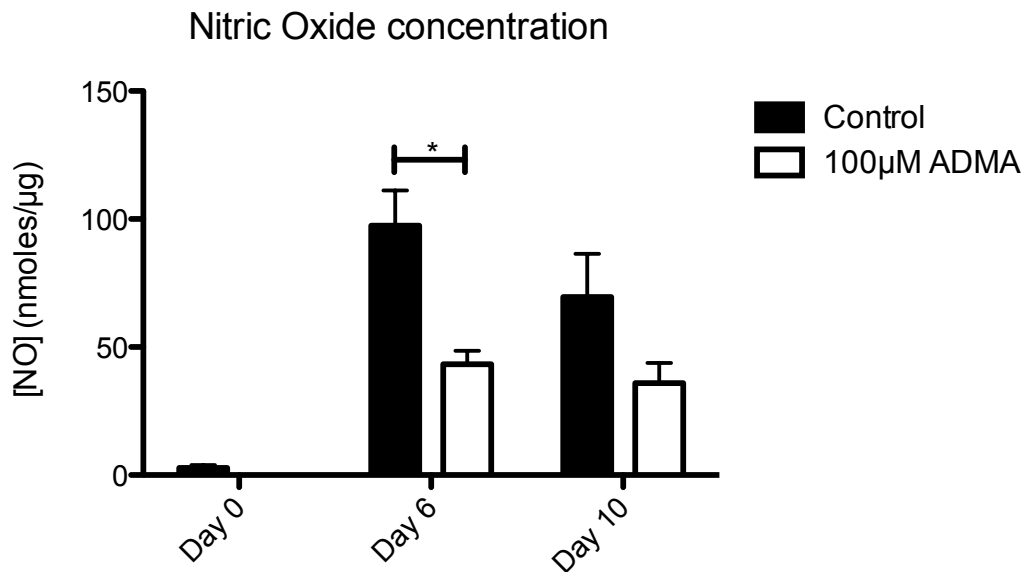


Figure 26. Intracellular nitric oxide concentration during differentiation in control and 100μM ADMA samples (Control Day 0 (2.796 ± 1.173), Control Day 6 (97.394 ± 13.788) vs 100μM ADMA Day 6 (43.314 ± 5.208), Control Day 10 (69.527 ± 16.527) vs 100μM ADMA Day 10 (35.995 ± 7.8)). On Day 6, ADMA treatment significantly decreased NO production by 54% compared to control samples. On Day 10, there was still a difference in NO levels maintained but due to a decrease in NO production in WT samples; the difference did not manage to reach statistical significance. n=3 Two-way ANOVA * $p < 0.05$.

4.3.3.4 Pluripotency Markers Expression Analysis

Oct4, *Sox2* and *Nanog* gene expression levels during differentiation were analyzed in order to identify a potential role of ADMA in maintaining pluripotency or blocking differentiation of embryonic stem cells in the absence of LIF. Analysis of all three markers showed no significant difference in expression of these three pluripotency genes (Figure 27).

Protein levels of OCT4 were also assessed by western blot. And no significant difference was identified during differentiation between control and ADMA treated samples (Figure 28).

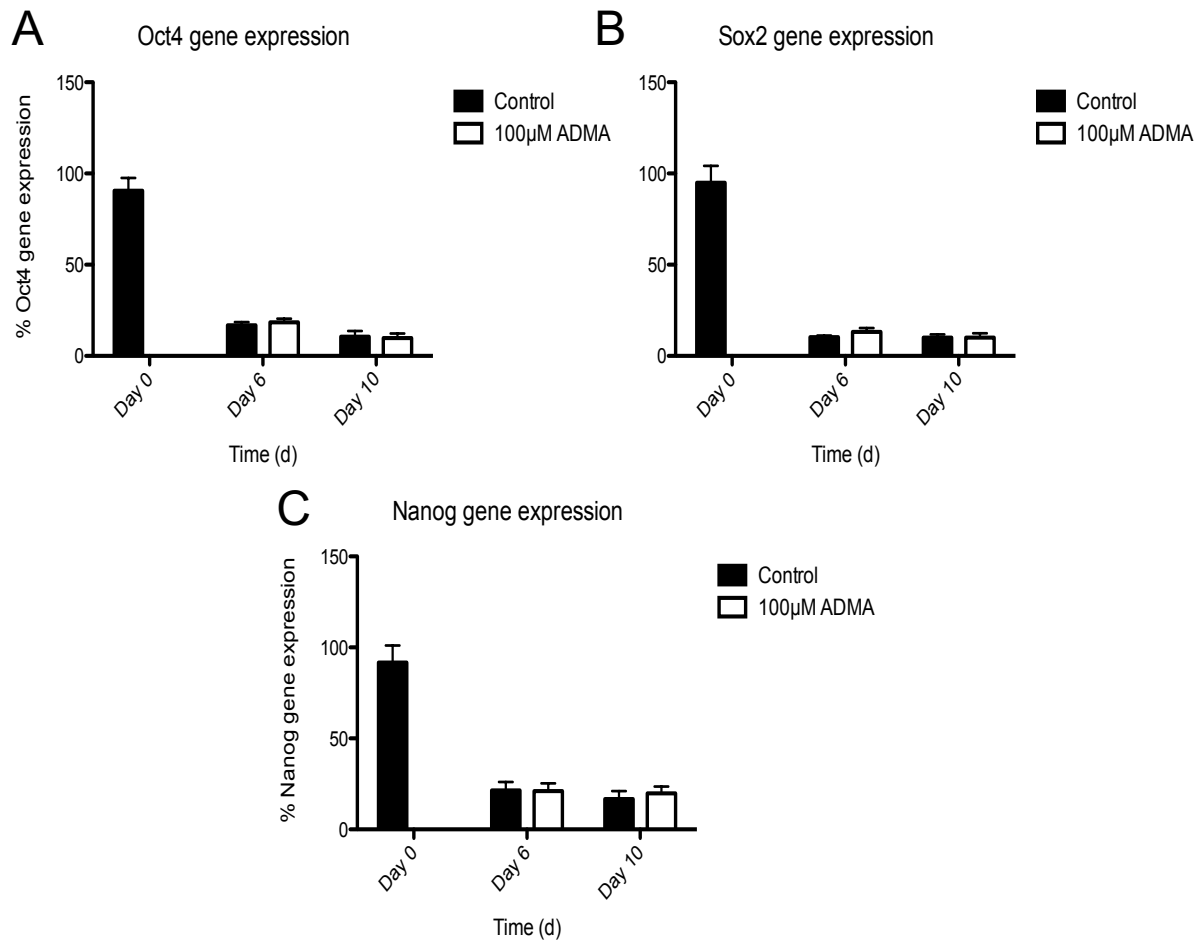


Figure 27. Pluripotency markers expression analysis during differentiation between control and 100μM ADMA treated embryoid bodies. **A.** *Oct4* expression analysis (Control Day 0 (90.536±6.969), Control Day 6 (16.783±1.783) vs 100μM ADMA Day 6 (18.399±2.019), Control Day 10 (10.548±3.080) vs 100μM ADMA Day 10 (9.849±2.494)) **B.** *Sox2* pluripotency marker mRNA levels (Control Day 0 (94.939±9.249), Control Day 6 (10.35±0.881) vs 100μM ADMA Day 6 (13.131±2.222), Control Day 10 (10.014±1.8) vs 100μM ADMA Day 10 (10.031±2.425)) **C.** *Nanog* expression pattern (Control Day 0 (91.670±9.331), Control Day 6 (21.426±4.692) vs 100μM ADMA Day 6 (21.105±4.258), Control Day 10 (16.649±4.464) vs 100μM ADMA Day 10 (19.769±3.838)). There was no significant difference between control and ADMA treated cells during differentiation in any of the pluripotency markers analyzed. n= 6 Two-way ANOVA ns.

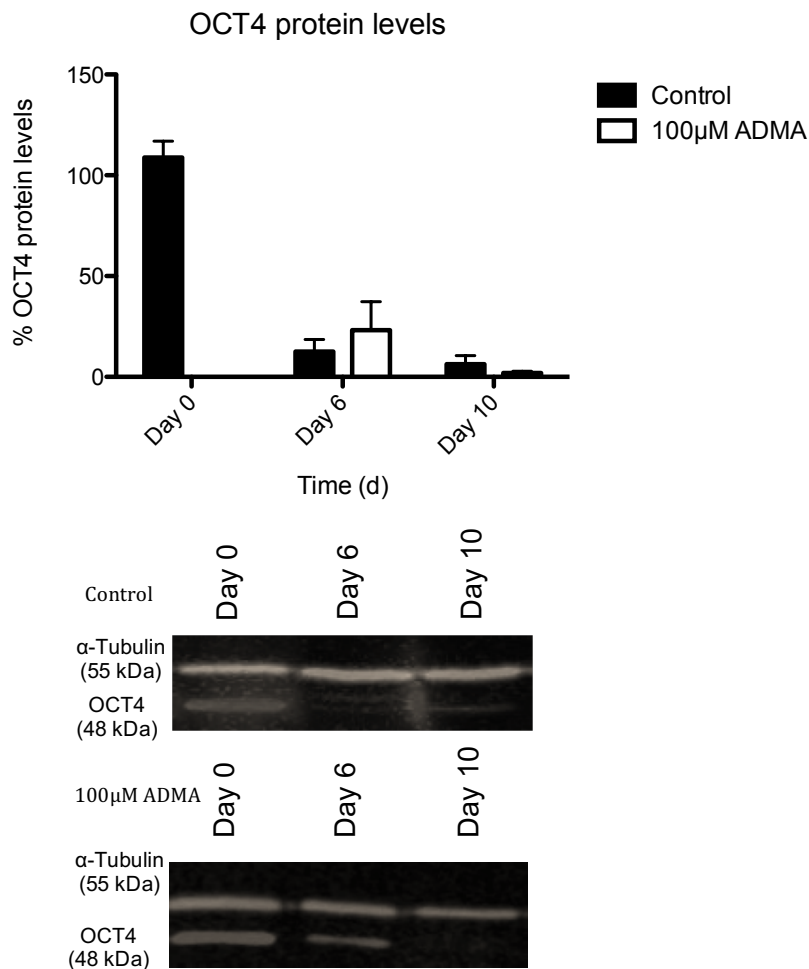


Figure 28. OCT4 protein levels during differentiation of control and 100µM ADMA treated embryoid bodies (Control Day 0 (108.769 ± 8.186), Control Day 6 (12.574 ± 6.010) vs 100µM ADMA Day 6 (23.136 ± 14.237), Control Day 10 (6.367 ± 4.250) vs 100µM ADMA Day 10 (1.962 ± 0.875)). Treatment with 100µM ADMA showed no significant effect on the protein levels of pluripotency marker OCT4 during differentiation. $n=4$ Two-way ANOVA ns.

4.3.3.5 Differentiation Markers Analysis

4.3.3.5.1 Differentiation of pluripotent stem cells to the three germ layers and the extra-embryonic tissue

Specific markers for each germ layer and an extra-embryonic marker were analyzed as a means to identify a strong direction towards a specific germ layer in ADMA treated cells. qPCR analysis of *Brachyury* (mesoderm), *Sox17* (endoderm) and *Tuj1* (ectoderm) showed no alteration in the specific commitment of embryonic stem cells towards one of these germ layers in ADMA treated samples (Figure 29). *Gcm1* extraembryonic tissue marker

showed a small trend in expression during Day 6 and Day 10 compared to control samples but was not statistically significant (Figure 29).

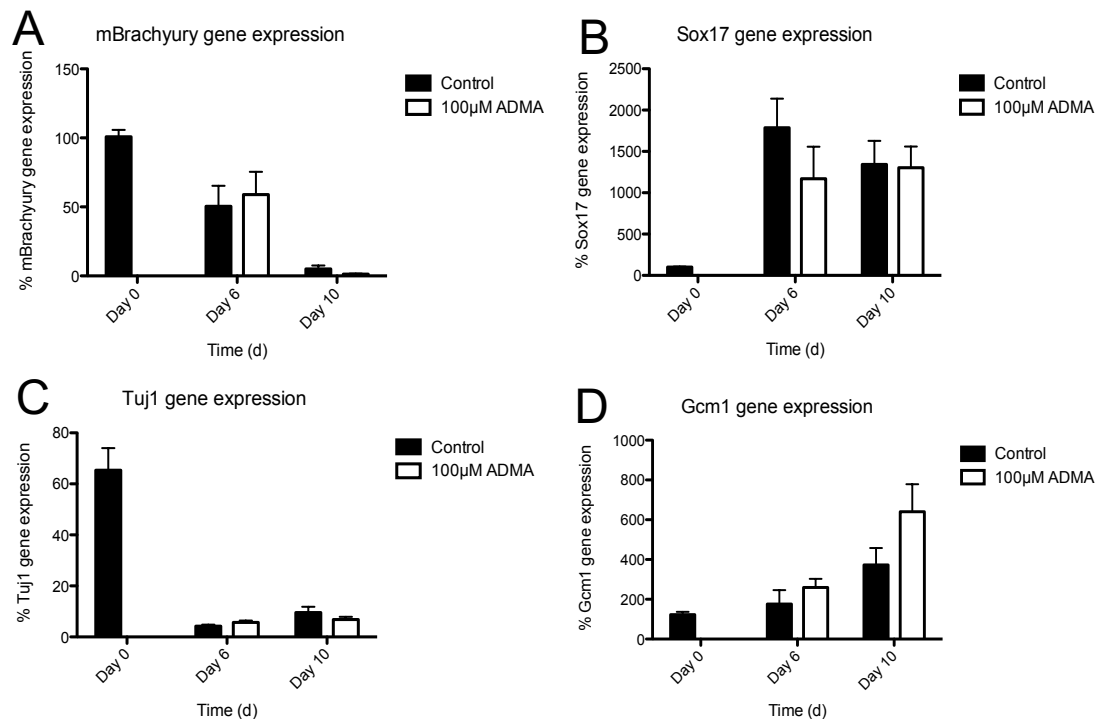


Figure 29. Germ layer (mesoderm, ectoderm, endoderm) and extraembryonic tissue associated gene expression levels analysis in control and 100µM ADMA treated embryoid bodies during 10 days of differentiation. **A.** mBrachyury expression (early mesoderm marker) (Control Day 0 (100.758±5.051), Control Day 6 (50.389±14.940) vs 100µM ADMA Day 6 (58.953±16.427), Control Day 10 (5.089±2.418) vs 100µM ADMA Day 10 (1.252±0.464)). **B.** Sox17 transcription factor mRNA levels (endoderm marker) (Control Day 0 (101.132±6.243), Control Day 6 (1787.114±352.357) vs 100µM ADMA Day 6 (1170.599±386.380), Control Day 10 (1342.635±286.360) vs 100µM ADMA Day 10 (1303.646±255.894)). **C.** *Tuj1* expression pattern (ectoderm marker) (Control Day 0 (65.383±8.665), Control Day 6 (4.218±0.647) vs 100µM ADMA Day 6 (5.686±0.772), Control Day 10 (9.589±2.228) vs 100µM ADMA Day 10 (6.855±1.034)). **D.** *Gcm1* expression analysis (extraembryonic tissue marker) (Control Day 0 (123.178±13.997), Control Day 6 (176.053±70.229) vs 100µM ADMA Day 6 (259.405±43.553), Control Day 10 (372.534±85.539) vs 100µM ADMA Day 10 (640.504±138.269)). There was no significant difference between control and ADMA treated samples in any of the germ layer and extraembryonic tissue associated markers expression pattern. n=6 Two-way ANOVA ns.

4.3.3.5.2 Nitric Oxide Regulatory Gene Expression during Differentiation

Expression levels of all NO regulatory genes were also analyzed during differentiation in order to investigate the effect of a NOS enzyme inhibitor on transcription level of these genes. *Ddah1* and *Ddah2* gene and protein expression were not altered by treatment with ADMA during differentiation (Figure 30,31). However, ADMA does affect the expression pattern of specific NOS isoforms during differentiation and particularly on Day 10. *nNOS* gene

expression levels showed no change with ADMA treatment during differentiation, however *eNOS* showed an increase in expression on Day 10 of differentiation in samples treated with ADMA (Figure 30) but no significant differences were measured on Day 6. The increase in gene expression on Day 10 was not reflected by an increase in protein expression (Figure 32). On the other hand, *iNOS* showed a significant decrease in expression on ADMA treated samples on Day 10 ($p<0.05$) (Figure 30).

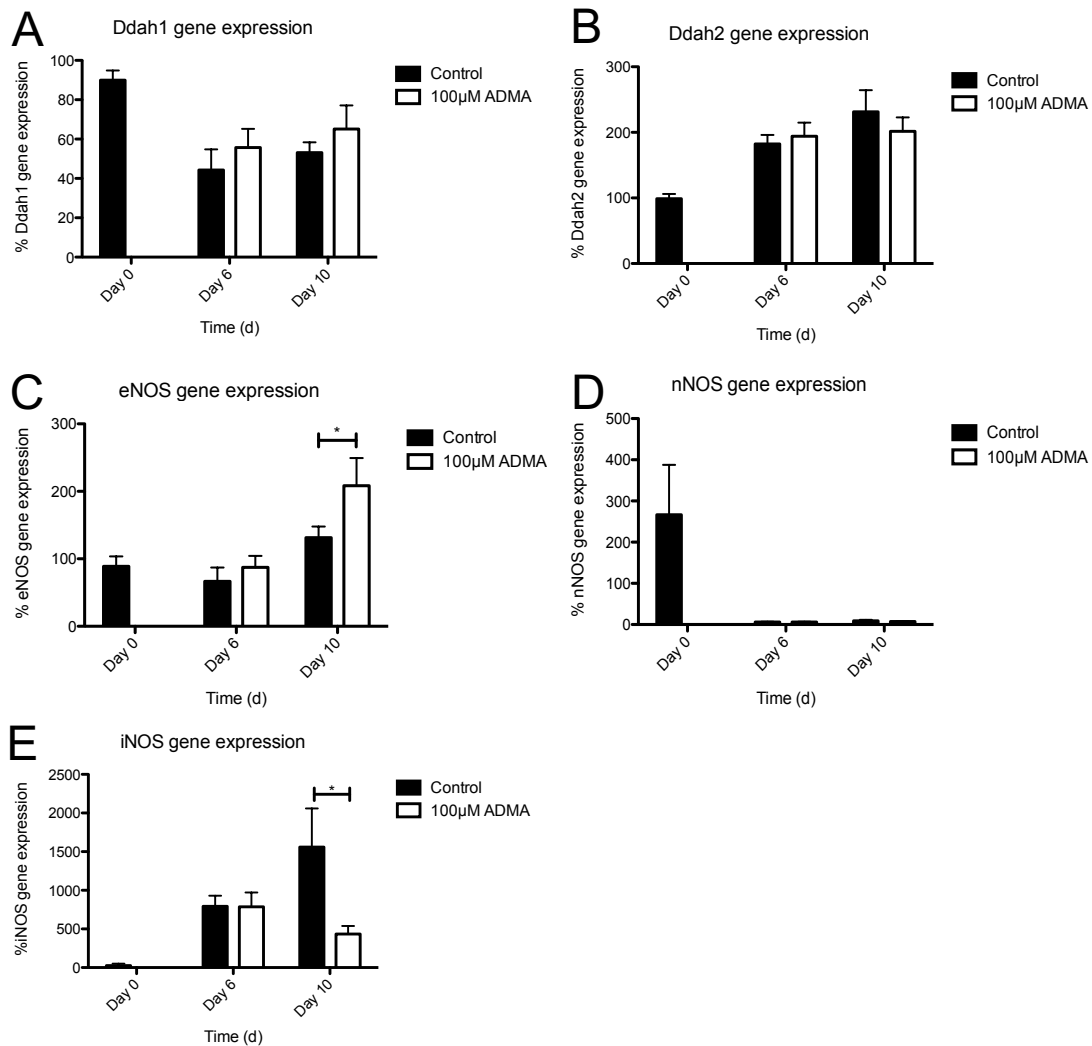


Figure 30. Expression analysis of nitric oxide regulation associated genes between control and 100µM ADMA treated samples. **A.** *Ddah1* mRNA levels (Control Day 0 (89.988 ± 4.896), Control Day 6 (44.261 ± 10.556) vs 100µM ADMA Day 6 (55.735 ± 9.443), Control Day 10 (53.088 ± 5.308) vs 100µM ADMA Day 10 (65.166 ± 11.993)). **B.** *Ddah2* gene expression (Control Day 0 (98.886 ± 7.307), Control Day 6 (182.317 ± 13.814) vs 100µM ADMA Day 6 (194.115 ± 20.659), Control Day 10 (231.280 ± 32.988) vs 100µM ADMA Day 10 (201.686 ± 21.097)). **C.** *eNOS* expression pattern (Control Day 0 (88.844 ± 14.703), Control Day 6 (66.542 ± 20.490) vs 100µM ADMA Day 6 (87.292 ± 17.008), Control Day 10 (131.481 ± 16.295) vs 100µM ADMA Day 10 (208.430 ± 41.060)). **D.** *nNOS* gene expression (Control Day 0 (82.395 ± 7.4), Control Day 6 (6.011 ± 0.973) vs 100µM ADMA Day 6 (5.9 ± 0.848), Control Day 10 (8.876 ± 2.622) vs 100µM ADMA Day 10 (7.311 ± 0.875)) and **E.** *iNOS* mRNA levels (Control Day 0 (25 ± 25), Control Day 6 (792.018 ± 137.926) vs 100µM ADMA Day 6 (785.791 ± 187.004), Control Day 10 (1558.533 ± 500.241) vs 100µM ADMA Day 10 (433.477 ± 105.961)). *Ddah1*, *Ddah2* and *nNOS*

gene expression analysis showed no significant difference between control and ADMA treated samples. On Day 10 of differentiation, *e*NOS showed significant upregulation in mRNA levels in the ADMA treated embryoid bodies. *i*NOS gene expression was significantly lower in ADMA treated embryoid bodies on Day 10 of differentiation. $n=6$ Two-way ANOVA * $p<0.05$.

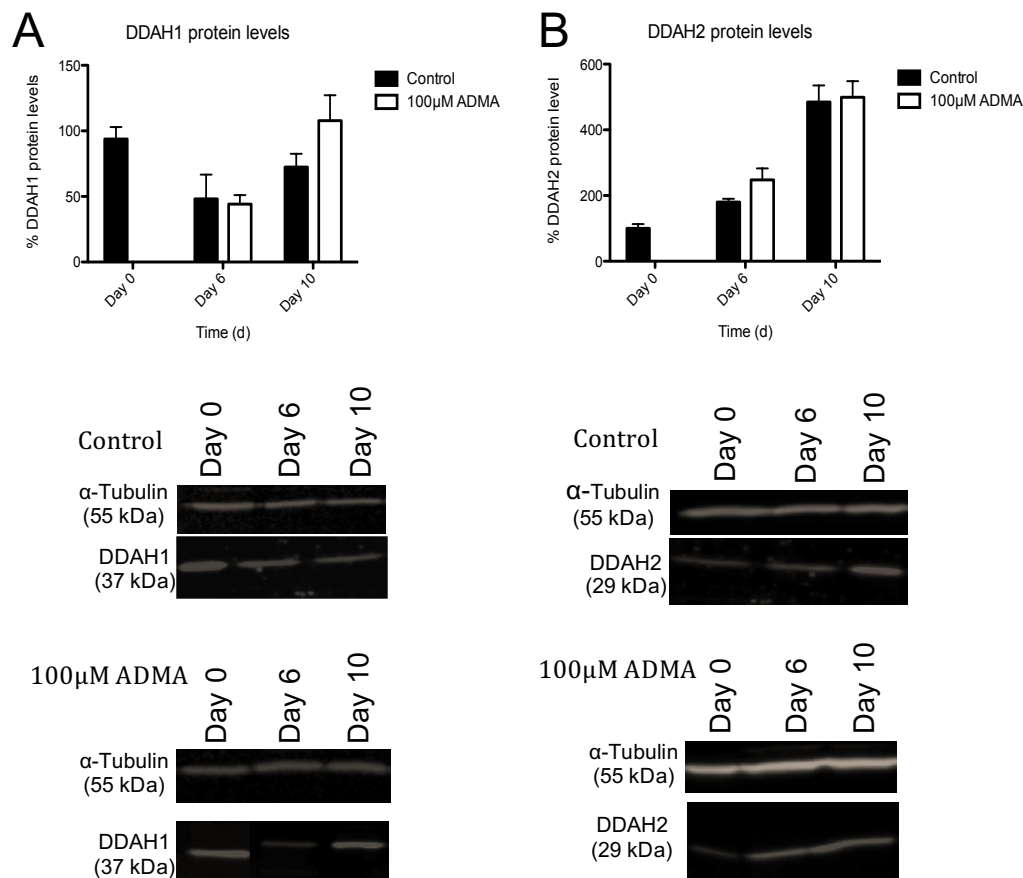


Figure 31. Protein levels of DDAH1 and DDAH2 genes during differentiation in control and 100µM ADMA treated samples. **A.** DDAH1 protein levels analysis (Control Day 0 (93.774 ± 9.222), Control Day 6 (48.182 ± 18.456) vs 100µM ADMA Day 6 (44.212 ± 6.849), Control Day 10 (72.492 ± 10.117) vs 100µM ADMA Day 10 (107.828 ± 19.375)). **B.** DDAH2 protein levels analysis (Control Day 0 (100.018 ± 13.149), Control Day 6 (180.389 ± 10.044) vs 100µM ADMA Day 6 (247.968 ± 34.813), Control Day 10 (484.756 ± 50.528) vs 100µM ADMA Day 10 (499.402 ± 48.674)). Treatment with ADMA had no effect on the protein levels of the DDAH isoforms during differentiation. $n=4$ Two-way ANOVA ns.

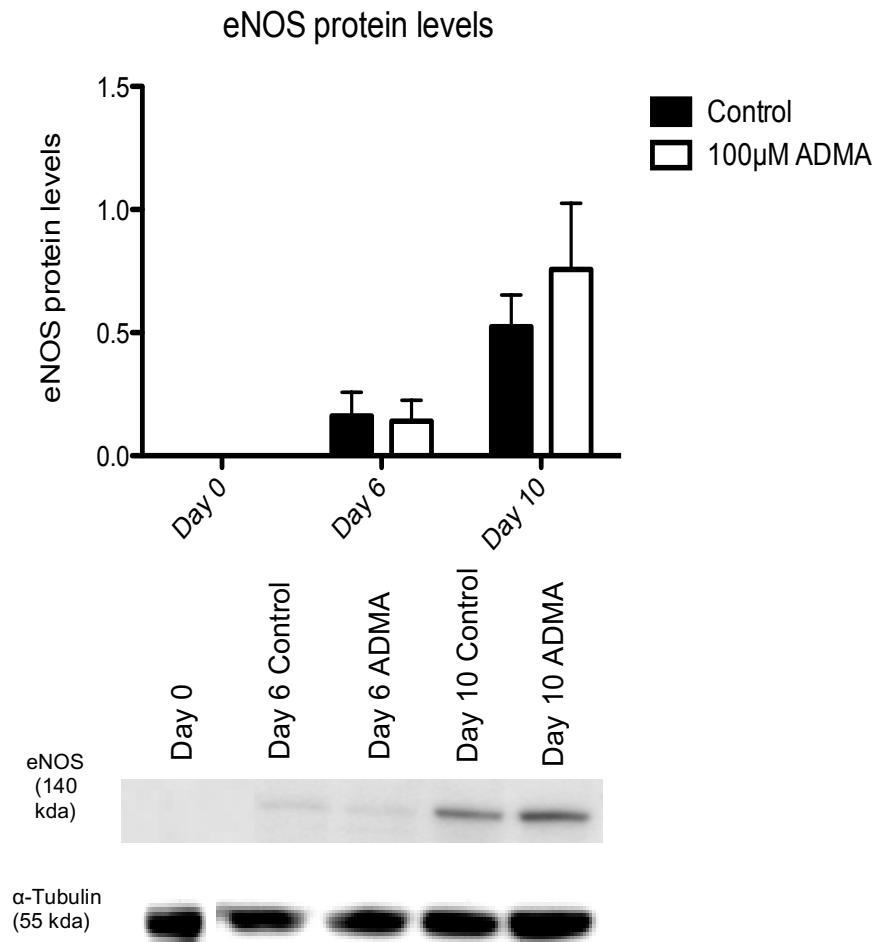


Figure 32. eNOS protein levels analysis by western blot on Day 0, Day 6 and Day 10 (Control Day 0 (0.000 ± 0.000), Control Day 6 (0.162 ± 0.096) vs 100µM ADMA Day 6 (0.141 ± 0.084), Control Day 10 (0.524 ± 0.128) vs 100µM ADMA Day 10 (0.757 ± 0.269)). No significant difference was measured between control and 100µM ADMA treated samples during differentiation. n=6 Two-way ANOVA ns.

4.3.3.5.3 Gene and Protein Expression of Cardiomyocyte Specific Markers during Differentiation

In order to further understand the effect of ADMA on the differentiation of embryonic stem cells to cardiomyocytes, both transcriptional factors and structural protein genes associated with cardiomyocyte differentiation were analyzed. ADMA had no significant effect on the expression levels of the early cardiac differentiation marker *Gata4* as measured by qPCR analysis but protein levels of GATA4 were significantly lower in ADMA treated cells throughout differentiation (Figure 33,34). *Nkx2.5* gene, a late stage ventricular differentiation marker, showed a significant decrease ($p < 0.01$) in gene and protein expression on Day 10 of differentiation (Figure 33,35).

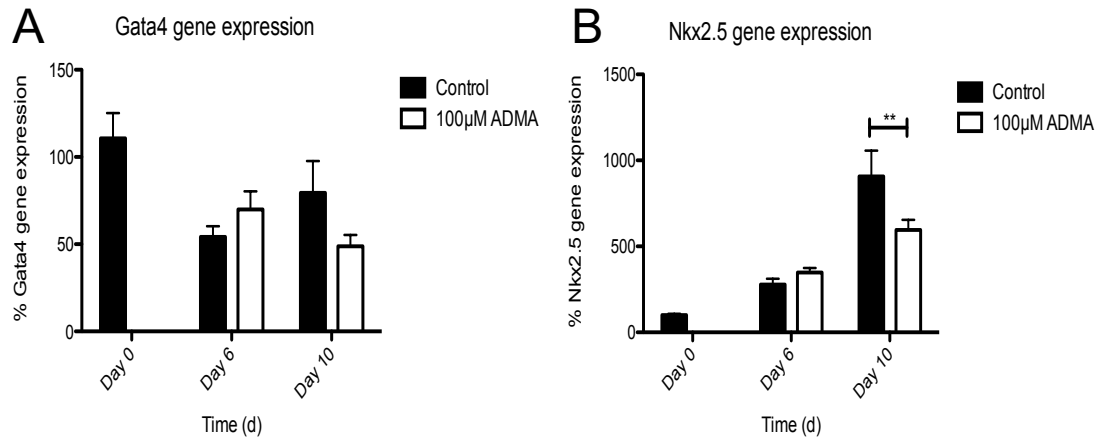


Figure 33. Early and late cardiac differentiation marker (transcription factors) expression pattern in control and 100µM ADMA treated embryoid bodies. **A.** *Gata4* mRNA levels (early cardiac differentiation marker) (Control Day 0 (110.731 ± 14.403), Control Day 6 (54.222 ± 6.119) vs 100µM ADMA Day 6 (69.963 ± 10.320), Control Day 10 (79.398 ± 18.349) vs 100µM ADMA Day 10 (48.805 ± 6.511)). **B.** *Nkx2.5* mRNA levels (late cardiac differentiation marker) (Control Day 0 (100.241 ± 7.276), Control Day 6 (277.523 ± 34.215) vs 100µM ADMA Day 6 (347.715 ± 26.324), Control Day 10 (907.154 ± 150.134) vs 100µM ADMA Day 10 (596.187 ± 58.730)). Treatment with ADMA showed no significant difference in expression levels of *Gata4* transcription factor. *Nkx2.5* was significantly downregulated on Day 10 of differentiation in ADMA treated embryoid bodies. n=6 Two-way ANOVA ** $p < 0.01$.

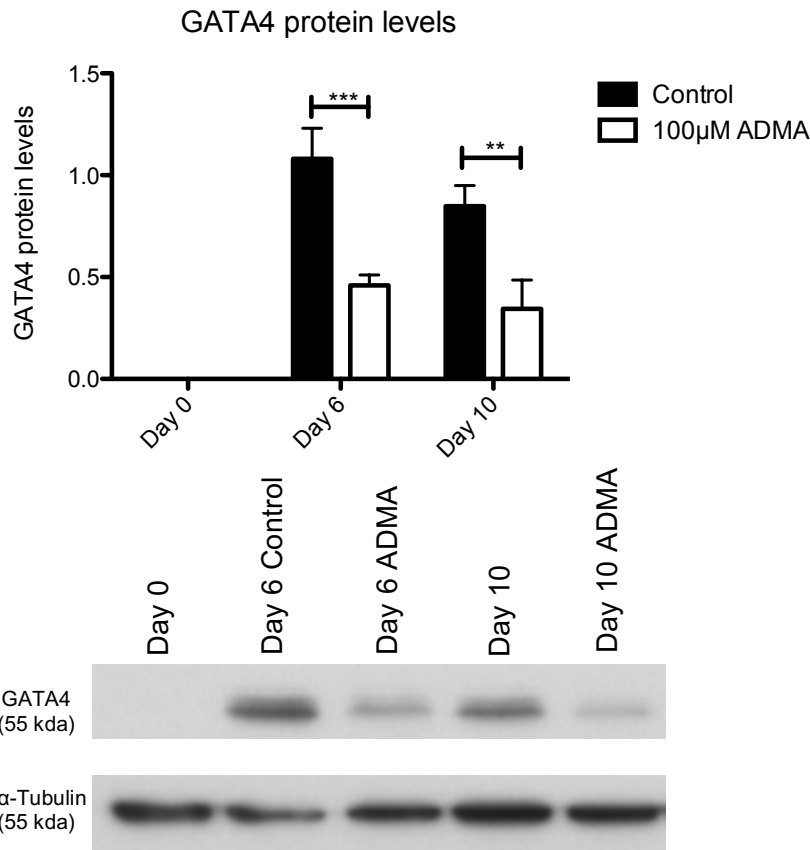


Figure 34. GATA4 protein levels analysis by western blot (Control Day 0 (0.000 ± 0.000), Control Day 6 (1.081 ± 0.15) vs 100µM ADMA Day 6 (0.459 ± 0.051), Control Day 10 (0.848 ± 0.101) vs 100µM ADMA Day 10 (0.344 ± 0.142)). ADMA treated cells showed significantly lower levels of GATA4 protein in both Day 6 and Day 10 of differentiation compared to control samples. n=6 Two-way ANOVA ** $p < 0.01$, *** $p < 0.001$.

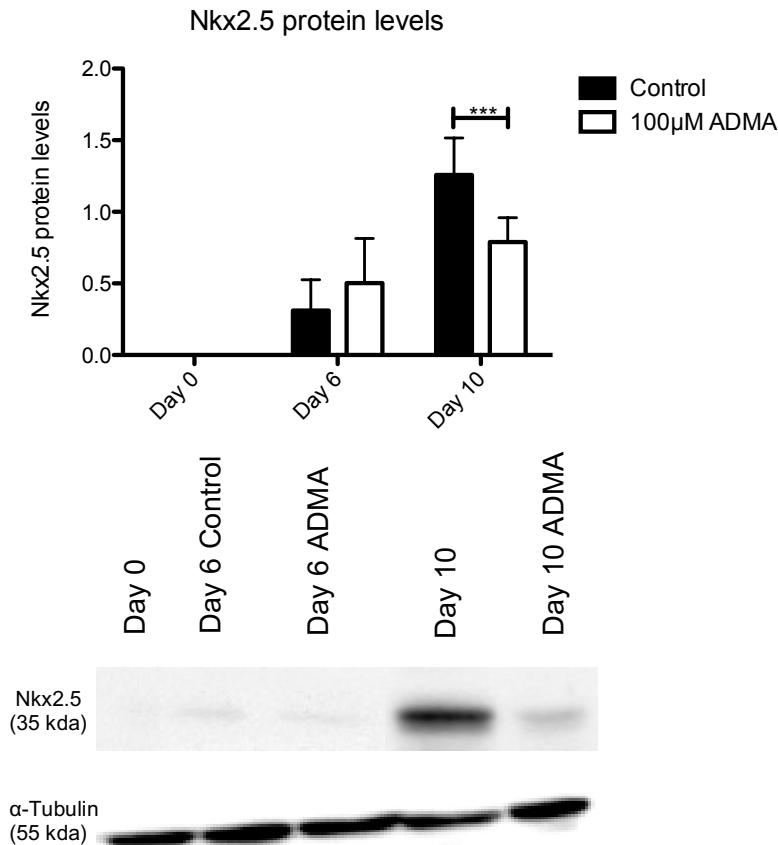


Figure 35. Nkx2.5 protein levels analysis by western blot during 10 days of differentiation (Control Day 0 (0.000 ± 0.000), Control Day 6 (0.312 ± 0.215) vs 100µM ADMA Day 6 (0.503 ± 0.312), Control Day 10 (1.258 ± 0.258) vs 100µM ADMA Day 10 (0.789 ± 0.171)). Nkx2.5 protein showed no significant difference between control and ADMA treated samples on Day 6 but Nkx2.5 protein levels were significantly lower in ADMA treated cells on Day 10 of differentiation. $n=6$ Two-way ANOVA *** $p < 0.001$.

Treatment of differentiating embryonic stem cells with 100µM ADMA had a significant impact on the expression of cardiomyocyte structural genes. Heavy myosin chain showed a significant decrease in the expression levels on Day 10 of differentiation ($p < 0.05$) (Figure 36). Cardiac troponin-T appeared to decrease on Day 10 but was not statistically significant (Figure 36). Finally, cardiac actin showed no change in the expression levels on both Day 6 and 10 of differentiation (Figure 36).

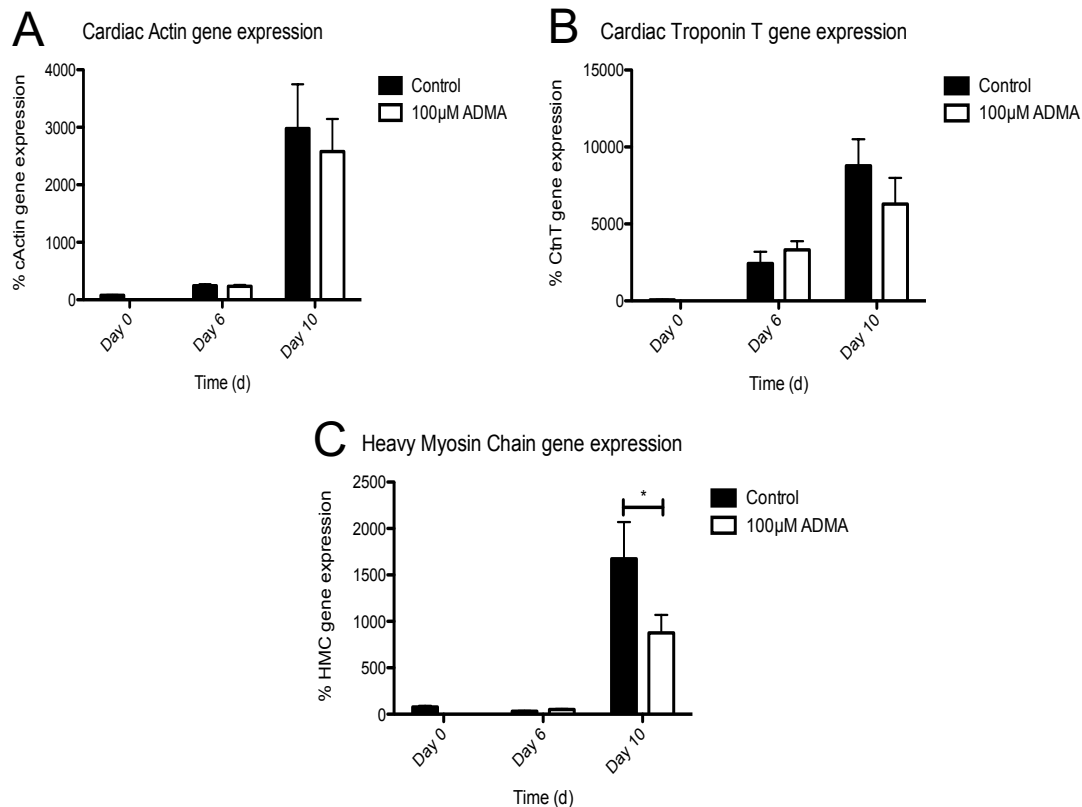


Figure 36. Cardiac structure associated gene expression levels analysis in control and 100μM ADMA during differentiation. **A-B.** Cardiac actin and cardiac troponin T showed no significant difference between control and ADMA treated samples Cardiac Actin (Control Day 0 (79.365±6.615), Control Day 6 (245.913±27.211) vs 100μM ADMA Day 6 (233.952±25.648), Control Day 10 (2978.52±770.093) vs 100μM ADMA Day 10 (2579.158±566.302)) Cardiac Troponin T (Control Day 0 (71.098±8.247), Control Day 6 (2426.561±768.431) vs 100μM ADMA Day 6 (3312.876±562.899), Control Day 10 (8786±1720.1) vs 100μM ADMA Day 10 (6298.859±1696.809)). **C.** Heavy myosin chain expression showed a decrease on Day 10 of differentiation in the ADMA treated embryoid bodies (Control Day 0 (78.075±12.820), Control Day 6 (32.143±3.722) vs 100μM ADMA Day 6 (50.292±7.374), Control Day 10 (1673±396.481) vs 100μM ADMA Day 10 (876.215±193.630)). n=6 Two-way ANOVA * p<0.05 *** p<0.001.

4.3.3.5.4 Embryonic Stem Cell Differentiation to Endothelial Cells and Early Hematopoiesis

In order to identify the role of ADMA in differentiation of embryonic stem cells, endothelial and early hematopoiesis gene expression markers were analyzed in order to understand if reduced levels of NO could force stem cells down this pathway of differentiation. CD34, a bone marrow and early hematopoiesis marker showed no difference between control and ADMA treated samples (Figure 37). Conversely, *Runx1* and CD31 genes showed differences in their expression levels in control compared to ADMA treated samples. *Runx1* (AML1), a transcription factor that plays a role in the maturation of blood cells, showed increased expression levels in ADMA treated samples on

Day 10 in both mRNA and protein levels (Figure 37,38). CD31, which has been associated with endothelial cell differentiation, showed a significant decrease in expression on Day 10 of differentiation (Figure 37). Finally, gene expression of *Acta2* gene showed a decrease expression in ADMA treated compared to control samples as shown by qPCR analysis (Figure 37). Western blot analysis of ACTA2 protein showed no significant difference between control and ADMA treated cells (Figure 39).

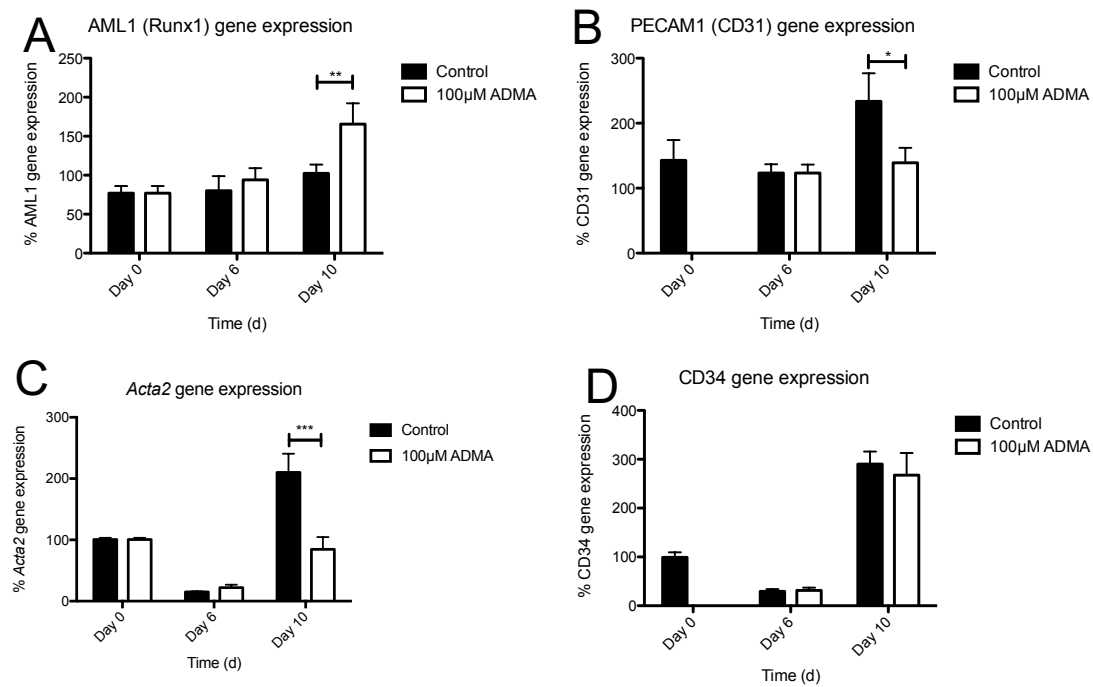


Figure 37. Expression analysis of hematopoiesis related markers in control and 100µM ADMA treated embryoid bodies during differentiation. **A.** *Runx1* (AML1) expression was significantly increased on Day 10 of differentiation in ADMA treated cells (Control Day 0 (77.016±9.104), Control Day 6 (80.196±18.759) vs 100µM ADMA Day 6 (93.995±15.121), Control Day 10 (102.470±11.077) vs 100µM ADMA Day 10 (165.453±26.739)). **B.** PECAM1 (CD31) gene expression was downregulated in ADMA treated embryonic stem cells on Day 10 of differentiation (Control Day 0 (142.823±31.390), Control Day 6 (123.348±13.448) vs 100µM ADMA Day 6 (123.046±13.005), Control Day 10 (233.683±43.310) vs 100µM ADMA Day 10 (138.902±22.985)). **C.** *Acta2* gene expression was significantly downregulated on Day 10 in ADMA treated samples (Control Day 0 (100.737±2.66), Control Day 6 (15.212±1.007) vs 100µM ADMA Day 6 (22.194±4.783), Control Day 10 (210.148±30.556) vs 100µM ADMA Day 10 (84.579±20.008)). **D.** CD34 hematopoietic marker showed no significant difference between control and ADMA treated samples (Control Day 0 (99.135±10.36), Control Day 6 (29.501±4.822) vs 100µM ADMA Day 6 (31.446±5.594), Control Day 10 (290.046±25.903) vs 100µM ADMA Day 10 (267.631±45.184)). n=6 Two-way ANOVA * p<0.05 ** p<0.01.

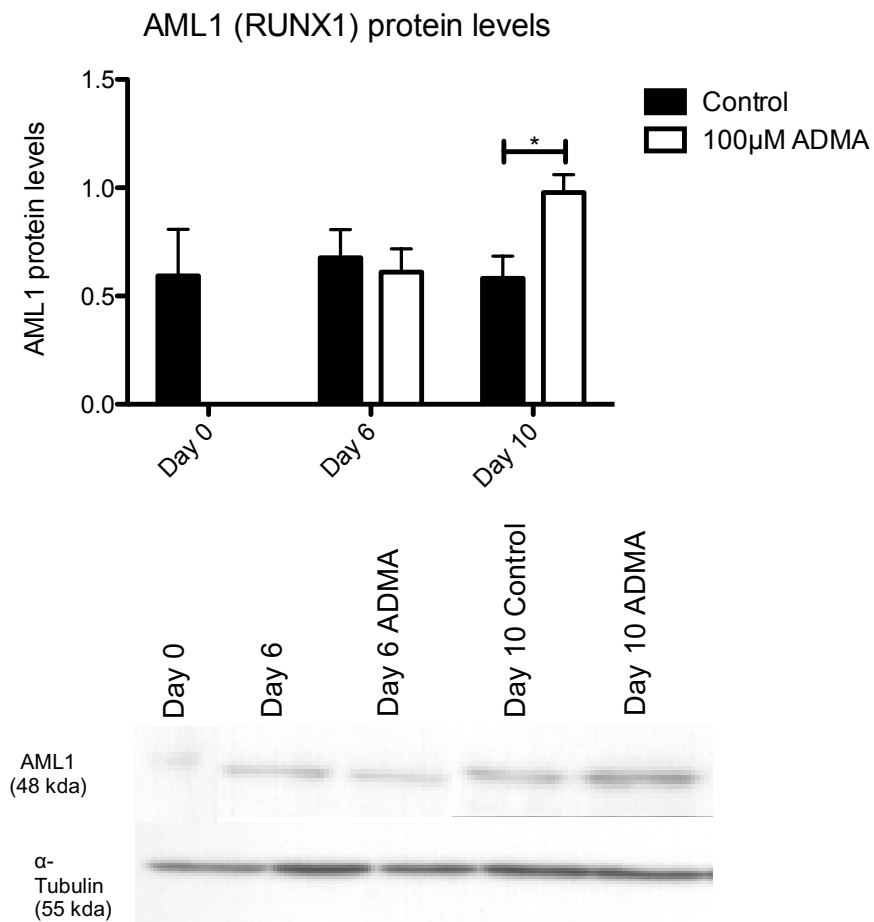


Figure 38. AML1 (Runx1) protein levels analysis by western blot during differentiation (Control Day 0 (0.593 ± 0.216), Control Day 6 (0.677 ± 0.13) vs 100µM ADMA Day 6 (0.61 ± 0.108), Control Day 10 (0.582 ± 0.103) vs 100µM ADMA Day 10 (0.978 ± 0.083)). AML1 gene showed increased protein levels on Day 10 of differentiation in 100µM ADMA treated samples. n=6 Two-way ANOVA * $p < 0.05$.

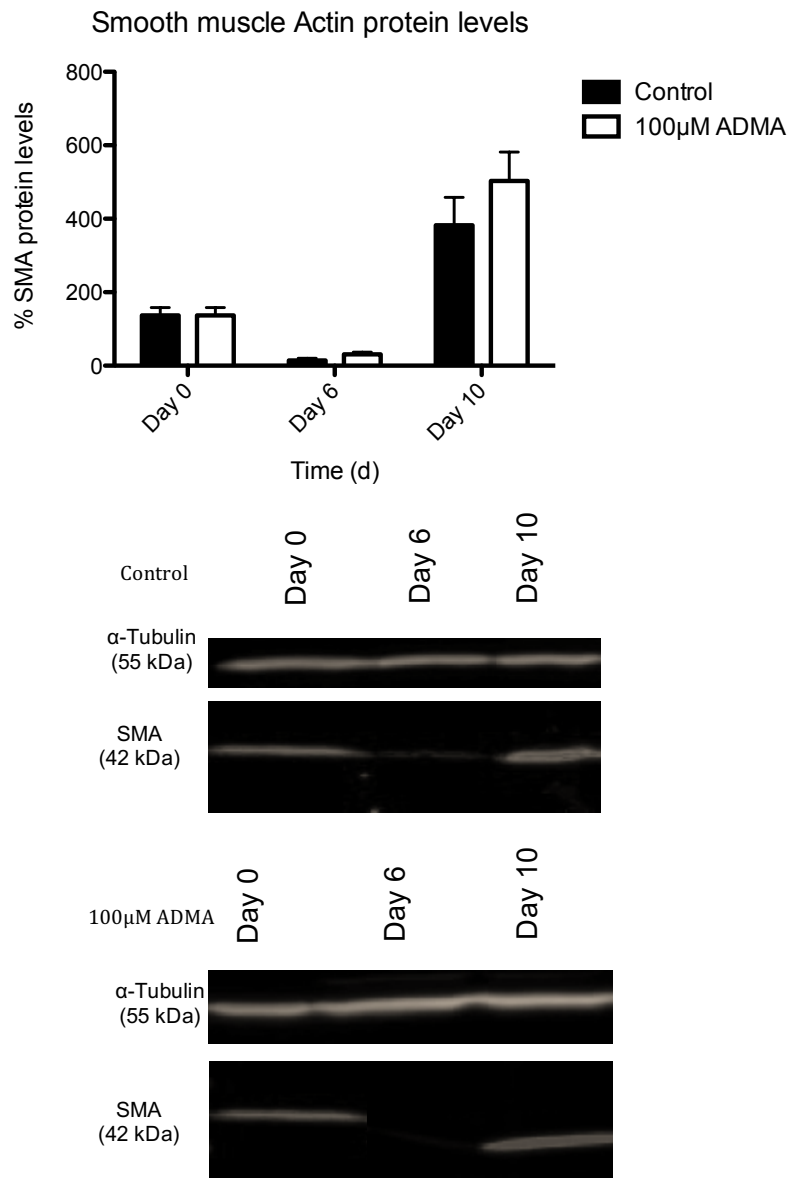


Figure 39. Smooth muscle actin protein levels analyzed by western blot during 10 days of differentiation (Control Day 0 (137.082 ± 21.299), Control Day 6 (14.584 ± 5.56) vs 100µM ADMA Day 6 (31.001 ± 6.076), Control Day 10 (382.054 ± 76.539) vs 100µM ADMA Day 10 (503.327 ± 78.209)). SMA protein levels showed no difference between control and 100µM ADMA treated cells. $n=6$ Two-way ANOVA ns.

4.5.4 Discussion

4.5.4.1 The Effect of NO Inhibition on Pluripotency of Embryonic Stem Cells

Treatment of embryonic stem cells with 100 μ M ADMA in the presence of LIF did not have a significant effect on the pluripotent state. AP staining along with mRNA and protein analysis of pluripotency markers showed no significant difference between control and treated samples. Also, analysis of differentiation markers of each one of the three germ layers did not reveal a specific differentiation fate acquisition of the treated cells compared to their relative controls. Finally, DDAH mRNA expression levels remained unchanged between control and treated samples.

According to previous approaches to identify a possible role of NO regulation via supplementation of NO donors (Beltran-Povea et al., 2015; Mora-Castilla et al., 2010), low levels of NO can maintain embryonic stem cells pluripotency through maintenance of expression of self-renewal genes (Beltran-Povea et al., 2015). On the other hand, high levels of NO can promote differentiation through downregulation of *Oct4* and *Nanog* genes leading to upregulation of differentiation markers such as Brachyury (Mora-Castilla et al., 2010). In our experimental approach, there was no upregulation of pluripotency genes and no change in differentiation markers. Our lack of expression difference of the pluripotency genes can be explained by the presence of LIF in our cell cultures and the duration of treatment. LIF presence in the culture media can efficiently maintain high expression of the pluripotency genes masking the effect of inhibition of NO production by ADMA. Also, it is possible that the effect of inhibition of NOS on the change in expression of the pluripotency or differentiation markers needs more than 4 days of treatment that was the course of our experiments. Finally, the expression pattern of pluripotent genes that was observed by (Beltran-Povea et al., 2015; Mora-Castilla et al., 2010) was the result of the supplementation of low and high concentrations of an NO donor. In our studies, we used a NOS inhibitor, ADMA, in order to study the effect of manipulation of endogenous levels of NO in pluripotency.

4.5.4.2 Expression of Pluripotency and NO-regulation Genes during Differentiation

Expression pattern of DDAH and NOS isoforms was assessed revealing a different temporal expression pattern for each gene. This is the first time that the expression pattern of all three NOS isoforms along with the two DDAH genes is being assessed. Analysis of pluripotency gene expression during differentiation showed downregulation of those markers and successful differentiation of the embryonic stem cells.

Oct4, a pluripotency regulator, forms a dimer with Sox2 in order to bind DNA (Boiani & Schöler, 2005). Downregulation of one of the factors is sufficient for the differentiation of embryonic stem cells. Loss of expression of both markers further reinforces the loss of pluripotency.

Ddah1 and *Ddah2* genes showed a differential expression pattern during differentiation to an environment promoting differentiation towards mesoderm and endoderm. *Ddah1* was downregulated by Day 6 and remained in the same levels on Day 10 when compared to pluripotent state. On the other hand, *Ddah2* gene expression was steadily increased throughout the 10 days of differentiation. These results were also confirmed at the protein level. Differential expression pattern of the two *Ddah* isoforms indicates a unique role for each one in relation to the development of various cell types. Expression analysis of *Ddah* genes in mice, have strongly associated *Ddah2* gene expression with the cardiovascular system (heart, immune system and endothelium) (Tran et al., 2000). Our results suggest a possible implication of *Ddah2* gene in differentiation of embryonic stem cells towards mesoderm/endoderm and early stages of development of the cardiovascular system.

NOS gene expression analysis during 10 days of differentiation of embryonic stem cells revealed a unique pattern for each one of the isoforms. eNOS expression levels remained unchanged when nNOS expression was depleted during differentiation and iNOS was steadily upregulated when compared to pluripotency. Differential expression of each of the NOS isoforms suggests different roles for eNOS and iNOS genes during embryonic development. nNOS expression depletion suggests that nNOS is not important during development of tissues from the mesoderm/endoderm germ layers. nNOS expression has been detected in adult cell types of the heart such as the

adult cardiomyocytes but it was absent from embryonic cardiomyocytes (Massion et al., 2005; Umar & van der Laarse, 2010). Our finding support that nNOS might be involved in physiological processes of adult but not embryonic cell types in the cardiovascular system. eNOS expression has been strongly associated with the cardiovascular system (Akyürek et al., 1996; Liu & Feng, 2012; Massion et al., 2005) and stable expression of the gene during differentiation reflects the importance of eNOS gene expression during embryonic development. Finally, iNOS steady upregulation through differentiation and eNOS continuous expression along with the increase in NO production during development indicates that iNOS and eNOS could be the main NOS isoforms for the production of NO during development. NO concentration increases rapidly by Day 6 indicating an important role of NO signaling in early stages of development. iNOS and eNOS can produce fast high amounts of NO. Our data agree with existing literature and suggest that during differentiation the extensive need for NO is covered by upregulation of iNOS gene expression.

Overall, we suggest that the *Ddah2* gene may play a role in differentiation of embryonic stem cells to mesoderm/endoderm. Further analysis with the use of *Ddah2*^{-/-} embryonic stem cells will help us identify a specific role for this isoform. Also, analysis of the three NOS isoforms revealed that iNOS is the major isoform responsible for the production of NO during differentiation of stem cells to mesoderm/endoderm.

4.5.4.3 Endogenous NO Level Manipulation during Differentiation

Embryoid body size during the first 2 days of differentiation was not affected by ADMA treatment, indicating that inhibition of NO production did not seem to affect the proliferation rate of the differentiating embryonic stem cells.

Analysis of nitric oxide concentrations during 10 days of differentiation verified that ADMA decreased the amount of NO produced by about 50%. Furthermore, analysis of control samples indicated a significant need for NO by Day 6 that was decreased in later stages of differentiation. A difference in NO levels between control and ADMA treated samples was still present on Day 10 but it was not significant due to a decrease in NO levels in controls indicating a smaller need for NO signaling. NO seems to play an important role in early

stages rather than in later stages of differentiation. This is the first time that endogenous NO levels were monitored throughout the first 10 days of embryonic stem cell differentiation.

Analysis of pluripotency markers *Sox2* and *Nanog* at the mRNA level and *Oct4* at both mRNA and protein level showed no effect of NO production inhibition on loss of pluripotency during differentiation. Furthermore, during 10 days of differentiation we recorded the expression of specific differentiation markers related to the three germ layers and the extraembryonic tissue. There was no effect of ADMA treatment on the expression pattern of those markers. As an early mesoderm marker, mBrachyury is upregulated in the first days of differentiation and then it is subsequently downregulated. mBrachyury, was analyzed at a late stage of mesoderm differentiation therefore its expression appears to be in lower levels when compared to pluripotency. Analysis of mBrachyury levels in early days of differentiation will help us clarify if there is a possible effect of NOS inhibition on mesoderm differentiation. Finally, *Gcm1*, an extraembryonic tissue marker, showed higher, but not significantly, levels of expression in ADMA treated samples on Day 10.

Expression pattern of *Ddah1* and *Ddah2* genes at the mRNA and protein levels remained unchanged by ADMA treatment. There has been no direct correlation between expression of DDAH isoforms and ADMA levels indicating that an increase in intracellular ADMA levels is not sufficient to drive the expression of *Ddah1* and *Ddah2* genes.

nNOS gene expression remained unchanged in ADMA treated samples when eNOS appeared significantly increased and iNOS showed a significant decrease on Day 10 in ADMA treated samples. eNOS upregulation could be seen as a compensatory mechanism to the ADMA treatment. When high levels of ADMA inhibit NO synthesis upregulation of eNOS gene expression may overcome the inhibition of the existing eNOS protein molecules. eNOS protein levels showed a slight non-significant increase on Day 10 marking a possible delay between transcription and translation of the eNOS mRNA in response to the ADMA treatment. On the other hand, iNOS loss of expression in ADMA treated samples could be linked to the differentiation fate that the embryonic stem cells are acquiring in low levels of NO. In normal conditions, iNOS is highly expressed providing high levels of NO required for differentiation of embryonic stem cells to heart related cells types (Kanno et al., 2004). By inhibiting NO

synthesis in early stages, differentiating cells might acquire different cell fates that do not require high concentration of NO for their later stages of differentiation.

4.5.4.4 Effect of Manipulation of Endogenous NO Synthesis on the Expression of Cardiac Specific Markers during Differentiation

Cardiac differentiation was monitored by mRNA and protein analysis of cardiac-specific transcription factors such as *Gata4* and *Nkx2.5* (Durocher et al., 1997; Lyons et al., 1995; Molkentin et al., 1997) and proteins of the contraction machinery of differentiated pluripotent stem cells to cardiomyocytes. ADMA treatment had a significant effect on both mRNA and protein levels of *Nkx2.5* when a significant difference in expression of *Gata4* gene was only detected on the protein level. The difference in mRNA and protein levels of *Gata4* gene indicates a possible post-transcriptional effect of NO inhibition on *Gata4* expression. Lower expression levels of both genes indicate lower differentiation of embryonic stem cells towards cardiomyocytes. NO has been shown to be an important signaling pathway in differentiation of embryonic stem cells to cardiomyocytes (Kanno et al., 2004). However, this is the first time that *Gata4*, an early cardiomyocyte differentiation marker, has been analyzed showing an early effect of NO inhibition on embryonic stem cells. *Gata4* expression is upregulated in cells acquiring a cardiac progenitor cell fate (forming the heart tube) and comes prior to *Nkx2.5* expression (Molkentin et al., 1997; Rojas et al., 2008). Analysis of myosin heavy chain gene expression also showed a reduced expression, which further supports our findings. Finally, cardiac troponin T and cardiac actin showed a non-significant reduction in expression. These data suggest that inhibition of NO synthesis affects cardiomyocyte differentiation by regulating the expression of specific but not all transcription factors and contraction proteins. Our data indicate that NO signaling is involved in early stages of cardiac differentiation affecting the expression levels of transcription factors such as *Gata4* and *Nkx2.5*.

4.5.4.5 Effect of Manipulation of Endogenous NO Synthesis on the Expression of Endothelial and Hematopoietic Specific Markers during Differentiation

Reduced expression of cardiac-specific markers in ADMA treated samples indicate a lower cardiac differentiation rate than non-treated controls, which lead us to try and identify the new differentiating pathway that those cells acquire. Analysis of hematopoietic markers showed an increase in expression of *Aml1* gene in ADMA treated samples but not CD34. Increase in expression of AML1 was also verified by western blot analysis. *Aml1* gene is an hematopoietic specific marker and has been strongly associated with hematopoiesis initiated at the hemangioblast (Choi et al., 1998; Swiers, De Bruijn, & Speck, 2010). The hemangioblast is comprised of multipotent precursor cells and has the potential to give rise to endothelial and hematopoietic cells (Choi et al., 1998). Hemangioblast formation is also linked to yolk sac formation. Our analysis of the number of EB sacs shows an increase in yolk-like sac formations in ADMA treated samples consistent with a possible increase in hematopoiesis. Analysis of endothelial cell differentiation specific markers such as CD31 showed a decrease in expression in ADMA treated samples on Day 10 of differentiation. These data further support the idea that low levels of NO direct the hemangioblast differentiation to a hematopoietic phenotype rather than endothelial differentiation. CD34, a bone marrow specific marker, showed no changes in expression further supporting the idea that normal bone marrow hematopoiesis is not affected by NO inhibition but hemangioblast differentiation is. Smooth muscle actin showed a decrease in mRNA expression in ADMA treated samples, which was not verified by our protein analysis suggesting that smooth muscle differentiation was not affected by NOS inhibition. Overall, we suggest that inhibition of NO synthesis in early embryonic development stages affects the differentiation of hemangioblast to a hematopoietic rather than an endothelial fate but does not affect the bone marrow.

4.6 Chapter 4 Key Findings

1. NO levels play an important role during development of mesoderm/endoderm.
2. Low NO levels decrease differentiation levels of mouse embryonic stem cells to cardiomyocytes as confirmed by analysis of cardiac specific markers.
3. Low NO levels seem to affect the differentiation of the hemangioblast but do not seem to have an effect on bone marrow.
4. Low NO levels push the cells to acquire a hematopoietic fate and in parallel the reduce endothelial differentiation of the hemangioblast.

Chapter 5

5. The Effect of Endogenous and Exogenous NOS Inhibitors on Coronary Flow and Tissue Survival during an Ischemia-Reperfusion Injury

5.1 Introduction

NO signaling during an ischemia-reperfusion injury has been shown to play an important role not only in protection of the myocardium but in other organs too (Parmentier et al., 1999; Schulz et al., 2004; Ward et al., 2005). Endogenous and exogenous inhibitors of NOS enzymes have been also associated with protection against an ischemia injury in the heart (Casadei & Sears, 2003) or the brain (Parmentier et al., 1999).

Elevated plasma levels of ADMA have been associated with higher risk of cardiovascular events and mortality (Maas, Böger, & Lüneburg, 2009). Also, increased plasma ADMA levels in patients who had survived a myocardial infarction has been linked to a higher chance of a second MI incident (Schnabel et al., 2005). Finally, high baseline plasma ADMA levels can predict future cardiovascular risk, establishing ADMA as an independent risk factor and novel biomarker (Schnabel et al., 2005).

L-NMMA, an endogenous inhibitor, has been also associated with cardiac function (Casadei & Sears, 2003). Isolated guinea pigs hearts infused with 10 μ mol/l L-NMMA showed lower cardiac output, lower maximum left ventricular pressure and cardiac contractility (dP/dt) (Casadei & Sears, 2003).

Finally, L-NAME and 1400W, exogenous NOS inhibitors were used to assess the effect of NO inhibition on ischemia-reperfusion survival. L-NAME was chosen as a potent inhibitor of all three NOS isoforms and it has been shown to affect hemodynamics *in vivo* (Szigeti, Simon, Parratt, & Vegh, 2004). 1400W is a highly selective inhibitor of iNOS (Garvey et al., 1997) and iNOS enzyme inhibition by 1400W has been shown to have a protective role during an ischemic brain injury (Parmentier et al., 1999).

NOS enzyme inhibition has been shown to affect the cardiovascular system and to play various roles during an ischemic injury. In order to

understand the role of the three NOS isoforms and the methylarginines during an ischemia-reperfusion injury, we perfused isolated mouse adult hearts using a Langendorff apparatus with specific concentration of each inhibitor and methylarginines and assessed tissue survival of the myocardium using TTC staining.

5.2 Experimental Design

As previously described (Chapter 3), hearts were isolated from 8-week-old male C57/BL6 mice and were connected to a Langendorff apparatus to be perfused. Once the hearts were connected to the Langendorff apparatus, they were perfused using perfusion buffer (control samples) or perfusion buffer supplemented with 10 μ M SDMA, 10 μ M ADMA, 10 μ M L-NMMA, 25 μ M L-NAME or 5 μ M 1400W. Following 15 minutes of perfusion (equilibration period), hearts underwent 30 minutes of ischemic injury followed by 45 minutes of reperfusion. Before and after the ischemic injury, the coronary flow was measured and at the end of the ischemia-reperfusion challenge, heart tissue was sliced, TTC stained and fixed with 4% PFA. Analysis of TTC staining was used as an indicator for tissue survival after the ischemia-reperfusion injury.

5.3 Results

Coronary flow was used as an indicator of the effect of various endogenous and exogenous NO inhibitors on the coronary vessels.

In control hearts, coronary flow higher than 2ml/min before ischemia was considered as sufficient and hearts with a coronary flow below that measurement were discarded.

10 μ M SDMA was used in the perfusate buffer as a secondary control to identify a potential dose-related toxic effect of the inhibitors on the heart tissue as SDMA is not a NOS inhibitor. SDMA treatment had no effect on both coronary flow and tissue survival verified by TTC staining (Figure 40,41).

10 μ M ADMA treatment significantly reduced coronary flow before ischemia compared to control (** p <0.001) (Figure 40). After ischemia, ADMA did not have an effect on coronary flow, which remained at the same levels as before the ischemic injury. TTC staining of ADMA perfused hearts showed no

significant effect of ADMA perfusion in tissue survival after ischemia/reperfusion injury (Figure 40,41).

Hearts perfused with 10 μ M L-NMMA suffered a significant reduction in coronary flow before ischemia compared to controls (** $p < 0.001$) (Figure 40). After ischemia, there was a further significant decrease in coronary flow when compared to control hearts (* $p < 0.05$) (Figure 40). Staining with 1% TTC did not show any significant effect of L-NMMA perfusion on tissue survival (Figure 40,41).

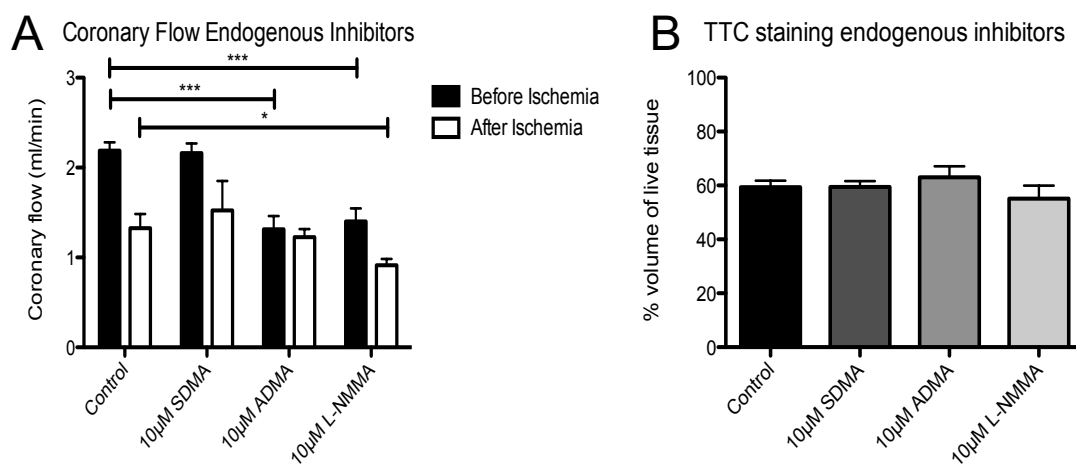


Figure 40. Coronary flow and TTC staining analysis of endogenous NOS inhibitors. **A.** Coronary flow analysis of control and endogenous NO inhibitor treated hearts before and after ischemic injury (Control Before Ischemia (2.188 $\pm$ 0.093) vs After Ischemia (1.325 $\pm$ 0.159), 10 μ M SDMA Before Ischemia (2.163 $\pm$ 0.105) vs After Ischemia (1.525 $\pm$ 0.327), 10 μ M ADMA Before Ischemia (1.313 $\pm$ 0.148) vs After Ischemia (1.225 $\pm$ 0.092), 10 μ M L-NMMA Before Ischemia (1.4 $\pm$ 0.145) vs After Ischemia (0.913 $\pm$ 0.072)). 10 μ M ADMA significantly decreased coronary flow before ischemia compared to control samples. The same effect was recorded in the coronary flow of 10 μ M L-NMMA treated hearts (vs Control). After ischemia, there was a significant decrease in coronary flow only in 10 μ M L-NMMA treated hearts compared to control hearts. $n=8$, t-test * $p < 0.05$, *** $p < 0.001$. **B.** TTC staining quantification of control and endogenous NO inhibitor treated hearts after an ischemia-reperfusion injury (Control (59.38 $\pm$ 2.399), 10 μ M SDMA (59.45 $\pm$ 2.170), 10 μ M ADMA (63.06 $\pm$ 4.037), 10 μ M L-NMMA (55.14 $\pm$ 4.806)). There was no significant difference between Control and endogenous inhibitor treated hearts. $n=8$, t-test, ns.

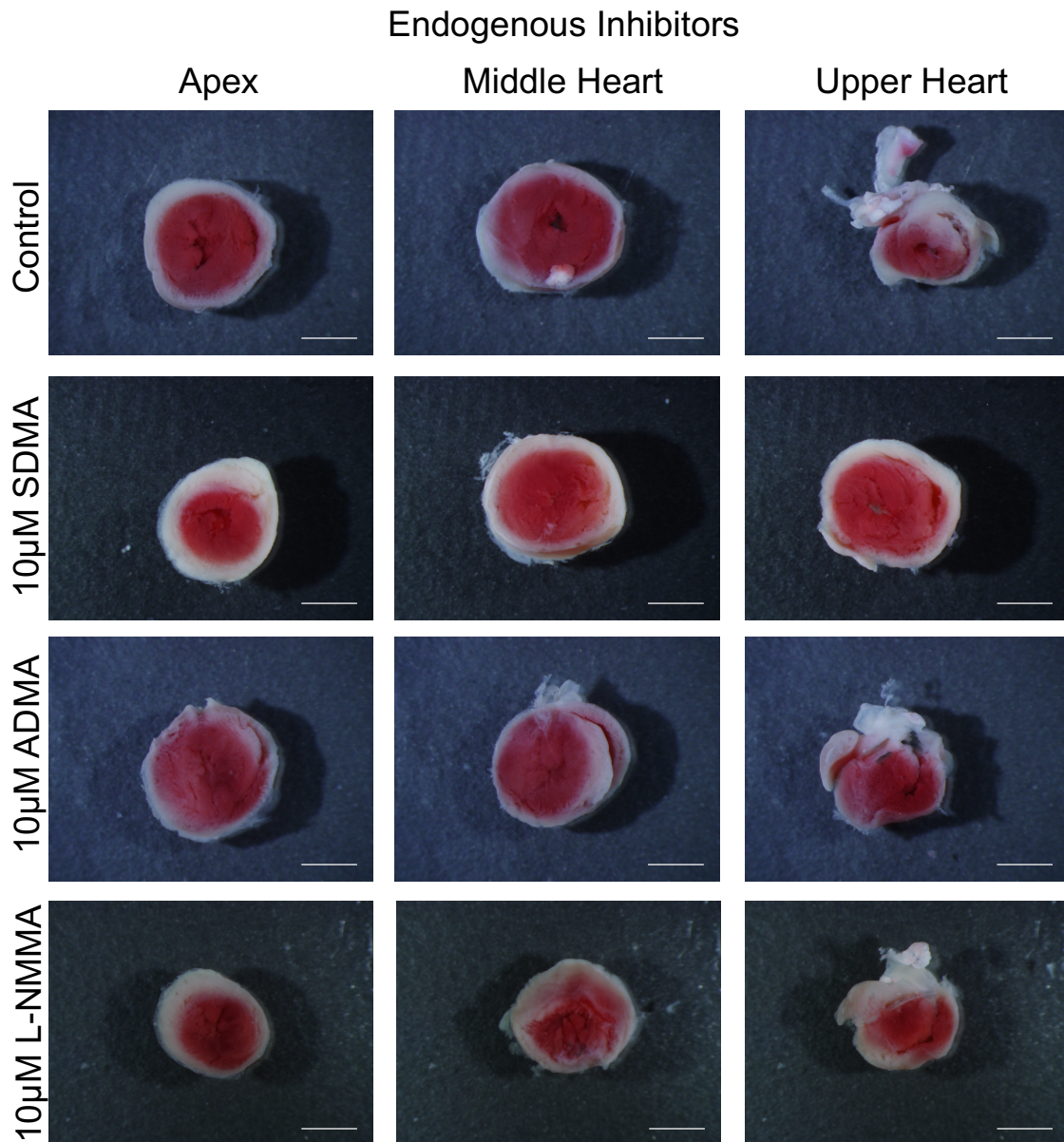


Figure 41. Slices of Control, 10μM SDMA, 10μM ADMA and 10μM L-NMMA TTC stained hearts. Red: metabolically active tissue, white: metabolically inactive tissue. Left: Apex, Middle: Middle Heart, Right: Upper Heart.

L-NAME (25μM) had a significant effect on both coronary flow and tissue survival as measured by TTC staining. L-NAME significantly reduced coronary flow before ischemia (** $p < 0.001$) and further reduced it after ischemic injury when compared to control hearts (* $p < 0.05$) (Figure 42). TTC staining showed a significant (14%) reduction in heart tissue survival treated with 25μM L-NAME compared to control samples (** $p < 0.01$) (Figure 42,43).

Finally, hearts were also perfused with 5μM 1400W, an iNOS specific inhibitor. Treatment with 1400W reduced coronary flow before ischemia significantly (* $p < 0.05$) but it did not significantly affect the coronary flow during reperfusion (Figure 42). TTC staining revealed a very small reduction in tissue

survival of heart treated with 1400W when compared to untreated samples (Figure 42,43).

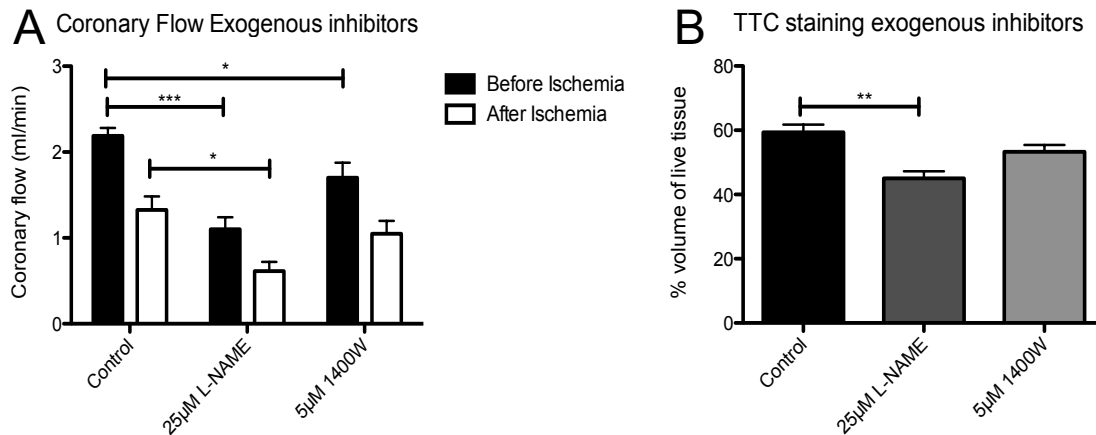


Figure 42. Coronary flow and TTC staining analysis of Control and synthetic NOS inhibitors **A.** Coronary flow analysis of control and exogenous NO inhibitor treated hearts before and after ischemic injury (Control Before Ischemia (2.188 ± 0.093) vs After Ischemia (1.325 ± 0.159), 25µM L-NAME Before Ischemia (1.1 ± 0.141) vs After Ischemia (0.613 ± 0.108), 5µM 1400W (iNOS specific inhibitor) Before Ischemia (1.7 ± 0.176) vs After Ischemia (1.05 ± 0.149)). Before ischemia, both 25 µM L-NAME and 5µM 1400W showed a significant decrease in coronary flow compared to control hearts. After ischemia, only 25µM L-NAME treatment had a significant effect on coronary flow. $n=8$, t-test, * $p<0.05$, *** $p<0.001$. **B.** TTC staining quantification of control and exogenous NO inhibitor treated hearts after an ischemia-reperfusion injury (Control (59.38 ± 2.399), 25µM L-NAME (45.05 ± 2.162), 5µM 1400W (53.3 ± 2.153)). 25µM L-NAME treated hearts showed lower tissue survival when compared to control hearts. $n=8$, t-test, ** $p<0.01$.

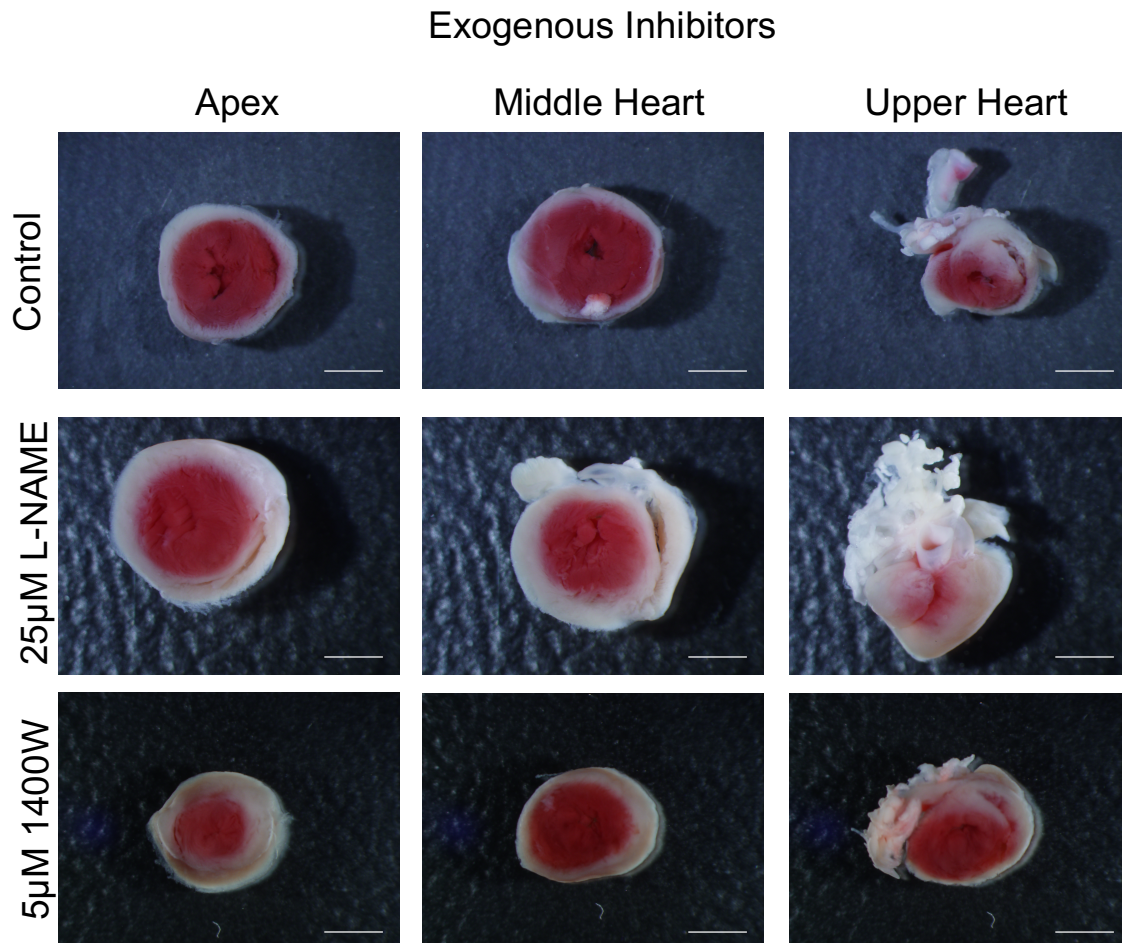


Figure 43. Slices of Control, 25μM L-NAME, 5μM 1400W TTC stained hearts. Red: metabolically active tissue, white: metabolically inactive tissue. Left: Apex, Middle: Middle Heart, Right: Upper Heart.

5.4 Discussion

5.4.1 The Effect of Methylarginines on Cardiac Tissue Survival after an Ischemia/Reperfusion Injury

Adult mouse hearts were perfused on a Langendorff apparatus with 10μM of SDMA, ADMA and L-NMMA in order to identify the effect of methylarginines on cardiac tissue survival after an ischemia/reperfusion injury. Heart perfusion with methylarginines had various effects on coronary flow with the exception of SDMA (it was used as a secondary control) but did not affect the survival of cardiac tissue. Previous experiments by others have shown an effect of methylarginines on blood pressure via NO level manipulation (Papapetropoulos et al., 1997; Shesely et al., 1996) but it is the first time that

the effect of different methylarginines on coronary flow and tissue survival after an ischemia/reperfusion injury is tested under the same conditions.

Adult hearts perfused with 10 μ M ADMA on a Langendorff apparatus showed reduced coronary flow before ischemia but did not show any further reduction in their coronary flow after ischemia. This observation suggests that ADMA has a limited effect on vascular homeostasis after an ischemic injury.

ADMA and L-NMMA are naturally occurring methylarginines and even though they are considered non-specific NOS inhibitors, it has been reported that they have higher affinity for eNOS and nNOS than iNOS (Vitecek et al., 2012). In contrast to ADMA treatment, perfusion with L-NMMA, had an effect on coronary flow before and after ischemia and it had a small non-significant effect (4%) on survival of the heart after an ischemia/reperfusion injury when compared to control. This absence of further reduction in the coronary flow after the ischemic insult could be an indication of a difference in action of those inhibitors.

Overall, we report that 10 μ M of ADMA and L-NMMA did not have an effect on the survival of cardiac tissue after an ischemia/reperfusion injury. There is a difference in coronary flow post ischemia in the two treatments suggesting a difference action pathway of the two methylarginines.

5.4.2 The Effect of Non-Endogenous Inhibitors on Cardiac Tissue Survival after an Ischemia/Reperfusion Injury

Ischemia/Reperfusion injury had a severe effect on both coronary flow and tissue survival. Coronary flow was significantly reduced before and after the ischemic injury, showing the highest reduction of all treatments. Also, tissue survival was significantly reduced and it was the only treatment that had a significant effect on tissue survival when compared to all other treatments. L-NAME is a potent non-specific inhibitor of all three NOS enzymes (Vitecek et al., 2012) therefore the large effect that it has on coronary flow and tissue survival can be an indication of the importance of NO production during an ischemia/reperfusion injury.

Production of NO can have a positive or negative effect on the survival of cardiac tissue during an ischemia/reperfusion injury depending on the time of production, the levels and the enzyme that is produced by (Bolli, 2001; Farah &

Reboul, 2015; Zweier & Talukder, 2006). More specifically, NO signaling depends on cardiomyocyte subcellular localization with reticulum sarcoplasmic and mitochondrial proteins being the main targets of S-nitrosylation (Murphy et al., 2012; Sun et al., 2012; Sun, Steenbergen, & Murphy, 2006). Moreover, NO can react during the reperfusion phase with O_2^- and produce peroxynitrite (Pacher, Beckman, & Liaudet, 2007). As a result an increase of NO bioavailability during reperfusion could have a harmful effect (Farah & Reboul, 2015). Also, during and after ischemia there is a change in NO availability and selection of S-nitrosation targets. Mitochondrial complex I S-nitrosation has been observed only after an ischemic injury and does not occur in normal hearts (Chouchani et al., 2013). Finally, it has been suggested that eNOS during ischemia is uncoupled and NO availability during that time is dependent on NO metabolites such as nitrite and S-nitrosothiols (Elrod et al., 2008; Farah et al., 2013; Singh et al., 1996). In the light of these examples, on high important localization and time dependent availability of NO is, we suggest that the concentration and the perfusion time of cardiac tissue with non-endogenous inhibitors might have a different effect on tissue survival.

1400W, an iNOS specific inhibitor (Garvey et al., 1997), showed a significant decrease in coronary flow when compared to control before an ischemic insult but not after. The effect of iNOS on tissue survival after an ischemia/reperfusion injury is not significant but shows a decrease of 6% when compared to the control. Selective inhibition of iNOS through perfusion with 1400W shows an effect on coronary flow before ischemia suggesting an involvement of iNOS enzyme in coronary flow regulation. Previous studies report that NO synthesis by iNOS in a preconditioned heart during an ischemia/reperfusion injury can have a beneficial effect on cardiac survival (Bolli, 2001). In our model, we recorded a negative effect of iNOS inhibition on tissue survival which might suggest that iNOS has a beneficial effect only when combined with tissue preconditioning which was not included in this study.

It is important to mention that the effect that we see on tissue survival could be an effect of the different concentrations of the inhibitors that were used. Perfusion with 10 μ M of ADMA was selected since it has been reported that pathophysiological levels of ADMA in humans can reach up to 10 μ M (Masuda et al., 1999). In order to be able to directly compare the effect of L-NMMA to ADMA since both of them are endogenous inhibitors of NOS enzymes

we treated the hearts with the same amount of the two substances (10 μ M) (Caplin & Leiper, 2012). On the other hand, we used pharmacological levels of L-NAME and 1400W as they are not endogenously produced and we wanted to achieve complete inhibition of the NOS enzymes. 1400W was used in a low concentration as it has been reported that it can have a cytotoxic effect on high concentrations (Garvey et al., 1997). All inhibitors were used in concentrations that have been shown to achieve inhibition of NO synthesis (Garvey et al., 1997; Vallance & Leiper, 2002; Vitecek et al., 2012).

Overall, we observed that perfusion with inhibitors with higher affinity for eNOS and nNOS (ADMA and L-NMMA) seemed to have no or little effect on tissue survival after an ischemia/reperfusion injury despite an effect on coronary flow. Finally, perfusion with a non-selective inhibitor of all three NOS isoforms caused a significant reduction in tissue survival suggesting an important role of NO signaling in cardioprotection.

5.5 Chapter 5 Key Findings

1. Selective inhibition of the NOS isoforms has no or little effect on tissue survival after an ischemia/reperfusion cardiac injury despite an effect on coronary flow.
2. Inhibition of all three NOS isoforms during an ischemia/reperfusion injury of the myocardium shows a significant reduction in tissue survival.

Chapter 6

6. Nitric Oxide (NO) and Methylarginine Regulation during *ex vivo* Global Ischemia-Reperfusion in WT *Ddah2*^{-/-} and *Ddah1*^{-/-} Hearts

6.1 Nitric Oxide (NO) and Methylarginine Regulation during *ex vivo* Global Ischemia-Reperfusion Injury

6.1.1 Introduction

NO as a master regulator, has been associated with a variety of roles in the cardiovascular system such as regulating cardiac contractility (Khan et al., 2003; Massion et al., 2005), effecting blood pressure (Achan et al., 2003; Leiper et al., 2007; Tran, Leiper, & Vallance, 2003) and even development of the heart (Kanno et al., 2004; Y. Liu & Feng, 2012).

Since its discovery, the Langendorff apparatus has been widely used in cardiovascular physiology studies and especially in those studying the effects of ischemia and reperfusion on the heart (Headrick et al., 2001; Liao, Podesser, & Lim, 2012; Reichelt et al., 2009).

In this experiment, we used a Langendorff apparatus to study the regulation of NO regulation-associated genes, methylarginine and NO levels during an ischemia-reperfusion injury of the mouse heart.

6.1.2 Experimental Design

C57/BL6 8-week old male mice were used in this experiment. Hearts were isolated and connected to the Langendorff apparatus. They were first perfused for 15 minutes in order to equilibrate and then, they undergo 30 minutes of ischemia followed by 45 minutes of reperfusion. Samples were collected before initiation of ischemia; at commence of reperfusion and at the end of reperfusion for mRNA, protein and mass spectrometry analysis. L-arginine, methylarginines and NO concentration were measured along with mRNA and protein levels of NO regulation genes.

6.1.3 Results

6.1.3.1 Concentration Analysis of L-arginine, Methylarginines and NO during Global Ischemia-Reperfusion Injury in WT Hearts

L-arginine showed a significant increase in cardiac tissue at the end of ischemia when compared to the normal cardiac state in the Langendorff apparatus (Figure 44). The increase in concentration after ischemia was slightly decreased at the end of reperfusion but remained higher when compared to continuous perfusion samples (Figure 44). A similar pattern of concentration fluctuations was observed in ADMA concentration during ischemia-reperfusion. ADMA was initially increased ($**p<0.01$) after ischemia in comparison to perfused hearts but it was significantly ($***p<0.001$) decreased after reperfusion when compared to ischemia samples (Figure 44). SDMA demonstrated a unique concentration pattern during injury as it was the only one of the methylarginines measured that was gradually decreased in concentration reaching significant levels when compared to normal heart state after the end of reperfusion ($**p<0.01$) (Figure 44). Finally, L-NMMA concentration remained unchanged before and after ischemia but it was significantly lower at the end of reperfusion (Figure 44).

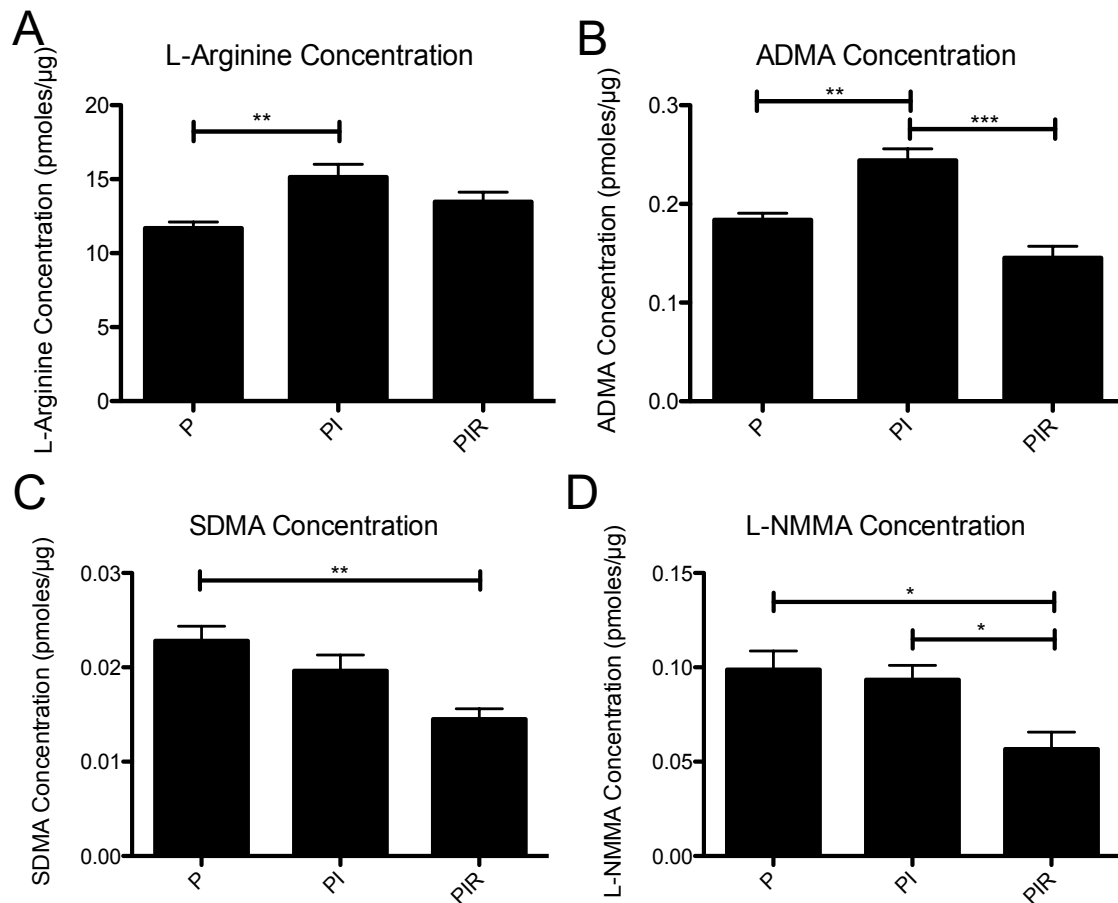


Figure 44. Mass spectrometry analysis of L-arginine and methylarginines (ADMA, SDMA and L-NMMA) during *ex vivo* global ischemia-reperfusion injury. **A.** L-arginine concentration (P (11.69±0.4285), PI (15.14±0.8613), PIR (13.47±0.6527)) shows a significant increase immediately after ischemia (PI) compared to normal cardiac state (P). **B.** ADMA concentration (P (0.1839±0.006), PI (0.2442±0.011), PIR (0.1454±0.011)) increased after ischemia (PI) compared to control state of the heart (P). ADMA was significantly decreased in hearts at the end of reperfusion (PIR) when compared to hearts that have only undergone ischemia (PI). **C.** SDMA concentration (P (0.022±0.001), PI (0.01963±0.0016), PIR (0.01451±0.0011)) was gradually decreased over time until it reached significance in samples collected after the end of reperfusion (PIR) compared to perfused hearts (P). **D.** L-NMMA concentration (P (0.098±0.0098), PI (0.093±0.0067), PIR (0.056±0.009)) remained stable after ischemia (PI) but was decreased after the end of reperfusion (PIR) when compared to both control (P) and after ischemia state of the heart (PI). n=8, One-way ANOVA with Bonferroni's post-test, * p<0.05, ** p<0.01, *** p<0.001. All concentrations were normalized to the protein concentration of each sample and concentration is shown as pmoles per μg of protein.

No significant changes were observed in NO concentration in the heart tissue during ischemia-reperfusion injury (Figure 45) despite the differences in methylarginine concentration such as ADMA and L-NMMA that are known NOS inhibitors (Figure 44).

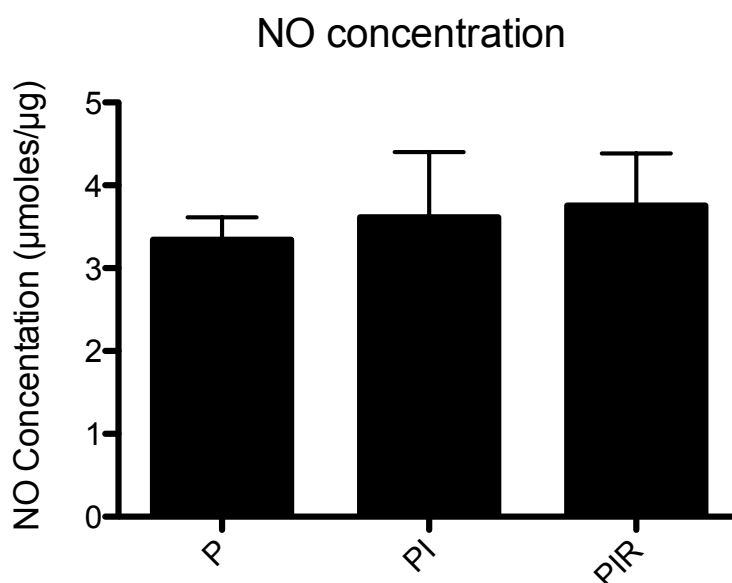


Figure 45. NO concentration in control heart tissue during ischemia-reperfusion injury. No significant difference was evident in [NO] during ischemia-reperfusion injury (P (3.343±0.2715), PI (3.613±0.7875), PIR (3.754±0.6324)). n=8, One-way ANOVA with Bonferroni's post-test, ns. NO concentration was measured as pmoles of NO normalized to μg of protein per sample.

6.1.3.2 NO Regulation-Associated Gene Expression Analysis

qPCR analysis of NO regulation-associated genes revealed their expression pattern during an ischemia-reperfusion injury of the myocardium.

Both *Ddah1* and *Ddah2* expression was downregulated during an ischemia-reperfusion injury with the decrease in expression of *Ddah1* being immediate after ischemia when for *Ddah2* was more gradual reaching significance only after reperfusion (Figure 46).

Measurement of NOS isoform mRNA levels qPCR showed a distinct pattern of expression for each protein. *eNOS* mRNA levels showed a small increase after ischemia without this increase reaching significant levels ($p>0.05$), an effect on expression that was reversed during reperfusion reaching expression levels lower than in perfused hearts (Figure 46). *nNOS* isoform was the only isoform that presented no changes in expression during ischemia-reperfusion injury (Figure 46). Finally, *iNOS* gene demonstrated a small increase in expression after ischemia followed by an acute decrease in expression at the end of reperfusion (** $p<0.01$) (Figure 46).

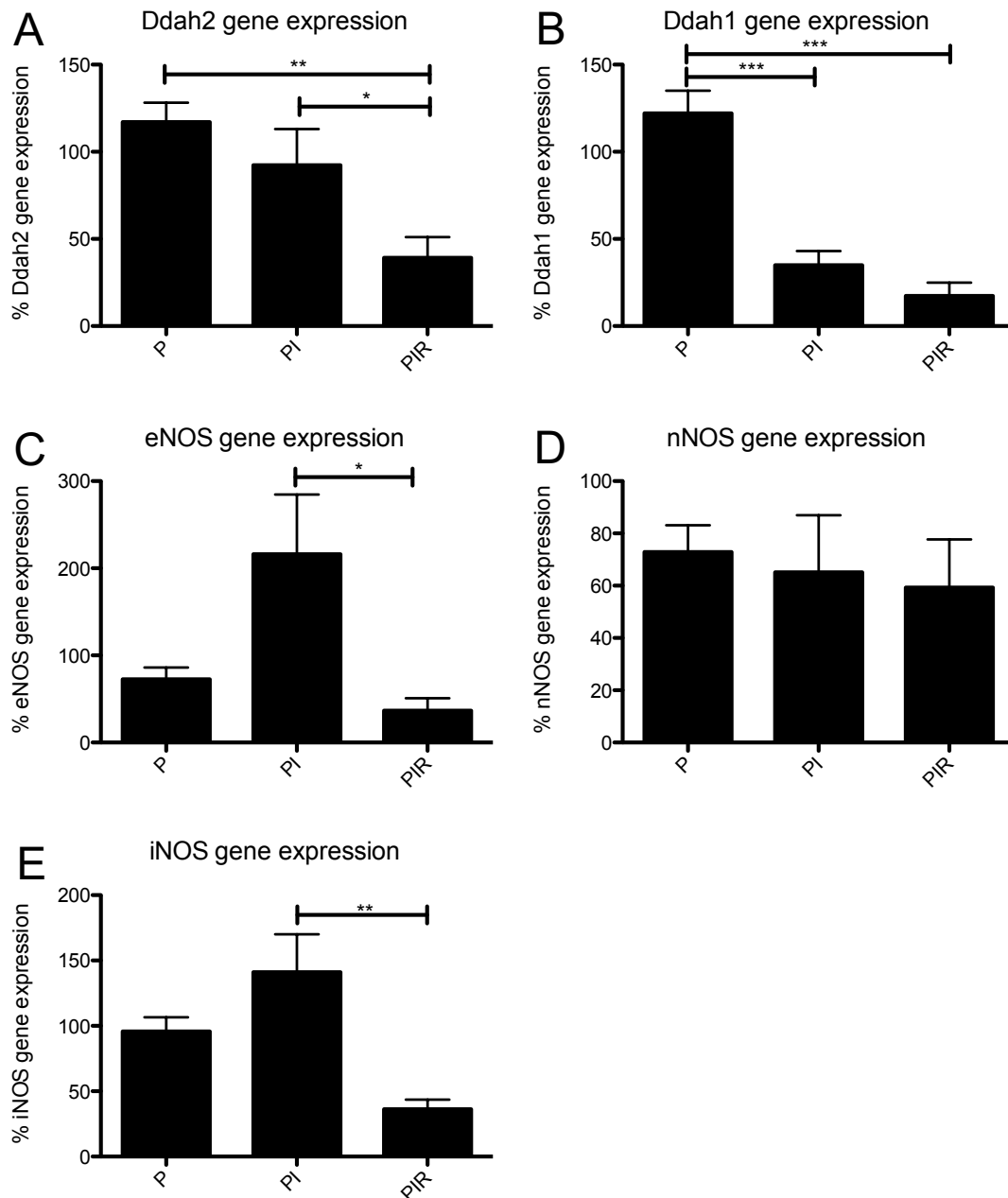


Figure 46. mRNA quantification of NO regulation-associated genes by qPCR during an ischemia-reperfusion injury of the myocardium. **A.** *Ddah2* expression (P (117±11.15), PI (92.21±20.75), PIR (39.19±11.86)) was steadily downregulated during ischemia and reperfusion. Only after the end of reperfusion (PIR) expression of *Ddah2* gene was significantly different when compared to expression during perfusion (P) or after ischemia (PI). **B.** *Ddah1* gene expression (P (122±13.01), PI (34.92±8.1), PIR (17.28±7.708)) was significantly downregulated ($p<0.001$) after ischemia (PI) and remained significantly downregulated even after reperfusion (PIR). **C.** qPCR analysis of *eNOS* mRNA levels (P (72.65±13.44), PI (216.2±68.56), PIR (36.56±14.41)) showed an increase in expression after ischemia (PI) which was completely reversed after the end of reperfusion (PIR). **D.** *nNOS* was the only of the NOS isoforms that exhibited no difference to its expression pattern during myocardial ischemia-reperfusion injury (P (72.89±10.22), PI (65.12±21.80), PIR (59.27±18.42)). **E.** Gene expression pattern of *iNOS* was similar to *eNOS* isoform with the increase in expression after ischemia (PI) compared to perfusion being smaller (P (95.69±10.96), PI (141.1±28.97), PIR (36.25±7.325)). $n=8$, One-way ANOVA with Bonferroni's post-test, * $p<0.05$, ** $p<0.01$, *** $p<0.001$. All gene expression levels were normalized to the expression of the house-keeping gene, *Gapdh*.

6.2 The Effect of *Ddah1* Gene in Myocardial Ischemia-Reperfusion Injury

6.2.1 Introduction

The *Ddah1* gene has been shown to play a vital role in regulation of ADMA and L-NMMA levels in mammals (Jacobi et al., 2005; Leiper et al., 2007; Nandi et al., 2012) and as a result *Ddah1* can have an effect on manipulation of NO production by removal of these endogenous NOS inhibitors.

Expression pattern analysis of the *Ddah1* in adult mammalian tissues revealed that *Ddah1* gene is expressed at least in mRNA level in the adult heart (Tran, Fox, Vallance, & Leiper, 2000). *In situ* hybridization of *Ddah1* mRNA showed that *Ddah1* gene is also expressed in the embryonic heart on day E9.5 of development (Breckenridge et al., 2010).

Ddah1 has also been implicated in myocardial infarction and hypoxia associated phenotypes. Immunolocalisation of *Ddah1* showed presence of *Ddah1* around the infarcted area after 7 days and increase enzymatic activities in rat hearts (G. a Gray et al., 2010). Finally, *Ddah1* was shown to be regulated by miR-21 in patients with pulmonary arterial hypertension and to be downregulated by hypoxia in cultured human pulmonary endothelial cells (Luo, Aslam, Welch, & Wilcox, 2015).

Ddah1 expression in the heart and regulation of its expression by hypoxia, along with increased activity of *Ddah1* around infarcted areas in rat hearts suggests a role of *Ddah1* during an ischemia-reperfusion injury of the myocardium.

6.2.2 Experimental Design

WT and *Ddah1*^{-/-} 8-week old male mice hearts were isolated and connected to a Langendorff apparatus in order to undergo a global ischemia-reperfusion injury. Hearts of both genotypes were loaded to the Langendorff apparatus and were perfused for 15 minutes to equilibrate to the new conditions. After equilibration, the hearts suffered 30 minutes of ischemia and 45 minutes of reperfusion. Coronary flow data were collected and analyzed as a marker for the physiological state of the hearts during the injury. Hearts

collected at the end of each timepoint, were sliced and TTC stained to reveal the affected area of the myocardium by the ischemia-reperfusion injury.

6.2.3 Results

6.2.3.1 *Ex vivo* Coronary Flow Analysis of WT and *Ddah1* KO Hearts

Coronary flow of WT and *Ddah1* KO hearts was measured before and after the induction of ischemia as a physiological marker of cardiac health and as a potential marker for the involvement of *Ddah1* in cardiac physiology during an ischemia-reperfusion injury. Both genotypes, exhibited a significant decrease in the coronary flow after an ischemic episode of 30 minutes (** $p < 0.001$) (Figure 47). Finally, there was no difference in coronary flow between WT and *Ddah1* KO hearts before or after ischemic injury (Figure 47).

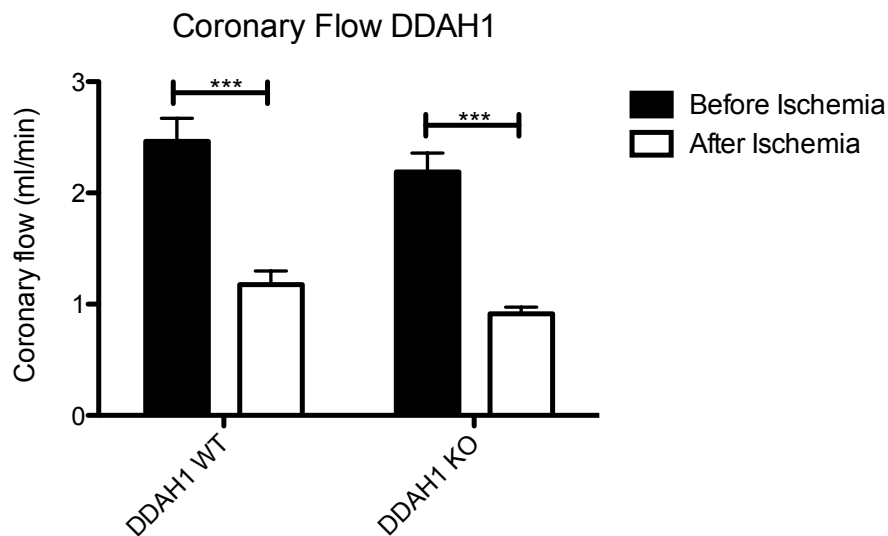


Figure 47. Coronary flow measurement of WT and *Ddah1* KO hearts before and after ischemia using a Langendorff apparatus (Before Ischemia WT (2.463 ± 0.208) vs *Ddah2*^{-/-} (2.188 ± 0.172), After Ischemia WT (1.175 ± 0.124) vs *Ddah2*^{-/-} (0.913 ± 0.061)). The pump connected to the Langendorff apparatus was set to a 5ml/min flow and the recordings were the flow that was allowed to go through the heart. All hearts with flow below 2 ml/min during the initial perfusion period were excluded from any further study and analysis. Coronary flow of WT and global *Ddah1* KO mice was significantly decreased after the ischemic injury. No significant difference was observed in the coronary flow between the two genotypes before and after ischemia. $n=8$, t-test *** $p < 0.001$.

6.2.3.2 Tissue Survival Analysis by TTC Staining of WT and *Ddah1* KO Hearts

Following an ischemia reperfusion injury of the myocardium, WT and *Ddah1* KO hearts were sliced and stained with TTC for assessment of tissue survival. Comparison between WT and *Ddah1* hearts showed no significant difference in survival between the two genotypes after a global *ex vivo* ischemia-reperfusion injury (Figure 48).

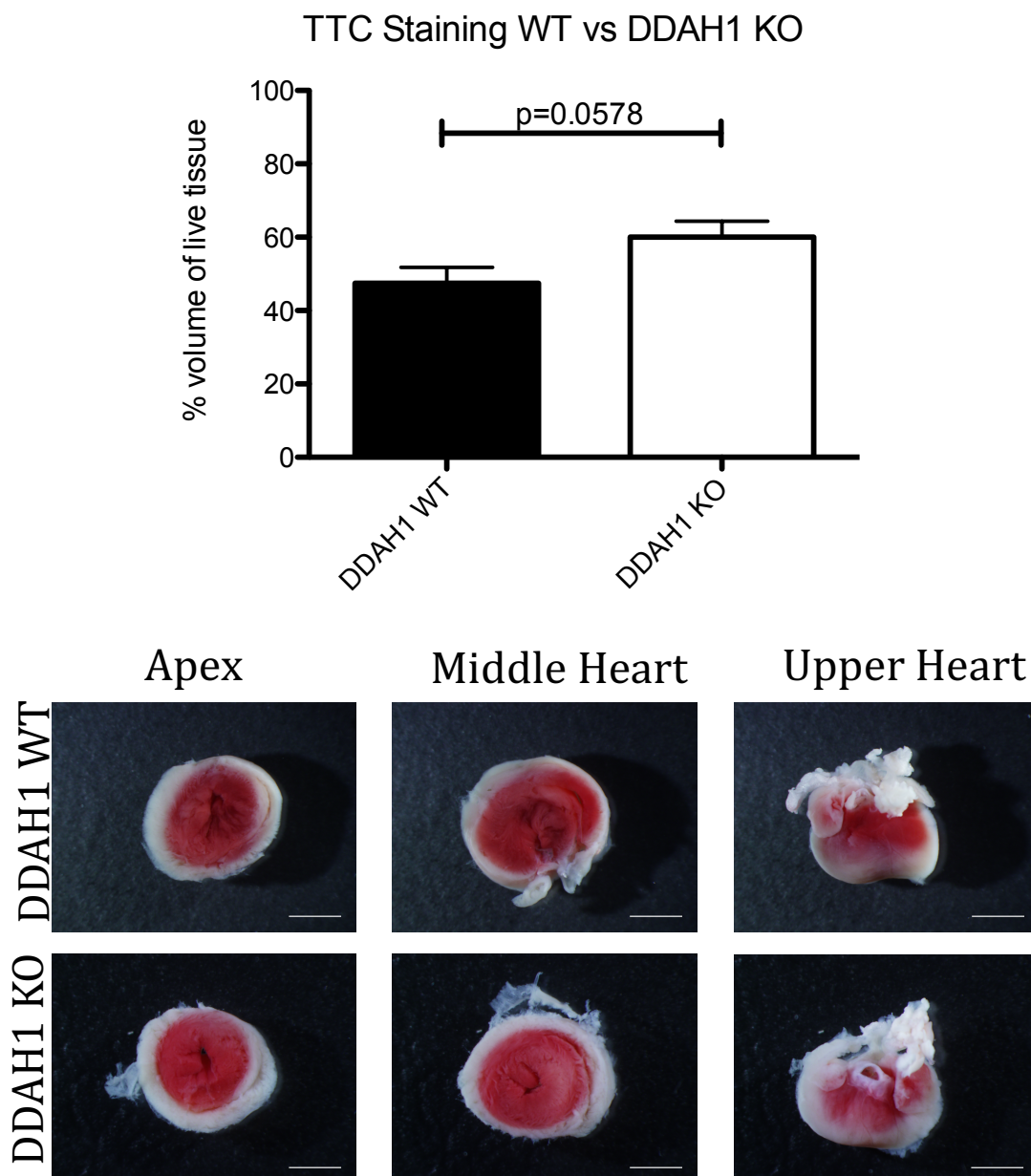


Figure 48. TTC staining of WT and *Ddah1* KO hearts after an ischemia-reperfusion injury using a Langendorff apparatus (WT (47.45±4.362) vs *Ddah1*^{-/-} (60.07±4.279)). TTC staining showed no significant difference in tissue survival between WT and *Ddah1* KO hearts. n=8, t-test ns.

6.3 The Role of *Ddah2* in an *ex vivo* Ischemia-Reperfusion Injury Model using a Langendorff Apparatus

6.3.1 Introduction

Ddah2 gene is involved in NO production via regulation of the endogenous NOS inhibitors levels, ADMA and L-NMMA (Lambden et al., 2015).

Even though *Ddah2* has a similar role as *Ddah1* gene, *Ddah2* expression pattern differs from that of *Ddah1* in mammals (Tran et al., 2000). *Ddah2* gene is the only isoform expressed in cells of the immune system such as macrophages and shows higher expression levels in tissues associated with the cardiovascular system like the heart, the aorta and the bone marrow (Lambden et al., 2015; Tran et al., 2000).

In adults, *Ddah2* is implicated in a variety of physiological and pathophysiological roles (Gray et al., 2010; Hasegawa et al., 2007; Lambden et al., 2015). More specifically, in endothelial cells, *Ddah2* has been shown to play an important role in VEGF regulation and as a result endothelial cell migration during angiogenesis (Hasegawa et al., 2006, 2007). In the immune system, *Ddah2*^{-/-} macrophages show less motility and as a result a reduction in immunological response compared to WT controls (Kelly et al., 2014).

Ddah2 has been associated with the cardiovascular system (Tran et al., 2000) and shows higher expression levels in the heart than *Ddah1*. Because of its expression pattern, *Ddah2* might be involved in the physiology of the heart during a global ischemia-reperfusion injury via regulation of NO levels or in a non-NO related mechanism.

6.3.2 Experimental Design

Hearts from WT and *Ddah2*^{-/-} 8-week old adult male mice were isolated and perfused on a Langendorff apparatus. Initial perfusion for equilibration of the hearts to the new conditions was 15 minutes (P). At the end of 15 minutes, both genotype hearts suffered a global ischemia injury for 30 minutes (PI). After ischemia, the tissues were perfused again for 45 minutes (PIR).

Coronary flow of WT and *Ddah2*^{-/-} hearts was measured before the ischemic injury (P) and at the end of reperfusion (PIR). Perfusate collected

during ischemia-reperfusion injury from both genotypes, was also analyzed by mass spectrometry for L-arginine and methylarginines levels. Heart tissue was collected for TTC staining to assess survival and for mRNA, protein and mass spectrometry analysis.

6.3.3 Results

6.3.3.1 Ex vivo Coronary Flow Analysis of WT and *Ddah2* KO Hearts

Coronary flow of WT and *Ddah2* KO hearts was measured before and after undergoing an ischemic injury. Both genotypes showed a significant reduction ($p < 0.001$) in coronary flow after ischemia but no difference was detected between WT and global *Ddah2* KO hearts before or after ischemia (Figure 49).

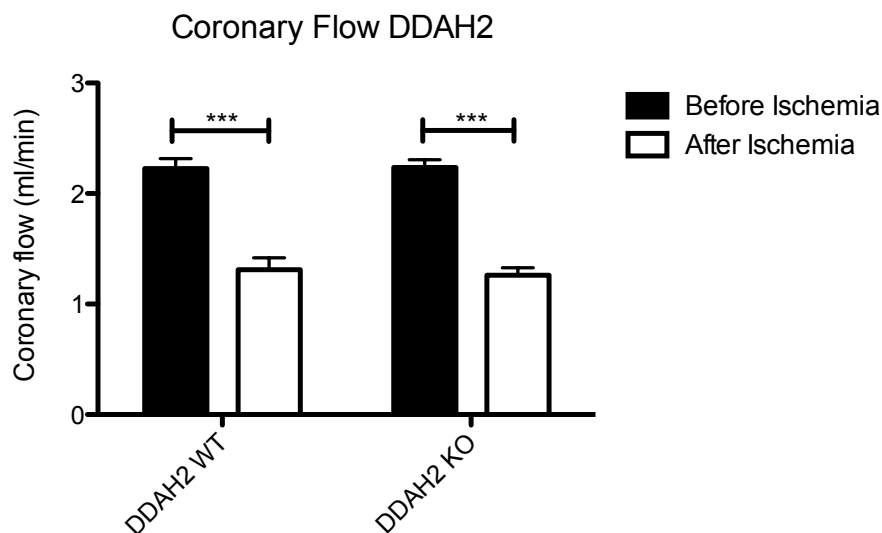


Figure 49. Coronary flow measurement of WT and global *Ddah2*^{-/-} hearts before and after an ischemic injury using a Langendorff apparatus (Before Ischemia WT (2.225±0.092) vs *Ddah2*^{-/-} (2.238±0.068), After Ischemia WT (1.313±0.106) vs *Ddah2*^{-/-} (1.263±0.065)). The pump connected to the Langendorff apparatus was set to a 5ml/min flow and the recordings were the flow that was allowed to go through the heart. All hearts with flow below 2 ml/min during the initial perfusion period were excluded from any further study and analysis. WT and *Ddah2*^{-/-} hearts demonstrated reduced coronary flow after ischemia. No significant difference was observed in coronary flow between the two genotypes before or after an ischemic injury. n=8 t-test *** $p < 0.001$.

6.3.3.2 Tissue Survival Analysis by TTC Staining of WT and Global *Ddah2*^{-/-} Hearts

After ischemia-reperfusion injury, hearts from both genotypes were sliced and stained with TTC to assess tissue survival. TTC staining analysis revealed significantly ($p < 0.001$) higher tissue survival of global *Ddah2* KO hearts when compared to equivalent WT controls (Figure 50).

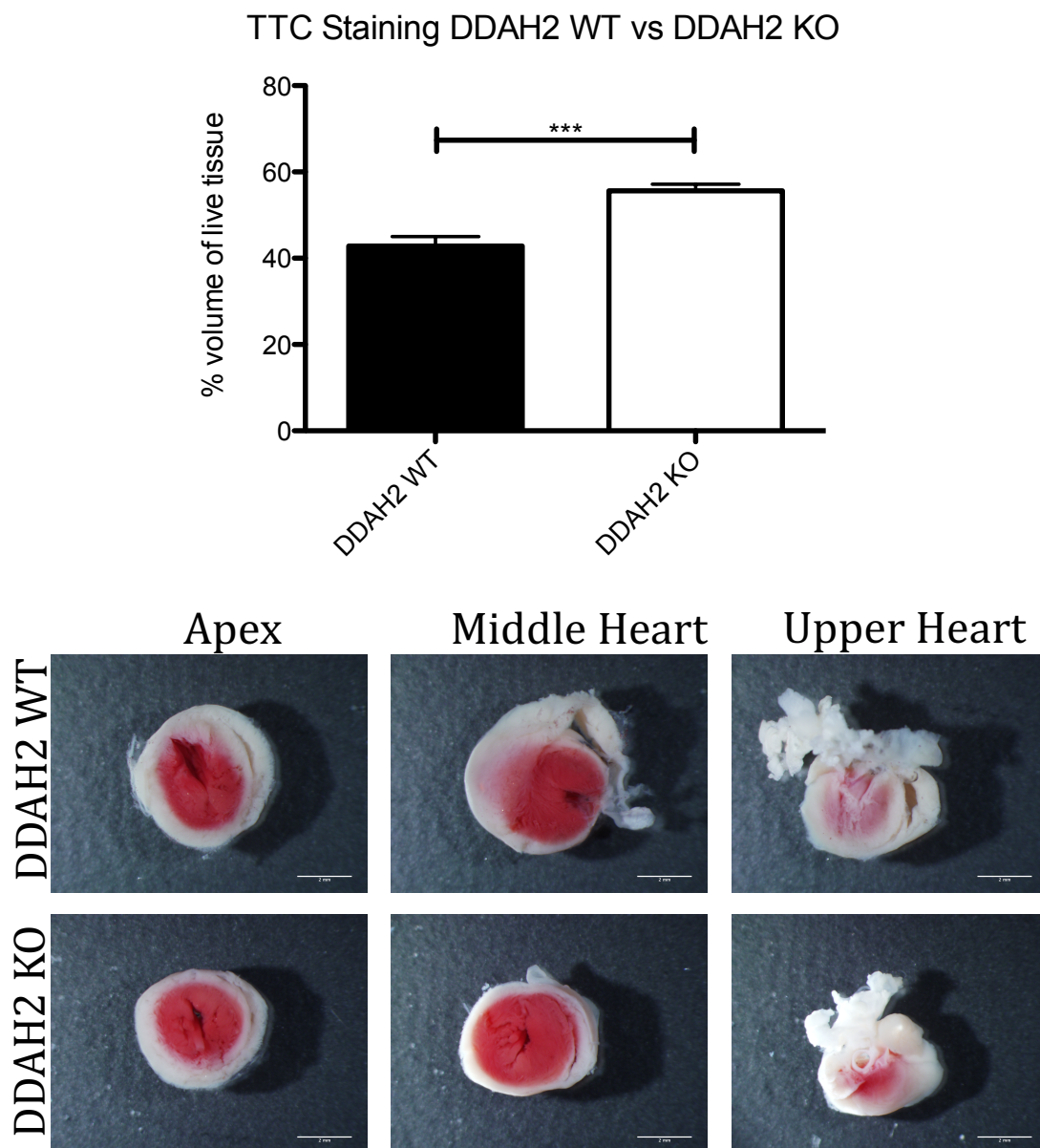


Figure 50. WT and *Ddah2*^{-/-} heart slices stained with TTC to assess tissue survival after an ischemia-reperfusion injury using a Langendorff apparatus (WT (42.86±2.155) vs *Ddah2*^{-/-} (55.66±1.530)). TTC staining revealed higher tissue survival levels of global *Ddah2*^{-/-} when compared to WT controls. n=8, t-test *** $p < 0.001$.

6.3.3.3 Analysis of L-arginine and Methylarginine Levels in Cardiac Perfusates

During ischemia-reperfusion injury, perfusate samples were collected during perfusion, after ischemia and during reperfusion (10, 20 and 45 minutes) and were analyzed using mass spectrometry.

L-arginine and methylarginine concentrations showed no significant difference during perfusion (P) and after ischemia (PI) between WT and global *Ddah2*^{-/-} heart perfusates (Figure 51). After the first 20 minutes of reperfusion, perfusates collected from WT hearts showed a significant increase in L-arginine, ADMA and SDMA concentration when compared to *Ddah2*^{-/-} perfusates (Figure 51). This rise in concentration might indicate increased proteolysis originating from increased tissue damage of WT hearts when compared to *Ddah2*^{-/-}. No significant difference in concentration of L-arginine, ADMA and SDMA was observed at 10 and 45 minutes of reperfusion (Figure 51). L-NMMA concentration was also analyzed but it was beyond detectable levels.

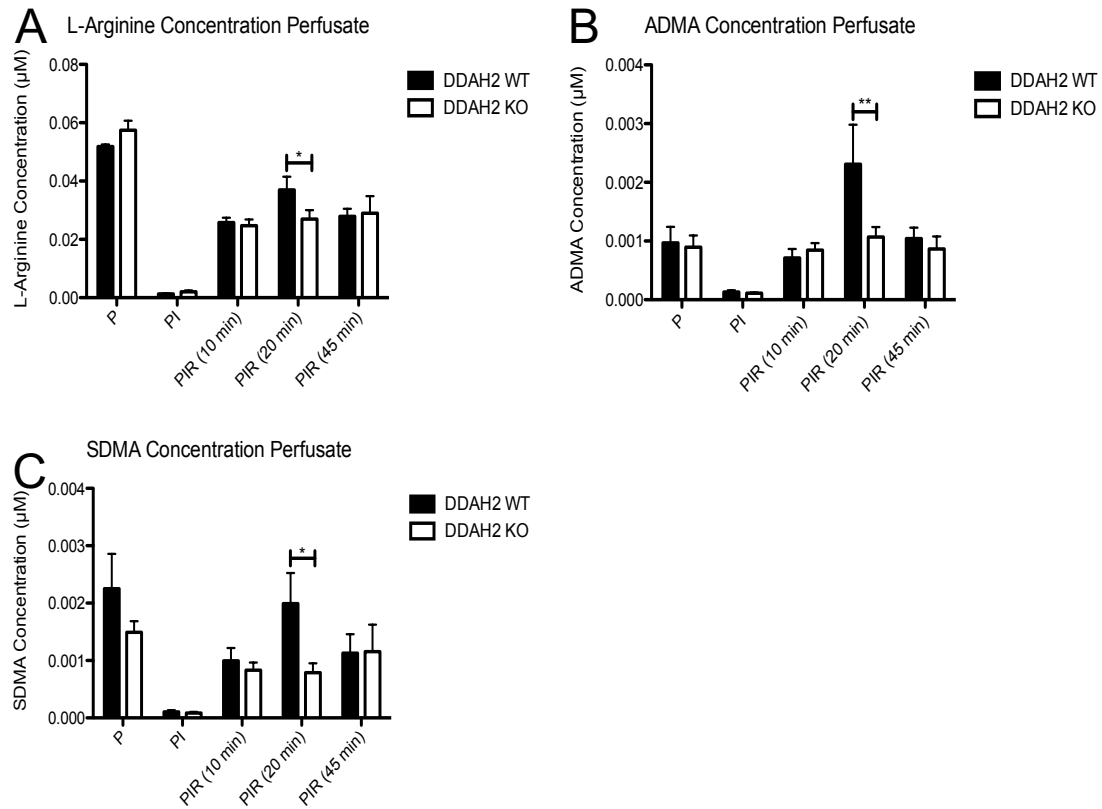


Figure 51. Perfusate analysis of WT and global *Ddah2* KO hearts during an ischemia-reperfusion injury using a Langendorff apparatus. Samples were collected before ischemia (P), after ischemia (PI), 10 minutes, 20 minutes and 45 minutes of reperfusion (PIR). L-NMMA concentration was also analyzed but it was beyond detectable levels. Perfusate analysis of L-arginine and methylarginines at 20 minutes of reperfusion showed a significant increase in concentration of WT hearts perfusate when compared to *Ddah2* KO samples. **A.** L-arginine concentration analysis showed an increase in concentration in WT perfusates at 20 minutes of reperfusion (P WT (0.052±0.0007) vs *Ddah2*^{-/-} (0.057±0.003), PI WT (0.001±0.0002) vs *Ddah2*^{-/-} (0.002±4.247e-004), PIR (10 min) WT (0.026±0.0017) vs *Ddah2*^{-/-} (0.025±0.002), PIR (20 min) WT (0.037±0.0045) vs *Ddah2*^{-/-} (0.027±0.003), PIR (45 min) WT (0.028±0.0026) vs *Ddah2*^{-/-} (0.029±0.006)). **B.** Concentration analysis of ADMA revealed a rise of ADMA at 20 minutes of reperfusion in perfusates collected from WT hearts (P WT (0.001±0.0003) vs *Ddah2*^{-/-} (0.0009±0.0002), PI WT (1.301e-004±3.2253e-005) vs *Ddah2*^{-/-} (0.0001±0.0001e-005), PIR (10 min) WT (0.001±0.0002) vs *Ddah2*^{-/-} (0.0008±0.0001), PIR (20 min) WT (0.002±0.0007) vs *Ddah2*^{-/-} (0.0011±0.0002), PIR (45 min) WT (0.001±0.0002) vs *Ddah2*^{-/-} (0.0009±0.0002)). **C.** SDMA concentration was significantly higher in WT perfusates when compared to *Ddah2*^{-/-} samples collected at 20 minutes of reperfusion (P WT (0.002±0.0006) vs *Ddah2*^{-/-} (0.001±0.0002), PI WT (1.036e-004±3.0960e-005) vs *Ddah2*^{-/-} (8.632e-005±1.9250e-005), PIR (10 min) WT (0.001±0.0002) vs *Ddah2*^{-/-} (0.001±0.0001), PIR (20 min) WT (0.002±0.0005) vs *Ddah2*^{-/-} (0.001±0.0002), PIR (45 min) WT (0.001±0.0003) vs *Ddah2*^{-/-} (0.001±0.0005)). n=8, Two-way ANOVA with Bonferroni's post-test, * p<0.05, ** p<0.01.

6.3.3.4 L-arginine and Methylarginine Concentration Analysis in WT and *Ddah2*^{-/-} Cardiac Tissue

L-arginine, ADMA, SDMA and L-NMMA concentrations were assessed during ischemia-reperfusion to identify a potential role of L-arginine or the methylarginines in protection of the myocardium in global *Ddah2*^{-/-} cardiac tissue. L-arginine concentration showed no difference between WT and *Ddah2*^{-/-} (Figure 52). ADMA and SDMA concentration in both genotypes followed the same pattern revealing no difference (Figure 52). Finally, L-NMMA concentration in heart tissue showed no significant difference between WT and *Ddah2*^{-/-} samples during perfusion (P) and after ischemia (PI), but a significant difference was detected at the end of reperfusion due to a decrease in L-NMMA concentration in WT hearts that was not apparent in DDAH2^{-/-} hearts (Figure 52).

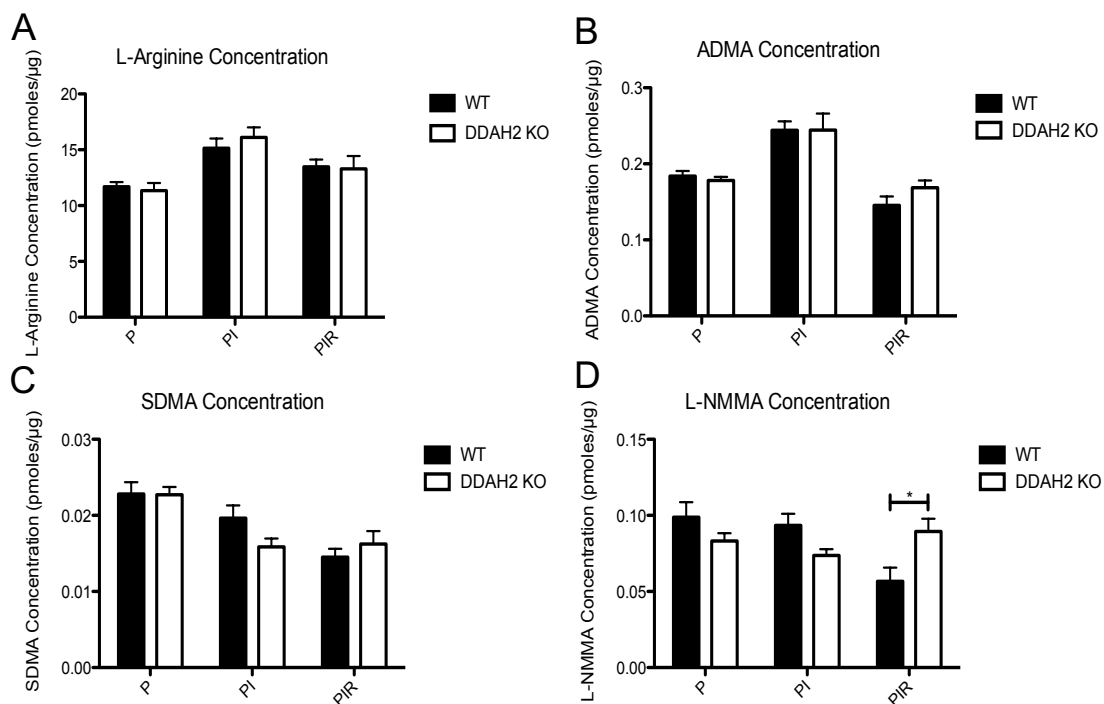


Figure 52. L-arginine, ADMA, SDMA and L-NMMA concentration of WT and global *Ddah2*^{-/-} cardiac tissue during a global ischemia-reperfusion injury. **A.** L-arginine concentration (P WT (11.686±0.429) vs *Ddah2*^{-/-} (11.348±0.670), PI WT (15.140±0.861) vs *Ddah2*^{-/-} (16.101±0.905), PIR WT (13.472±0.653) vs *Ddah2*^{-/-} (13.290±1.159)) **B.** ADMA concentration analysis L-arginine concentration (P WT (0.184±0.007) vs *Ddah2*^{-/-} (0.178±0.005), PI WT (0.244±0.012) vs *Ddah2*^{-/-} (0.244±0.022), PIR WT (0.145±0.012) vs *Ddah2*^{-/-} (0.169±0.010)) **C.** SDMA concentration L-arginine concentration (P WT (0.023±0.002) vs *Ddah2*^{-/-} (0.023±0.001), PI WT (0.020±0.002) vs *Ddah2*^{-/-} (0.016±0.001), PIR WT (0.015±0.001) vs *Ddah2*^{-/-} (0.016±0.002)) **D.** L-NMMA concentration analysis L-arginine concentration (P WT (0.099±0.0010) vs *Ddah2*^{-/-} (0.083±0.005), PI WT (0.093±0.008) vs *Ddah2*^{-/-} (0.074±0.004), PIR WT (0.057±0.009) vs *Ddah2*^{-/-} (0.089±0.008)). At the end of reperfusion, WT hearts showed a significant decrease in

concentration of L-NMMA in comparison to *Ddah2*^{-/-} hearts. n=8, Two-way ANOVA with Bonferroni's post-test, * p<0.05.

6.3.3.5 NO Concentration in WT and DDAH2 KO Cardiac Tissue

Nitrate and nitrite levels were analyzed in cardiac tissue during a global ischemia-reperfusion injury as a reflection of free NO molecules. Analysis showed no significant difference in NO levels between WT and *Ddah2* KO hearts during perfusion, after ischemia and at the end of reperfusion (Figure 53).

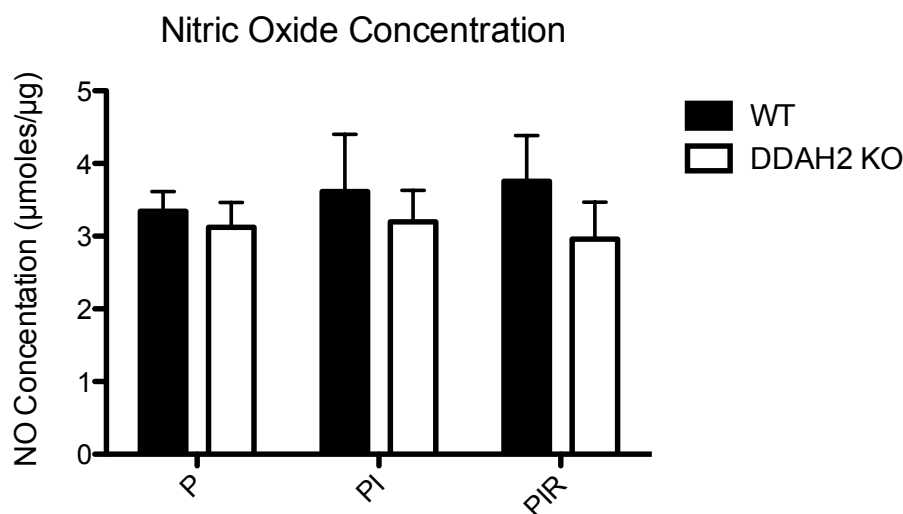


Figure 53. NO concentration in WT and global *Ddah2* KO heart tissue during a global ischemia-reperfusion injury using a Langendorff apparatus. Analysis of nitric oxide concentration showed no significant difference between WT and *Ddah2*^{-/-} cardiac tissue during ischemia-reperfusion injury L-arginine concentration (P WT (3.343±0.272) vs *Ddah2*^{-/-} (3.124±0.342), PI WT (3.613±0.787) vs *Ddah2*^{-/-} (3.199±0.432), PIR WT (3.754±0.632) vs *Ddah2*^{-/-} (2.959±0.510)). n=8, Two-way ANOVA with Bonferroni's post-test, ns.

6.3.3.6 DDAH Gene Expression Analysis of WT and DDAH2 KO Hearts

Expression pattern of the DDAH isoforms was assessed in cardiac tissue of WT and global *Ddah2* KO mice by qPCR and western blot during an ischemia-reperfusion injury *ex vivo*.

Ddah2 expression was significantly decreased throughout ischemia-reperfusion injury in *Ddah2*^{-/-} hearts when compared to WT controls in both transcriptional and translational levels, verifying that *Ddah2* gene expression was successfully interrupted in global *Ddah2* KO mice (Figure 54,55).

At the end of perfusion, *Ddah1* mRNA levels were slightly higher in WT hearts when compare to *Ddah2*^{-/-} tissue samples without this difference in expression being significant (Figure 54). After ischemia, expression of *Ddah1* gene in *Ddah2*^{-/-} hearts was reduced and remained unchanged until the end of reperfusion (Figure 54). After reperfusion, we recorded a significant difference in *Ddah1* expression on the mRNA level between WT and *Ddah2*^{-/-} hearts. Overall, *Ddah1* expression gradually decreases during global ischemia-reperfusion injury reaching very low levels at the end of reperfusion in WT hearts. By contrast DDAH1 expression is significantly elevated in *Ddah2*^{-/-} hearts during reperfusion. DDAH1 protein was present in low amounts in both WT and *Ddah2*^{-/-} hearts at all stages of ischemia-reperfusion injury. Analysis of Western blot results demonstrated no significant differences in DDAH1 protein levels between WT and *Ddah2*^{-/-} (Figure 56). Protein expression was barely detectable by a western blot in both genotypes during the ischemia/reperfusion injury, making any analysis inconclusive.

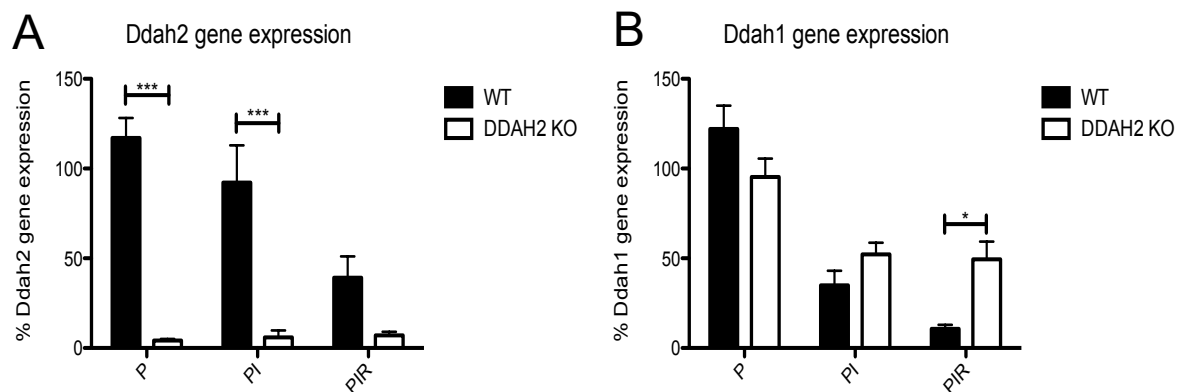


Figure 54. mRNA levels analysis by qPCR of *Ddah2* and *Ddah1* genes in WT and global *Ddah2* KO hearts during an ischemia-reperfusion injury. **A.** *Ddah2* expression levels were significantly downregulated in global *Ddah2* KO compared to WT tissues therefore verifying that *Ddah2* gene expression has been efficiently disrupted in global *Ddah2* KO animals (P WT (117.020±11.153) vs *Ddah2*^{-/-} (4.138±0.930), PI WT (92.214±20.754) vs *Ddah2*^{-/-} (5.901±3.869), PIR WT (39.192±11.865) vs *Ddah2*^{-/-} (7.049±2.023)). **B.** *Ddah1* mRNA levels were gradually downregulated during perfusion (P) and after ischemia (PI) in both genotypes. At the end of reperfusion (PIR), *Ddah1* mRNA levels remained unchanged in *Ddah2*^{-/-} hearts as compared to after ischemia levels but were decreased in WT tissues thus creating a significant difference in expression levels between the two genotypes (P WT (122.050±13.012) vs *Ddah2*^{-/-} (95.322±10.233), PI WT (34.919±8.100) vs *Ddah2*^{-/-} (52.193±6.454), PIR WT (10.675±2.224) vs *Ddah2*^{-/-} (49.444±9.872)). n=8, Two-way ANOVA with Bonferroni's post-test, * p<0.05, *** p<0.001.

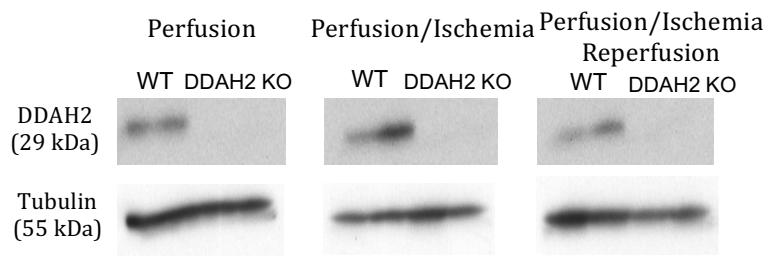
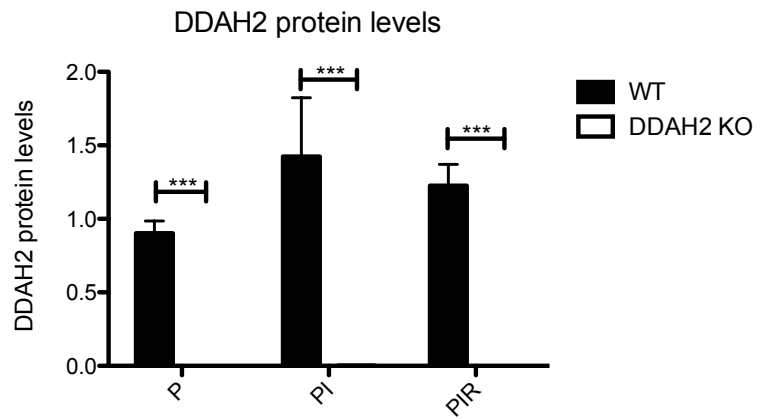


Figure 55. DDAH2 protein levels during ischemia-reperfusion *ex vivo* in WT and *Ddah2* KO hearts (P WT (0.903 ± 0.083) vs *Ddah2*^{-/-} (0.001 ± 0.0006), PI WT (1.424 ± 0.401) vs *Ddah2*^{-/-} (0.002 ± 0.0007), PIR WT (1.226 ± 0.144) vs *Ddah2*^{-/-} (0.001 ± 0.0003)). Analysis of DDAH2 protein levels showed that *Ddah2* gene expression was successfully disrupted and verified the results of *Ddah2* mRNA level analysis (Figure 54). DDAH2 protein seems to be slightly higher in WT hearts after ischemia. n=6, Two-way ANOVA, *** p<0.001.

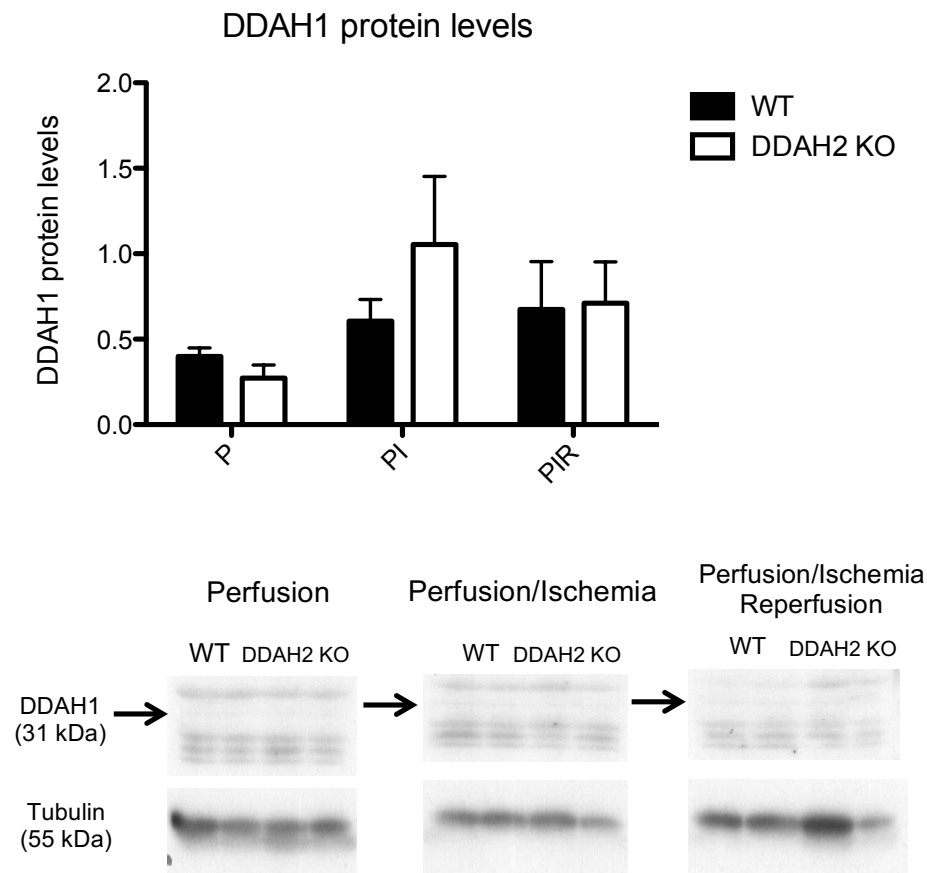


Figure 56. DDAH1 protein analysis by Western blot during ischemia-reperfusion injury of WT and *Ddah2*^{-/-} hearts *ex vivo* (P WT (0.400±0.049) vs *Ddah2*^{-/-} (0.272±0.076), PI WT (0.606±0.128) vs *Ddah2*^{-/-} (1.053±0.399), PIR WT (0.674±0.280) vs *Ddah2*^{-/-} (0.711±0.241)). DDAH1 protein was detected in very low amounts in WT and *Ddah2*^{-/-} hearts. Analysis did not reveal any statistically significant differences in DDAH1 expression between WT and *Ddah2* KO tissues. n=6, Two-way ANOVA, ns.

6.3.3.7 NOS Isoform Pattern Expression in WT and DDAH2 KO Cardiac Tissue during Ischemia-Reperfusion Injury

During ischemia-reperfusion injury, WT and global *Ddah2*^{-/-} heart NOS genes expression levels were measured to monitor the response of those genes to the injury.

In both genotypes, all three NOS isoforms are expressed during perfusion (Figure 57). After the ischemic insult, nNOS expression remained unchanged in WT hearts when eNOS and iNOS showed a non-significant tendency to increase their mRNA levels (Figure 57). At the end of 45 minutes of reperfusion, expression of eNOS and iNOS in WT samples were decreased reaching very low levels of expression (Figure 57). nNOS expression remained unchanged even after the end of reperfusion (Figure 57).

In *Ddah2*^{-/-} hearts, eNOS mRNA levels were gradually increased after ischemia and after reperfusion reaching significance when compared to WT samples only after reperfusion (Figure 57) due to a high decrease in eNOS expression in WT hearts. eNOS protein amount remained overall at the same levels during ischemia-reperfusion in both WT and *Ddah2*^{-/-} hearts (Figure 57). WT hearts showed a minor non-significant decrease in protein expression at the end of reperfusion in comparison to *Ddah2*^{-/-} hearts that maintained expression of eNOS protein at the same levels throughout injury (Figure 58). The increase observed in mRNA levels of eNOS gene during reperfusion in *Ddah2*^{-/-} hearts was not reflected in protein expression.

iNOS expression followed a similar to eNOS expression pattern reaching a significant difference in expression between WT and *Ddah2*^{-/-} hearts after reperfusion (Figure 57). nNOS in *Ddah2*^{-/-} heart tissue showed a small trend of upregulation after ischemia and reperfusion but due to high variability in expression this increase was not significant when compared to WT hearts.

iNOS protein analysis revealed that iNOS expression was below detectable levels by a Western blot and therefore it could not be further quantified (Figure 57).

Overall, nNOS expression remained unchanged in WT hearts during an ischemia/reperfusion injury of the myocardium with a small trend of upregulation in *DDAH2*^{-/-} hearts. eNOS and iNOS demonstrated a similar pattern in both WT and *Ddah2*^{-/-} hearts. After ischemia, gene expression showed a small increase in both genotypes but there was a significant difference in expression after reperfusion with *Ddah2*^{-/-} hearts further increasing their eNOS and iNOS expression and WT hearts losing a significant amount of gene expression.

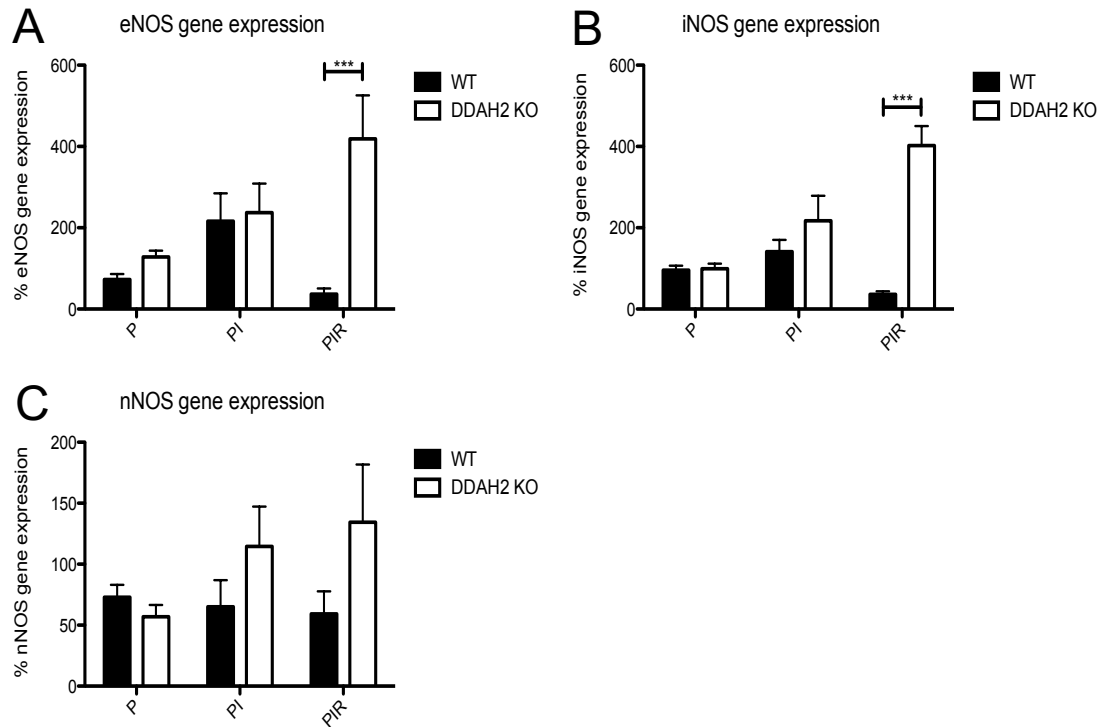


Figure 57. NOS isoforms mRNA levels assessment by qPCR in WT and *Ddah2*^{-/-} heart tissue during a global ischemia-reperfusion injury. **A.** *eNOS* gene expression pattern (P WT (72.654±13.436) vs *Ddah2*^{-/-} (128.150±15.445), PI WT (216.184±68.562) vs *Ddah2*^{-/-} (237.420±71.432), PIR WT (36.652±14.411) vs *Ddah2*^{-/-} (418.861±106.881)). Initially, *eNOS* gene expression was higher in *Ddah2*^{-/-} hearts compared to WT controls but this difference did not reach significance. After ischemia, both genotypes showed an increase in *eNOS* gene expression. *eNOS* gene expression at the end of reperfusion was elevated in *Ddah2*^{-/-} when it was significantly downregulated in WT tissue. **B.** *iNOS* expression pattern was similar to that of *eNOS* gene. No significant difference was present in expression levels during perfusion (P) and after ischemia (PI). After ischemia, *iNOS* gene was upregulated in both genotypes. At the end of reperfusion, *iNOS* mRNA levels in WT hearts were significantly reduced when there was a further increase in expression in *Ddah2*^{-/-} samples (P WT (95.687±10.957) vs *Ddah2*^{-/-} (99.184±12.656), PI WT (141.146±28.974) vs *Ddah2*^{-/-} (217.490±61.271), PIR WT (36.247±7.325) vs *Ddah2*^{-/-} (402.128±48.257)). **C.** *nNOS* expression was gradually upregulated in *Ddah2*^{-/-} tissues but remained the same in WT controls. *nNOS* increase in expression after ischemia and at the end of reperfusion in *Ddah2*^{-/-} samples did not reach significance due to high variation in expression of *nNOS* gene in *Ddah2*^{-/-} hearts (P WT (72.893±10.219) vs *Ddah2*^{-/-} (56.968±9.610), PI WT (65.123±21.800) vs *Ddah2*^{-/-} (114.512±32.678), PIR WT (59.266±18.421) vs *Ddah2*^{-/-} (134.372±47.322)). n=8, Two-way ANOVA with Bonferroni's post test, *** p<0.001.

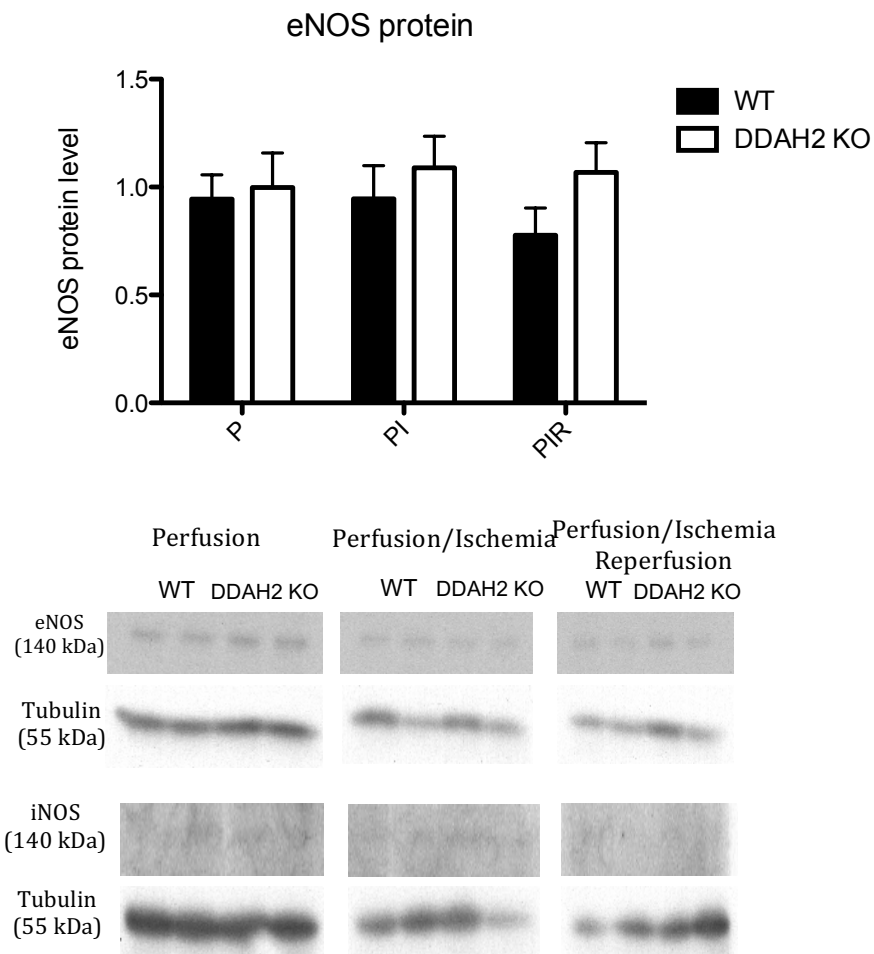


Figure 58. *eNOS* and *iNOS* western blot analysis of WT and *Ddah2*^{-/-} hearts during ischemia-reperfusion injury *ex vivo* (P WT (0.944±0.114) vs *Ddah2*^{-/-} (0.998±0.160), PI WT (0.945±0.154) vs *Ddah2*^{-/-} (1.089±0.146), PIR WT (0.777±0.125) vs *Ddah2*^{-/-} (1.068±0.137)). *eNOS* protein levels remained relatively unchanged in both genotypes. A minor decrease was observed after reperfusion in WT hearts but none of the results was significantly different. *iNOS* protein levels were barely detected by Western blot and it was impossible to quantify and assess. n=6, Two-way ANOVA, ns.

6.3.3.8 Expression Pattern of Cardiac, Mitochondrial and Hypoxia Related Markers in WT and DDAH2 KO Hearts

Myosin Heavy Chain (MHC) and *Cytb* were selected as assessment markers of the overall health of the cells as MHC is part of the contraction apparatus and *Cytb* is involved in mitochondria respiration. *Glut1* gene was selected as a hypoxia marker to assess the effectiveness of hypoxia treatment and the response of the tissue to this stimulus.

The MHC gene was gradually decreased in mRNA levels during ischemia-reperfusion in WT tissues (Figure 59). On the other hand, *Ddah2*^{-/-} hearts followed the downregulation pattern but the effect was reversed to

perfusion levels of expression after reperfusion (Figure 59). A statistical significant difference was measured after reperfusion in the expression levels of MHC gene.

CytB, a mitochondria respiration marker, demonstrated a gradual decrease in mRNA expression in WT hearts throughout the ischemia/reperfusion injury. *Ddah2*^{-/-} hearts expressed lower levels of *CytB* at the end of perfusion without this difference being significant ($p>0.05$) (Figure 59). After ischemia, mRNA levels remained at the same level. Finally, at the end of reperfusion, *Ddah2*^{-/-} hearts increased the expression of *CytB* gene in levels higher than the ones recorded after perfusion, resulting in a significant difference in expression between the two genotypes (Figure 59).

Finally, *Glut1* gene, a hypoxia marker, was also downregulated in WT samples during ischemia/reperfusion suggesting that WT hearts were not able to initiate a hypoxic response. *Ddah2*^{-/-} hearts, expressed *Glut1* in similar levels to WT hearts at the end of perfusion (Figure 59). After ischemia there was a slight increase in *Glut1* expression in *Ddah2*^{-/-} samples, which remained at the same levels even after reperfusion of the tissue (Figure 59). After reperfusion, *Ddah2*^{-/-} maintained expression of *Glut1* to after ischemia levels leading to a significant difference in *Glut1* expression between WT and *Ddah2*^{-/-} hearts (Figure 59).

Overall, WT hearts showed a decrease in mRNA levels in MHC and *CytB* during an ischemia/reperfusion and they failed to upregulate *Glut1* expression after an ischemic insult. On the other hand, *Ddah2*^{-/-} hearts showed a trend to decrease the expression levels of MHC and *CytB* during the ischemic injury, a trend that was reversed upon induction of reperfusion. Finally, *Ddah2*^{-/-} hearts seem to slightly upregulate the expression of *Glut1* gene upon induction of anoxia.

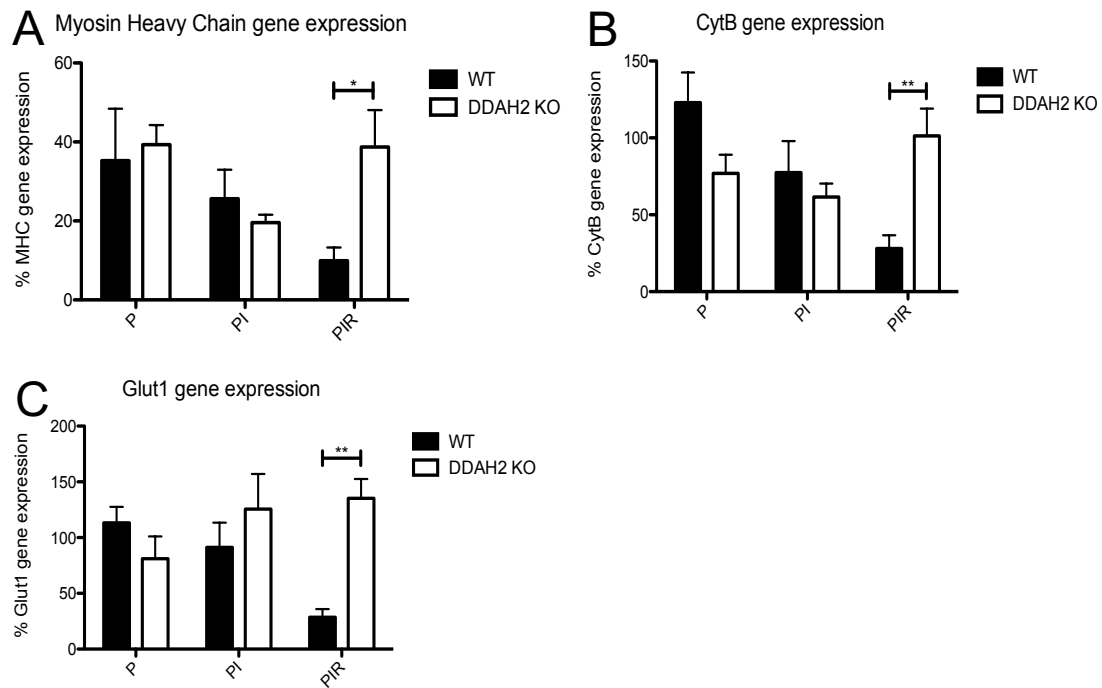


Figure 59. Expression level analysis of MHC, *Glut1* and *CytB* genes during ischemia-reperfusion injury of WT and global *Ddah2* KO myocardium *ex vivo*. **A.** MHC gene expression pattern showed a gradual downregulation after ischemic injury in both genotypes (P WT (35.260±13.164) vs *Ddah2*^{-/-} (39.311±4.945), PI WT (25.639±7.349) vs *Ddah2*^{-/-} (19.581±1.993), PIR WT (9.939±3.341) vs *Ddah2*^{-/-} (38.718±9.349)). This downregulation was reversed in *Ddah2*^{-/-} cardiac tissue but it was further increased in WT controls ($p < 0.05$). **B.** *CytB*, a mitochondria health marker, mRNA levels were higher in WT hearts compared to *Ddah2*^{-/-} hearts but not significant. WT heart expression was gradually decreased during ischemia-reperfusion. In comparison, *Ddah2*^{-/-} *Cytb* levels remained in similar levels during perfusion (P) and after ischemia (PI) but were slightly upregulated at the end of reperfusion (PIR) (P WT (122.987±19.604) vs *Ddah2*^{-/-} (76.980±12.071), PI WT (77.382±20.557) vs *Ddah2*^{-/-} (61.584±8.737), PIR WT (28.109±8.588) vs *Ddah2*^{-/-} (101.301±17.743)). **C.** *Glut1* gene, a hypoxia marker, in WT hearts was gradually downregulated with the highest decrease happening between beginning (PI) and end of reperfusion (PIR). *DDAH2*^{-/-} heart tissue showed a small increase after ischemia, which was maintained during reperfusion (P WT (113.183±14.470) vs *Ddah2*^{-/-} (81.053±20.059), PI WT (91.168±22.271) vs *Ddah2*^{-/-} (125.569±31.435), PIR WT (28.506±7.410) vs *Ddah2*^{-/-} (135.188±17.444)). $n=8$, Two-way ANOVA, * $p < 0.05$, ** $p < 0.01$.

6.3.3.9 Cardiac Troponin T and mTOR Protein Expression during Ischemia-Reperfusion Injury in WT and DDAH2 KO Cardiac Tissue

Cardiac Troponin T was selected as cardiomyocyte specific marker as it is part of the contraction apparatus. Western blot analysis of protein samples showed no significant differences in cardiac troponin T amounts in both genotypes during ischemia-reperfusion injury (Figure 60). Overall expression of cardiac troponin T was slightly elevated with the increase being a little bit higher in *Ddah2*^{-/-} but not significant compared to WT heart during perfusion.

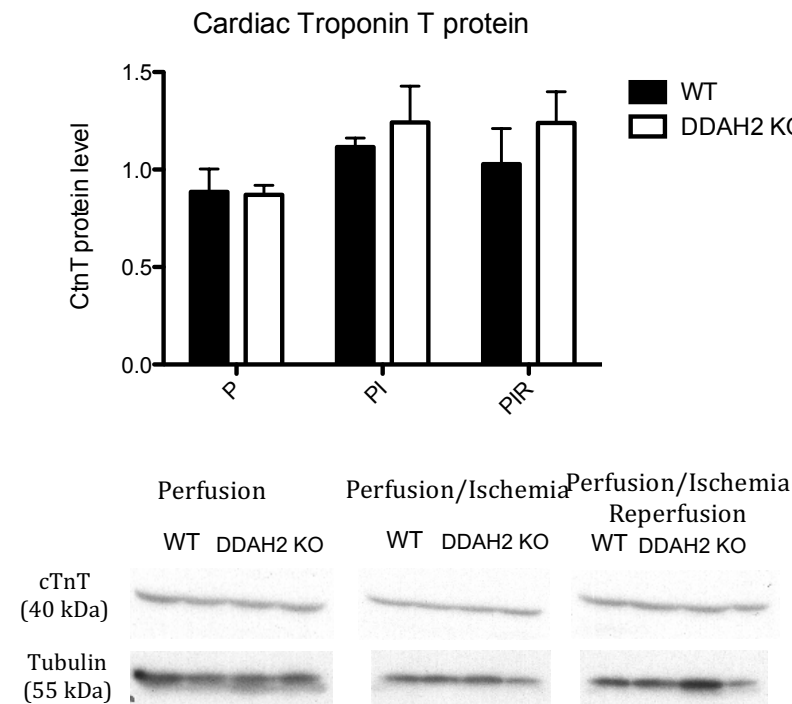


Figure 60. Protein expression of Cardiac Troponin T during ischemia-reperfusion injury of WT and *Ddah2*^{-/-} hearts (P WT (0.885±0.118) vs *Ddah2*^{-/-} (0.870±0.049), PI WT (1.115±0.046) vs *Ddah2*^{-/-} (1.242±0.186), PIR WT (1.027±0.183) vs *Ddah2*^{-/-} (1.240±0.159)). Cardiac troponin T was selected as a cardiac overall health indicator as it is a contraction apparatus associated protein. During injury and especially after the ischemic incident, cardiac troponin T was slightly upregulated in both genotypes. No differences in the amounts of cardiac troponin T were detected throughout ischemia-reperfusion. n=8, Two-way ANOVA, ns.

AKT was also analyzed as it is part of the RISK pathway and is placed upstream of eNOS (Mullonkal & Toledo-Pereyra, 2007). Analysis of AKT protein could implicate the RISK pathway through eNOS in cardiac protection of *Ddah2*^{-/-} hearts. AKT analysis by Western blot showed a significant difference in AKT levels at the end of reperfusion in WT and *Ddah2*^{-/-} hearts (Figure 61).

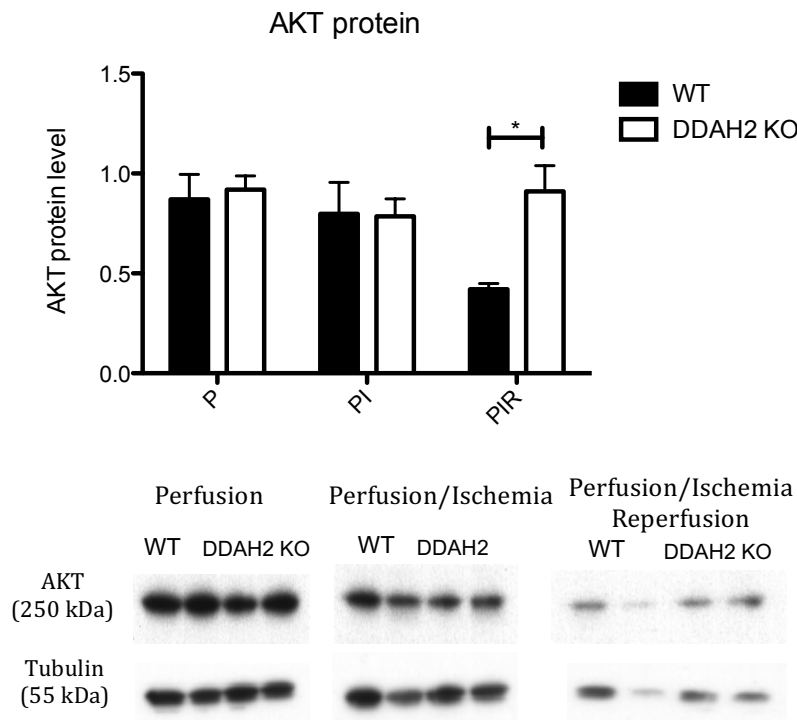


Figure 61. AKT expression pattern analyzed by Western blot during ischemia-reperfusion injury in WT and global *Ddah2* KO hearts (P WT (0.871 ± 0.125) vs *Ddah2*^{-/-} (0.920 ± 0.068), PI WT (0.798 ± 0.158) vs *Ddah2*^{-/-} (0.785 ± 0.088), PIR WT (0.421 ± 0.029) vs *Ddah2*^{-/-} (0.910 ± 0.129)). Expression analysis of AKT revealed a significant difference in protein expression between the two genotypes at the end of reperfusion. n=8, Two-way ANOVA, ns.

Overall, no significant difference was observed in protein amounts of Cardiac Troponin T but a significant difference in AKT levels was observed at the end of reperfusion since *Ddah2*^{-/-} hearts maintained their AKT protein levels during reperfusion.

6.4 Discussion

6.4.1 Characterization of NO Regulation Mechanism in WT Hearts during an Ischemia/Reperfusion Challenge

We first analyzed key elements of the NO signaling pathway in WT hearts during an *ex vivo* ischemia/reperfusion injury in order to record for the first time the variations that occur through the challenge. L-arginine and methylarginine concentration were measured by mass-spectrometry along with NO concentration and mRNA levels of major NO-regulation associated genes.

L-arginine and ADMA followed a similar pattern during the injury. An expected increase during ischemia was measured in both molecules due to cellular death and increased proteolysis induced by ischemia (anoxia) (Harris, 2002). After the end of reperfusion, L-arginine and ADMA levels were similar to perfusion (P) levels indicating that the system reached a new balance either by further processing or transferring into the blood stream (perfusate) the excess of molecules produced during the injury. On the other hand, SDMA and L-NMMA concentration showed a steady decline during an ischemia/reperfusion injury suggesting either a preference to asymmetric dimethylation of L-arginine in cardiac proteins or an effective cellular transfer mechanism for SDMA and L-NMMA into circulation.

This is the first time that the levels of L-arginine and methylarginines were measured during an ischemia/reperfusion model showing particular differences in their cellular levels. In the future, it will particularly interesting to identify the different mechanisms of action of those molecules and their transfer in and out of the cell as well as the effect of hypoxia on these processes.

Both *Ddah* genes showed a steady reduction in message, reaching the lost levels at the end of reperfusion. The reduction in mRNA levels of the *Ddah* genes suggests that *Ddah* gene expression might not be linked to a hypoxic response with the parallel destruction of the existing mRNA by cellular cell death and mRNA degradation. Finally, downregulation of *Ddah* genes could suggests that a cellular transfer mechanism might be involved in the reduction of methylarginine levels after the end of reperfusion.

6.4.2 NO Level and NOS Expression Analysis in WT Cardiac Tissue

NO signaling pathway has been shown to be involved in cellular responses to an ischemia/reperfusion injury with time of production to be a determining factor of the final outcome (Charlotte Farah & Reboul, 2015b; Schulz et al., 2004). Also, a cardioprotective role has been attributed to NO through ischemic preconditioning, claiming that production of NO can be induced by hypoxia in a distant area to the heart and through circulation to affect the cardiac muscle during an ischemia/reperfusion injury (Szigeti et al., 2004; Vegh, Szekeres, & Parratt, 1992; Zweier & Talukder, 2006).

NO analysis during ischemia/reperfusion injury showed that free NO levels did not change in WT cardiac tissue. These results suggest that production of NO by the heart was not affected by the ischemic insult or that the increase in ADMA levels after ischemia blocked any excess NO synthesis by the NOS enzymes.

eNOS and iNOS gene expression was significantly affected by the ischemia/reperfusion challenge but nNOS expression levels remained stable throughout the insult. nNOS stable expression suggests that this isoform might be involved in a reaction mechanism to an anoxic insult post-translationally but not on a transcriptional level. Upregulation of eNOS and iNOS genes indicate an involvement of these two isoforms in a cellular reaction to anoxia (ischemia). iNOS upregulation appears to be small as in an inflammation response iNOS expression has shown 100x fold increase (Lambden et al., 2015). After reperfusion eNOS and iNOS were significantly downregulated, a fact that could be attributed to cellular death and mRNA degradation. As previously mentioned, free NO levels remained unchanged during an ischemia/reperfusion challenge despite the fact that eNOS and iNOS expression levels were upregulated. These results suggest that there is a post-transcriptional regulation mechanism that regulates eNOS and iNOS protein levels or that the increase in ADMA concentration was sufficient to block any *de novo* NO synthesis.

6.4.3 Physiological Analysis and Tissue Survival in *Ddah1*^{-/-} during an Ischemia/Reperfusion Challenge

Ddah1^{-/-} hearts demonstrated no difference in coronary flow before and after an ischemic insult and deletion of *Ddah1* gene did not seem to have a significant effect on tissue survival as assessed by TTC staining. *Ddah1* is involved in blood pressure regulation through methylarginine, NOS endogenously produced inhibitors, conversion to L-citrulline (Breckenridge et al., 2010; Hasegawa et al., 2007; Iannone et al., 2014). In our experiments, we do not record a coronary flow difference between WT and *Ddah1*^{-/-} hearts. Various reasons can be responsible for these results. First of all, we are using *ex vivo* hearts perfused at a stable aortic pressure controlled by the Langendorff apparatus. Previous publications have shown that *Ddah1*^{-/-} mice are hypertensive. An absence of coronary flow differences between WT and *Ddah1*^{-/-}

^{-/-} hearts could be explained by the fact that once the hearts are excised from the animal circulation and the aortic pressure is not anymore controlled by the physiology of the animal but from the Langendorff apparatus, the role of *Ddah1* in blood pressure regulation is minimized. Also, the Langendorff apparatus measures coronary flow by subtracting the amount of liquid flowing to the heart from the amount of liquid pumped by the machine. This is not a sensitive method that could detect minor changes in coronary flow. Finally, the apparatus cannot measure coronary flow changes in the microvessels of the coronary circulation. More detailed analysis of the coronary flow will help us clarify if there is an effect of *Ddah1* deletion on coronary circulation.

Analysis of heart slices stained with TTC after an ischemia/reperfusion injury in WT and *Ddah1*^{-/-} hearts, the p value in the statistical test was not significant (p=0.0578). Also, in previous vivo experiments using a myocardial infarction model in *Ddah1*^{-/-} mice, carried out in collaboration with Dr. Gillian Gray, global *Ddah1*^{-/-} mice demonstrated a worst outcome after a myocardial infarction compared to WT mice (data not published). This phenotype was attributed to the fact that global *Ddah1*^{-/-} are chronically hypertensive and implicates the circulatory system in the survival of the cardiac muscle following a myocardial infarction since *Ddah1* is highly expressed in the endothelial cells (Tran et al., 2000). Since our focus in this study is to investigate the role of *Ddah* genes in the survival of the cardiac muscle following an ischemia/reperfusion injury isolating as much as possible other contributors such as the circulatory or the immune system by using the Langendorff apparatus, contributed to our decision to not further study the role of *Ddah1* in an ischemia/reperfusion model. Finally, because of the statistically non-significant difference between WT and *Ddah1*^{-/-} hearts and the results demonstrated in Chapter 7 that support that *Ddah1* gene is not expressed or is expressed in low levels in adult mouse cardiomyocytes we chose not to further analyze WT and *Ddah1*^{-/-} heart samples.

6.4.4 A Cardioprotective Role of DDAH2 Deletion during an Ischemia/Reperfusion Injury

6.4.4.1 The Effect of DDAH2 Deletion on Coronary Flow and Tissue Survival

Ddah2 gene expression has been detected in the cardiovascular system and has been shown to regulate blood pressure and reactions of the immune system especially during sepsis (Lambden et al., 2015; Tran et al., 2000). Measurement of coronary flow by the Langendorff apparatus showed no differences between WT and *Ddah2*^{-/-} hearts before and after an ischemic insult. *Ddah1* and *Ddah2* genes are both expressed in endothelial cells lining the inner side of the vessels and both have been shown to regulate blood pressure (Leiper et al., 2007; Tran et al., 2000). As a result of these previous observations, the absence of a *Ddah2*^{-/-} phenotype might be attributed to the same reasons described in Section 6.2 for DDAH1 KO hearts.

Tissue survival analysis by TTC staining showed a significantly higher tissue survival in *Ddah2*^{-/-} hearts when compared to WT indicating a cardioprotective role of DDAH2 deletion on cardiac survival during an ischemia/reperfusion injury. Due to this observation, we further analyzed WT and *Ddah2*^{-/-} hearts in order to verify and further characterize this phenotype.

In our TTC staining, we observed that metabolically inactive tissue was observed only in the outer layers of the heart. In our experiments, we use a global ischemia/reperfusion injury model using a Langendorff apparatus compared with the majority of the literature that uses ligation of the left anterior descending aorta in combination with the Langendorff apparatus (Ahmed et al., 2011; Wang Wang et al., 2013). The same phenotype was observed in a global ischemia/reperfusion model of an isolated rat heart with the same ischemia duration (30 minutes) (Jia et al., 2016). Even though it seems that this phenotype is associated with the global ischemia/reperfusion model, there is no explanation in literature about the molecular mechanisms that could lead to that phenotype.

6.4.4.2 NO Signaling Pathway Regulation during Ischemia/Reperfusion in WT and *Ddah2*^{-/-} Hearts

Perfusate analysis using mass spectrometry revealed an upregulation of L-arginine and methylarginine concentration 20 minutes after the initiation of reperfusion only in WT hearts but not in *Ddah2*^{-/-} samples. L-NMMA concentration was below detectable levels in perfusates. Combined with the higher tissue survival in *Ddah2*^{-/-} hearts, this increase in L-arginine and methylarginines suggests that *Ddah2*^{-/-} hearts are relatively protected from cell death compared to WT hearts releasing to coronary circulation lower amounts of aminoacids. This finding is particularly interesting as we identify for the first time a possible cardioprotective role of DDAH2 deletion during an ischemia/reperfusion injury. Also, we managed to precisely identify the time point (20 minutes after initiation of reperfusion) where maximum cell death and proteolysis occur analyzing the amount of aminoacids released into coronary circulation. Finally, based on these observations we can conclude that *Ddah2* inhibition by pharmacological or other factors could be a possible therapeutic approach. It seems that *Ddah2* deletion beneficial effects occur during reperfusion rather than before or during ischemia. Medical treatment can be provided during reperfusion as it mainly occurs in the hospital rather than intervention in earlier stages of ischemia/reperfusion that is not possible.

Tissue timepoint specific analysis showed no difference in L-arginine, ADMA and SDMA concentration. *Ddah* gene expression pattern and protein analysis verified the successful disruption of *Ddah2* gene expression in *Ddah2*^{-/-} hearts and showed that *Ddah1* expression was maintained in mRNA but not protein levels in *Ddah2*^{-/-} hearts after reperfusion when compared to WT samples, which might be a general reflection of higher tissue survival or a compensatory mechanism of *Ddah2* deletion. In WT samples, *Ddah1* expression was gradually downregulated indicating a negative effect of the ischemia/reperfusion injury on *Ddah1* expression. Protein analysis of DDAH1 did not verify our gene expression pattern in both WT and *Ddah2*^{-/-} but DDAH1 protein levels in both genotypes were really low therefore we cannot extract safe results from its analysis. L-NMMA measurement in cardiac tissue revealed higher levels of L-NMMA at the end of reperfusion in *Ddah2*^{-/-} when compared to WT hearts. L-NMMA was not detectable in perfusates and tissue analysis did

not include sample collection at 20 minutes of reperfusion therefore we did not have comparable samples. Since, perfusate analysis revealed the time of highest death it will be important to analyze WT and *Ddah2*^{-/-} cardiac tissue at 20 minutes into reperfusion in order to verify our observations in perfusates. Finally, in Chapter 5, we perfused WT hearts with 10μM of L-NMMA in order to test if the cardioprotective effect of *Ddah2* deletion was propagated through L-NMMA concentration in circulation. Cardiac perfusion with L-NMMA has shown in the past an increase in vasoconstriction and cardiac dysfunction (Casadei & Sears, 2003). As shown in our results, perfusion with high levels of L-NMMA did not have any effect on tissue survival of WT hearts. We still cannot conclude that the *Ddah2* cardioprotective effect is not mediated through L-NMMA because we tested the effect by supplementing extracellular L-NMMA to increase intracellular levels rather than increasing intracellular levels by arginine methylation and subsequent proteolysis.

NO levels were stable in both WT and *Ddah2*^{-/-} and did not show a difference throughout the ischemia/reperfusion challenge despite the gradual upregulation of eNOS and iNOS gene expression. It is possible that NO incorporated in proteins or stored in nitrosothiols might be the key factor to the cardioprotective effect we measure. eNOS and iNOS gene expression upregulation comes in agreement with previous published results supporting a cardioprotective role of NO production during an ischemia/reperfusion injury (Schulz et al., 2004; Vegh et al., 1992). In order to further verify our results, we analyzed eNOS and iNOS protein levels. eNOS protein expression pattern did not verify our mRNA results and iNOS protein was beyond detectable levels by a Western blot therefore we could not extract any results. nNOS showed the same trend but due to high variability between samples, we cannot safely make any conclusion about the role of nNOS in the cardioprotection effect of *Ddah2* deletion during an ischemia/reperfusion injury. eNOS and iNOS upregulation in *Ddah2*^{-/-} might be related to *Ddah2* gene expression disruption but either is a part of a reaction cellular mechanism to anoxia that needs more time to be set in full action or that is regulated post-transcriptionally and during translation. These results along with methylarginine analysis in WT and *Ddah2*^{-/-} cardiac tissue suggest that the cardioprotective effect of *Ddah2* deletion may be independent of the NO stored in nitrates/nitrites and can be propagated by other cellular storing compounds such as nitrosothiols. Further experiments,

including analysis of earlier timepoints in reperfusion and measurement of nitrosothiols levels during ischemia/reperfusion injury will help us identify a possible mechanism that propagates the cardioprotective effect of *Ddah2* deletion. Previous publications have suggested a cardioprotective role of NO in early reperfusion stages through the regulation of the opening of the mitochondrial pores and subsequent protection of cells from an oxidative stress caused by reperfusion (Ong et al., 2015; Rasola & Bernardi, 2011).

6.4.4.3 The Effect of *Ddah2* Gene Expression Disruption on Cardiac and Hypoxia Markers during an Ischemia/Reperfusion Injury

To further characterize the effect of *Ddah2* gene deletion on cardiac tissue survival during an ischemia/reperfusion injury, we analyzed the expression of contraction, mitochondria and hypoxia-associated markers. Myosin heavy chain and cytb gene expression was maintained and showed an increase at the end of reperfusion in *Ddah2*^{-/-} hearts. In contrast, WT hearts steadily downregulated the expression of those genes. The expression pattern of those genes might reflect the highest tissue survival recorded by TTC staining in *Ddah2*^{-/-} hearts. Also, upregulation of those genes could be related to a repair cellular mechanism initiated during reperfusion (Kim et al., 2012) that could have a cardioprotective effect propagated by *Ddah2* deletion. Analysis of cardiac troponin T protein levels showed no difference in protein levels between WT and *Ddah2*^{-/-} hearts suggesting a post-transcriptional regulation of cardiac Troponin T mRNA or that protein degradation of Troponin T needs more time in order to be visible on a Western Blot.

Glut1 has been shown to be upregulated by hypoxia (Ward et al., 2005) and has been extensively used as a hypoxia marker as it is more stable than HIF1a and is located downstream of it in a hypoxic response signaling pathway (Harris, 2002; Ward et al., 2005). Interestingly, in our experiments we detected an increase in *Glut1* expression in *Ddah2*^{-/-} hearts upon induction of hypoxia but this increase was not present in WT hearts. This result combined with the cardiac tissue survival indicate that either *Ddah2*^{-/-} managed to upregulate

hypoxia related genes earlier than WT hearts or that cell death and mRNA degradation was induced in WT than *Ddah2*^{-/-} hearts faster blocking any hypoxia induced cellular reaction by cardiac cells.

6.4.4.4 The Role of the Akt/eNOS Pathway in the Cardioprotective Effect of *Ddah2* Deletion

In an attempt to identify a possible pathway through which *Ddah2* deletion is propagating its cardioprotective effect during an ischemia/reperfusion injury, we chose to investigate the role of Akt in cardioprotection.

The RISK pathway has been extensively characterized as one of the cardioprotective pathways, with the involvement of NO signaling, during an ischemia/reperfusion injury (Heusch et al., 2008). This pathway is recruited in early reperfusion stages. Briefly, phosphoinositide 3 kinase (PI3K) activates Akt via phosphorylation. Downstream, Akt activates eNOS also through phosphorylation, increasing NO synthesis in the myocardium. NO signaling is transferred to the mitochondria priming the close of the MPTP and therefore the protection of mitochondria from oxidative stress (Heusch et al., 2008; Schulz et al., 2004).

AKT protein level analysis showed a significant difference between WT and *Ddah2*^{-/-} hearts at the end of reperfusion. This evidence could suggest that AKT is involved in the cardioprotection phenotype that we see in *Ddah2*^{-/-} hearts. Though, we will have to analyze the levels of P-Akt before we could extract any safe conclusions about the role of Akt in the cardioprotective effect of *Ddah2* deletion in an ischemia/reperfusion injury, as P-Akt is the active form of Akt, which causes the activation of eNOS and therefore propagates NO synthesis.

In order to identify potential biochemical pathways that could be implicated in protection of the myocardium propagated by deletion of the *Ddah2* gene we need to take in further consideration the time length of the ischemia/reperfusion injury model and our focus in the immediate effect that it has on the myocardium compared to other models that look into the long-term effects of such an injury. Our ischemia/reperfusion model is consisted of a half an hour ischemia period and a 45-minute reperfusion phase. In this time length, *de novo* protein synthesis is difficult to change protein levels especially of

proteins associated with the contractility machinery and might not play a major role in the protection of the myocardium propagated by deletion of the *Ddah2*. Alternatively, analyzing protein modifications such as protein phosphorylation that could change the activity of proteins in a small-time frame could lead in the identification of pathways involved in cardioprotection. Unfortunately, due to an error in experimental design, phosphatase inhibitors were not used at the point of sample collection therefore rendering analysis of phosphorylation levels impossible at this point. In future experiments, analysis of phosphorylation levels could lead in identifying biochemical pathways involved in cardioprotection associated with *Ddah2* deletion.

6.5 Chapter 6 Key Findings

- *Ddah1* deletion has no significant effect on tissue survival after an ischemia/reperfusion injury.
- *Ddah2* deletion has a significant cardioprotective role during an ischemia/reperfusion injury.

Chapter 7

7. Expression Pattern of DDAH Isoforms in Isolated Mouse Adult Cardiomyocytes and the Role of *Ddah2* Gene during a Hypoxia-Normoxia Challenge

7.1 Introduction

Analysis of the adult mouse heart cell types has shown that cardiomyocytes are the predominant cell type present in adult heart tissue (Banerjee et al., 2007a). Myocytes consist approximately 55% of the total cell population with endothelial cells, fibroblasts and vascular smooth muscle cells to be present in significant amounts (Banerjee et al., 2007b).

Ddah1 and *Ddah2* gene expression pattern varies in mammals and different tissues has been shown to express both or one of the isoforms (Tran et al., 2000). Dot blot analysis showed that both isoforms were expressed on mRNA level in the heart (C. T. Tran et al., 2000a). *Ddah1* mRNA levels were lower than *Ddah2* suggesting that *Ddah2* gene is the predominant isoform expressed in the heart (C. T. Tran et al., 2000a).

NO regulation has been shown to play a major role in maintaining a healthy myocardium (Massion et al., 2003). It has been shown to improve left ventricular diastolic function (Paulus, Frantz, & Kelly, 2001) and to dictate the velocity of shortening in adult mouse cardiomyocytes especially after β -adrenergic stimulation (P. B. Massion et al., 2004).

During an ischemia-reperfusion injury, NO has been attributed a protective role via the RISK pathway (Hausenloy, 2004; Mullonkal & Toledo-Pereyra, 2007). More specifically, *Akt* phosphorylates *eNOS* protein and NO production is promoted in the myocardium (Dimmeler et al., 1999). Production of NO protects the myocardium in the first minutes of reperfusion by inhibiting the opening of mPTP (Rasola & Bernardi, 2011).

Due to heterogeneity of cell populations in heart tissue, isolation and analysis of mouse adult cardiomyocytes could give us an insight in *Ddah1* and *Ddah2* expression pattern in this particular cell type versus a global expression of the heart tissue. Also, as we suggested in the previous chapter, WT and global *Ddah2*^{-/-} hearts with an *ex vivo* ischemia-reperfusion model showed higher

tissue survival by TTC staining assessment in *Ddah2*^{-/-} hearts when compared to WT. Since TTC staining can only distinguish between metabolic active and inactive tissue, it was important to distinguish if cardiomyocytes, being the most important cell type in the heart, are the cells, which are protected against ischemia-reperfusion injury.

7.2 Experimental Design

Adult mouse cardiomyocytes were isolated from male 8-week-old WT and global *Ddah2*^{-/-} mice using a Langendorff apparatus. Briefly, the heart was excised and the aorta was used to connect the heart to the Langendorff apparatus. The heart was perfused to equilibrate and was digested using liberase. After adequate digestion, the ventricles were isolated and further processed for isolation of cardiomyocytes.

Once cells were successfully isolated, they were cultured over-night and they were collected for DDAH1 and DDAH2 protein analysis or they were subsequently used for a hypoxia/normoxia challenge.

Cardiomyocytes were placed for 3 hours in a hypoxia chamber with 5% O₂, 5% CO₂ at 37°C. After hypoxia challenge, the cells were placed in a normal incubator to equilibrate to normoxic levels for 1 hour. mRNA samples for qPCR analysis were collected before hypoxia (BH), after hypoxia (AH) and after normoxia (AN).

After hypoxia/normoxia challenge, *Ddah1* and *Ddah2* expression analysis along with eNOS, iNOS and specific cellular health and hypoxia markers (cardiac troponin T, *CytB*, *Glut1*) was carried out in order to monitor expression changes in the mRNA level between WT and *Ddah2*^{-/-} cardiomyocytes.

7.3 Results

7.3.1 Expression Pattern of DDAH Isoforms in Isolated Adult Cardiomyocytes

Protein analysis of WT adult mouse cardiomyocytes revealed that DDAH2 protein is expressed but DDAH1 protein was below detectable levels

and was not able to be measured by a Western blot (Figure 62). *Ddah2*^{-/-} cardiomyocytes were used as a negative control and showed specificity of our DDAH2 antibody. WT kidney tissue was used as a positive control for both DDAH1 and DDAH2 expression.

DDAH2 is the predominant isoform expressed in adult mouse cardiomyocytes.

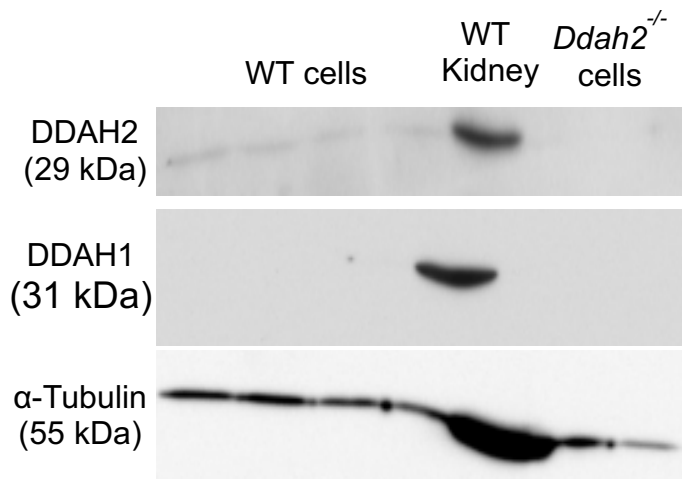


Figure 62. Protein expression analysis of DDAH2 and DDAH1 isoforms in isolated adult mouse cardiomyocytes. WT kidney tissue expresses high amounts of both isoforms and it was used as a positive control. WT and *Ddah2*^{-/-} cells were isolated and were blotted for DDAH1 and DDAH2 expression. Analysis showed that DDAH2 is the only isoform detected in adult mouse cardiomyocytes and there was no upregulation of DDAH1 protein levels upon knocking out of DDAH2 isoform. α-Tubulin was used as a loading control. WT (n=4), *Ddah2*^{-/-} (n=2).

7.3.2 Gene Expression Profile of WT and *Ddah2*^{-/-} Adult Mouse Cardiomyocytes during a Hypoxia-Normoxia Challenge

Following a hypoxia/normoxia challenge on WT and *Ddah2*^{-/-}, mRNA samples were collected and analyzed during the process to determine the expression pattern of NO regulatory genes alongside with specific cellular and hypoxic markers.

Ddah1 gene expression analysis revealed a small gradual increase during hypoxia/normoxia challenge in WT cardiomyocytes. The same effect was not present in *Ddah2*^{-/-} cardiomyocytes, which showed a steady mRNA expression of *Ddah1* gene throughout the challenge (Figure 63).

Ddah2 gene expression was also monitored in WT and *Ddah2*^{-/-} cardiomyocytes to verify the successful disruption of *Ddah2* gene expression but also to document the expression pattern of *Ddah2* during a

hypoxia/normoxia challenge. Analysis of mRNA levels in WT and *Ddah2*^{-/-} cardiomyocytes proved the successful disruption of *Ddah2* gene expression throughout the challenge (* p<0.05, ** p<0.001) (Figure 63). In WT cardiomyocytes, *Ddah2* mRNA levels gradually increased through hypoxia and normoxia without that increase to reach statistical significance (Figure 63).

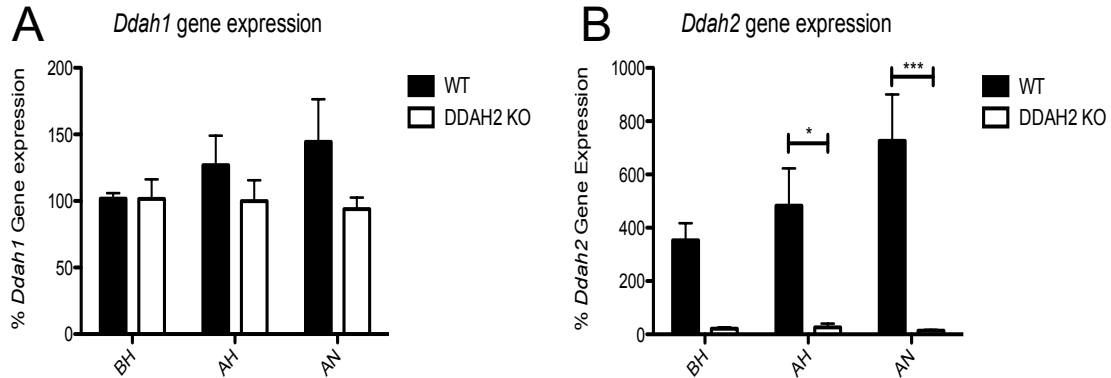


Figure 63. *Ddah1* and *Ddah2* expression analysis of WT and *Ddah2*^{-/-} cardiomyocytes during a hypoxia/normoxia challenge. **A.** *Ddah1* expression pattern analysis showed a small increase in WT compared to *Ddah2*^{-/-} cardiomyocytes. *Ddah2*^{-/-} cardiomyocytes did not change their *Ddah1* mRNA levels throughout the challenge (BH WT (101.766±4.153) vs *Ddah2*^{-/-} (101.577±14.706), AH WT (126.959±22.102) vs *Ddah2*^{-/-} (99.986±15.557), AN WT (144.483±31.898) vs *Ddah2*^{-/-} (93.954±8.672)). **B.** *Ddah2* mRNA level analysis revealed that in *Ddah2*^{-/-} cardiomyocytes expression of *Ddah2* gene was successfully interrupted (BH WT (352.639±64.618) vs *Ddah2*^{-/-} (20.892±5.250), AH WT (483.288±139.268) vs *Ddah2*^{-/-} (26.559±13.512), AN WT (726.362±174.310) vs *Ddah2*^{-/-} (13.615±3.095)). Also, as *Ddah1* gene, *Ddah2* show a trend of slight upregulation during hypoxia/normoxia challenge without reaching significant levels. n=6, Two-way ANOVA, * p<0.05, *** p<0.001.

Following *Ddah1* and *Ddah2* gene expression analysis, *eNOS* and *iNOS* mRNA levels were monitored as the NOS isoforms associated with cardiomyocytes in health (*eNOS*) and inflammation (*iNOS*).

eNOS expression levels were consistently lower in *Ddah2*^{-/-} cardiomyocytes compared to WT throughout the challenge (Figure 64). This difference in *eNOS* expression was consistent but it did not become significant at any timepoint.

iNOS gene followed *eNOS* expression pattern during hypoxia/normoxia challenge in WT and *Ddah2*^{-/-} cardiomyocytes. *iNOS* levels remained lower throughout the process in *Ddah2*^{-/-} cardiomyocytes with a small non-significant upregulation after hypoxia (Figure 64). Expression levels did not change at any point in WT cells.

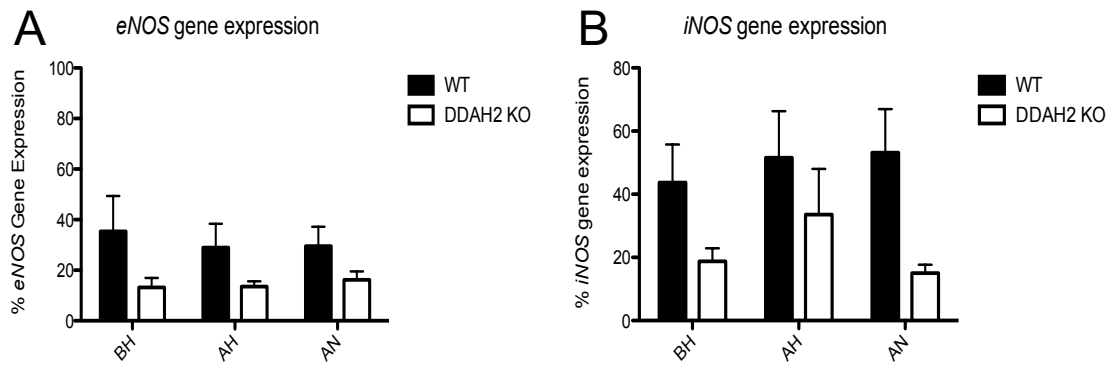


Figure 64. *eNOS* and *iNOS* mRNA levels of WT and *Ddah2*^{-/-} cardiomyocytes during a hypoxia/normoxia challenge. **A.** *eNOS* gene expression revealed no significant difference between WT and *Ddah2*^{-/-} cardiomyocytes even though expression levels of *eNOS* remained in slightly higher levels throughout the challenge (BH WT (35.350±14.063) vs *Ddah2*^{-/-} (13.209±3.722), AH WT (28.924±9.415) vs *Ddah2*^{-/-} (13.524±2.103), AN WT (29.582±7.608) vs *Ddah2*^{-/-} (16.212±3.349)). **B.** *iNOS* gene showed consistently higher levels of expression in WT compared to *Ddah2*^{-/-} cardiomyocytes without reaching significance. After hypoxia, *Ddah2*^{-/-} *iNOS* expression was slightly upregulated but that upregulation was reversed after normoxia treatment for 1 hour (BH WT (43.683±12.099) vs *Ddah2*^{-/-} (18.780±4.125), AH WT (51.589±14.688) vs *Ddah2*^{-/-} (33.597±14.464), AN WT (53.192±13.795) vs *Ddah2*^{-/-} (15.037±2.642)). n=6, Two-way ANOVA, ns.

Cardiac Troponin T (*cTnT*) gene was slightly upregulated after hypoxia and remained at higher levels during normoxia (Figure 65) in WT cardiomyocytes. This upregulation was not present in *Ddah2*^{-/-} cardiomyocytes. After normoxia, there was a difference in expression of *cTnT* between WT and *Ddah2*^{-/-} cardiomyocytes with higher expression levels maintained by WT cardiomyocytes but it was not significant (Figure 65).

CytB, a mitochondria specific marker, was also analyzed during hypoxia/normoxia challenge in WT and *Ddah2*^{-/-} cardiomyocytes. Analysis revealed that gene expression was upregulated by hypoxia and remained in the same levels after normoxia in WT samples when it did not show any changes in *Ddah2*^{-/-} cardiomyocytes (Figure 65). High variation (±200%) in *CytB* mRNA levels especially in WT cardiomyocytes after hypoxia prevented any result from reaching statistical significance.

Finally, *Glut1*, a hypoxia marker was analyzed by qPCR to show an effective induction of hypoxic response by our challenge and also to monitor any expression differences of hypoxic regulating genes. mRNA levels of *Glut1* gene were successfully upregulated in WT cardiomyocytes but there was no change in *Glut1* expression in *Ddah2*^{-/-} (Figure 65). After normoxia, this difference between WT and *Ddah2*^{-/-} gene expression reached significant levels (* p<0.05) (Figure 65).

Overall, WT cardiomyocytes showed a small increase in all analyzed markers except the NOS genes through the hypoxia/normoxia challenge. In contrast, *Ddah2*^{-/-} cardiomyocytes did not upregulate any marker and remained unaffected by hypoxia challenge.

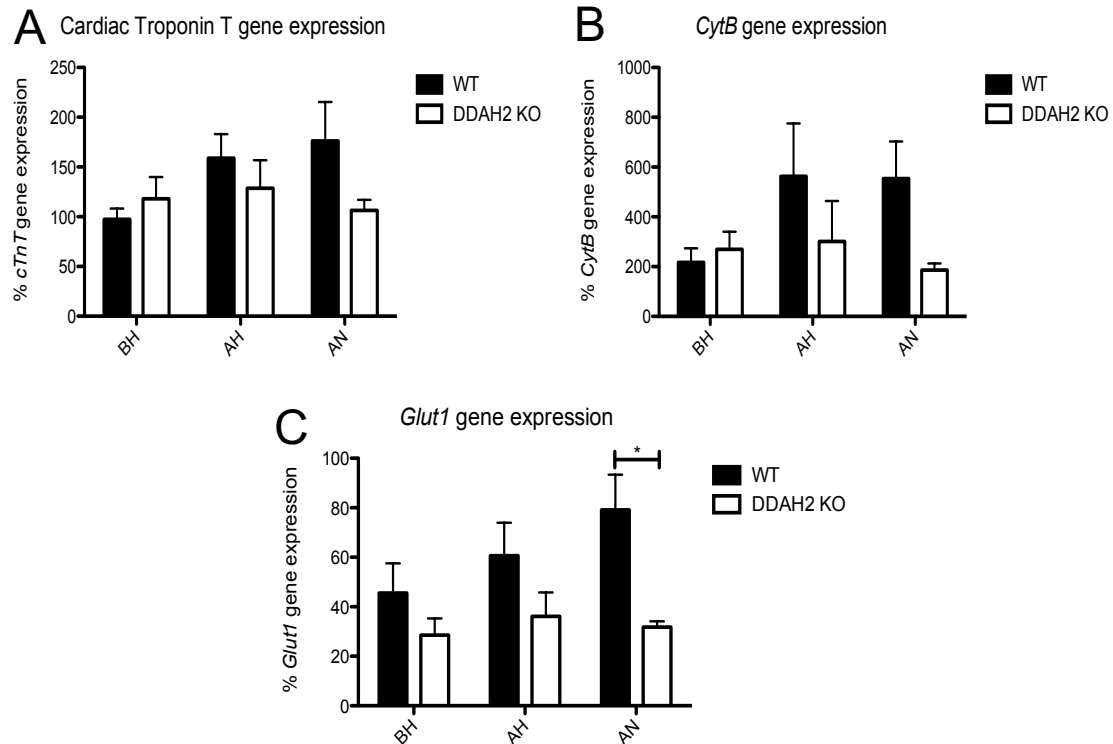


Figure 65. Expression analysis of *cTnT*, *CytB* and *Glut1* by qPCR. **A.** *cTnT* gene expression did not show any significant differences between WT and *Ddah2*^{-/-} (BH WT (97.389±10.827) vs *Ddah2*^{-/-} (118.092±21.868), AH WT (158.876±24.182) vs *Ddah2*^{-/-} (128.630±28.156), AN WT (176.095±39.239) vs *Ddah2*^{-/-} (106.335±10.618)). Expression of *cTnT* (a cardiac specific marker) was gradually upregulated in WT cells but this trend was not followed by the *Ddah2*^{-/-} cardiomyocytes. **B.** *CytB* (a mitochondrial specific marker) showed an initial increase in expression in WT cardiomyocytes after hypoxia, an increase that was maintained after normoxia. *Ddah2*^{-/-} cardiomyocytes showed no changes in their *CytB* expression. Due to high variability in expression between samples, none of the trends were significant (BH WT (216.736±56.915) vs *Ddah2*^{-/-} (269.942±70.774), AH WT (233.953±51.554) vs *Ddah2*^{-/-} (139.791±23.234), AN WT (443.762±124.638) vs *Ddah2*^{-/-} (185.903±27.238)). **C.** *Glut1*, a hypoxia related gene, was gradually upregulated in WT cardiomyocytes but its expression did not change in *Ddah2*^{-/-} even after hypoxia challenge. After normoxia for 1 hour, the difference in expression between WT and *Ddah2*^{-/-} reached significance ($p < 0.05$) (BH WT (45.552±11.984) vs *Ddah2*^{-/-} (28.558±6.705), AH WT (60.663±13.329) vs *Ddah2*^{-/-} (36.116±9.688), AN WT (79.091±14.259) vs *Ddah2*^{-/-} (31.797±2.339)). $n=6$, Two-way ANOVA, * $p < 0.05$.

7.4 Discussion

7.4.1 Expression Pattern of DDAH Isoforms in Isolated Adult Mouse Cardiomyocytes

As suggested by our results, isolated adult mouse cardiomyocytes express only DDAH2 and not DDAH1 isoform on protein level. It has been previously reported that DDAH1 and DDAH2 isoforms can be both expressed in the same cell type but they can also be found independently from each other (Tran, Leiper, & Vallance, 2003; Tran et al., 2000). DDAH2 has been strongly associated with expression to the cardiovascular system being expressed in the heart, the endothelium and macrophages (Lambden et al., 2015; Tran et al., 2000). This is the first time that DDAH2 specific expression in protein levels is verified in adult mouse cardiomyocytes.

The role of DDAH2 in adult mouse cardiomyocytes has not yet been identified and it is interesting is the fact that adult mouse cardiomyocytes do not express DDAH1 isoform. DDAH2 has been attributed the same role as DDAH1, processing ADMA and L-NMMA into L-citrulline and therefore regulating NO production, only due to structural similarities but since DDAH2 has never been isolated and its activity has never been measured in vitro its physiological role remains unknown. Also, the differential expression of the two DDAH isoforms and the potential cardioprotective role of Ddah2 deletion could be a potential pharmacological target. A potential selective inhibitor for Ddah2 could have a cardioprotection role without affecting systems such as the circulatory system and physiological processes such as blood pressure regulation that is highly orchestrated by Ddah1 (Achan et al., 2003; Leiper and Nandi, 2011). These characteristics make Ddah2 a perfect candidate for pharmacological intervention during an ischemia/reperfusion injury.

Further experiments are needed to help us identify the role of DDAH2 in cardiac function. Differentiation of DDAH2^{-/-} embryonic stem cells to cardiomyocytes will help us identify the role of DDAH2 in the development of this particular cell type. Finally, analysis of gene expression between control and DDAH2^{-/-} isolated adult mouse cardiomyocytes will help us identify potential differences in expression of other genes.

7.4.2 Gene Expression Profile of WT and *Ddah2*^{-/-} Adult Mouse Cardiomyocytes during a Hypoxia-Normoxia Challenge

In Chapter 6.3, we demonstrated a possible protective role of *Ddah2* deletion in an ex vivo ischemia/reperfusion model of the adult mouse heart. The protective effect of *Ddah2* deletion was shown with a TTC staining, which is the most widely used method for the detection of metabolically active and inactive tissue but cannot distinguish between different cell types. Given that the most important cell type in the heart is the cardiomyocytes, we isolated WT and *Ddah2*^{-/-} adult mouse cardiomyocytes and used hypoxia treatment in order to investigate the role of *Ddah2* in protection of adult mouse cardiomyocytes during a hypoxia/normoxia challenge.

Ddah2 expression analysis verified that we successfully disrupted the expression of *Ddah2* genes in adult mouse cardiomyocytes. Interesting is the fact that during the hypoxia/normoxia challenge, *Ddah2* expression was significantly upregulated in WT adult mouse cardiomyocytes reaching its maximum levels after normoxia (AN) suggesting the involvement of *DDAH2* in a hypoxia/normoxia response. This is the first time that an upregulation in expression of *Ddah2* gene has been shown during a hypoxia/normoxia challenge in isolated adult cardiomyocytes.

A small non-significant increase in *Ddah1* gene expression in WT cardiomyocytes was observed but it was completely absent from *Ddah2*^{-/-} cardiomyocytes. A significant upregulation in *Ddah1* expression was seen after a period of 24h *in vivo*, which could explain the absence on significance in our results since we only treated our cells for 3h in hypoxia.

In our analysis, we included eNOS and iNOS gene expression but not nNOS due to limited sample availability. eNOS and iNOS expression levels were consistently lower in *Ddah2*^{-/-} cardiomyocytes but due to high variability between samples it was not significant. Due to this high variability, we cannot draw any conclusions about the expression pattern of eNOS and iNOS gene during hypoxia/normoxia challenge.

Analysis of cardiac, mitochondrial and hypoxia specific markers showed a trend for upregulation in WT, which was absent in *Ddah2*^{-/-} cardiomyocytes. *Glut1* was the only gene that was significantly upregulated after normoxia.

These data suggest that *Ddah2*^{-/-} cardiomyocytes do not respond to the hypoxia challenge at least at the mRNA level. This lack of reaction could be either because they do not sense hypoxia or that even when they are in a low oxygen environment they do not need to initiate a hypoxic response due to sufficient oxygen availability. In WT cardiomyocytes, a trend in upregulation of cardiac troponin T and *CytB* could indicate a response to cellular damage caused by low oxygen levels.

DDAH2 has been shown to be involved in VEGF regulation in endothelial cells and potentially to be involved in the hypoxic mechanism (Hasegawa et al., 2006). In our experiments, we attempted to analyze the expression levels of VEGF in WT and *Ddah2*^{-/-} cardiomyocytes. Attempts to identify samples that could give us a standard curve for our qPCR analysis were not successful and in combination with limited sample availability, we were not able to analyze at this point the levels of VEGF expression. The role of *Ddah2* gene in a hypoxic response in cardiomyocytes needs to be identified as it could be used as a target for the development of drugs that could minimize the effect of hypoxia/normoxia on cardiomyocytes after a myocardial infarction.

7.4.3 Comparative Discussion for Chapters 6 and 7

During the analysis and subsequent comparison of mRNA expression data between our *ex vivo* and *in vitro* model of an ischemia/reperfusion injury, we came across an interesting observation analyzing the same gene expression patterns. The same genes showed almost contradicting expression patterns during the same time points (Figure 66). This observation has to be carefully taken into account in our overall comparison of our findings.

First of all, we have to analyze the limitations and characteristics of each one of the two models. In our *ex vivo* experimental model, we analyzed the gene expression of the whole heart containing different cell types in various percentages with the most important one being: cardiomyocytes, endothelial cells and cardiac fibroblasts (Banerjee et al., 2007). Also, our *ex vivo* model is a global ischemia model meaning that for 30 minutes there is no perfusion of the coronary arteries to the heart causing anoxia to the cardiac tissue. After the ischemia period, we have a reperfusion stage, which lasted 45 minutes as it has been shown to be an efficient amount of time to initiate an oxidative response

(Ferrera et al., 2009). On the other hand, our *in vitro* model analyzes the gene expression pattern in isolated adult mouse cardiomyocytes as a single cell type culture not taking into account the effect of other cardiac cell types signaling on gene expression of cardiomyocytes. Also, our *in vitro* system simulates the effect of hypoxia rather than anoxia on cardiomyocyte gene expression since the cells are supplied with 5% O₂ during a three-hour period followed by one hour of normoxia. In the whole isolated heart system, cardiomyocytes are still part of intact myotubes that contract under the complete control of the electrophysiology of the heart. All the cells contract in perfect synchronization during the cardiac cycle. On the other hand, isolated cardiomyocytes, do not show any synchronization in the contraction cycle and all of them do not show a contractile phenotype. Moreover, the type of contraction differs in the two systems as isolated cardiomyocytes have a wave-like contraction that starts on one end of the cardiomyocyte and expands to the other end without a focal point to be identifiable in comparison with the synchronized contraction of myotubes in the whole heart (Engel, Fechner, Sowerby, Finch, & Stier, 1994).

In our isolated adult mouse cardiomyocyte gene expression analysis *Glut1* was upregulated in WT but not in *Ddah2*^{-/-} cardiomyocytes suggesting that *Ddah2*^{-/-} do not sense hypoxia or in low O₂ concentrations they do not need to initiate a hypoxic response in order to survive. In order to identify, if this effect is the reason why *Ddah2*^{-/-} show higher tissue survival after an ischemia/reperfusion injury, we created a cardiomyocyte specific DDAH2 KO mouse model using the Cre recombinase expression under the α -MHC promoter. Due to lack of time, we did not manage to characterize this model and use DDAH2 KO hearts in our *ex vivo* experiments. In our future experiments, isolating DDAH2 KO hearts and combining them with our *ex vivo* model of an ischemia/reperfusion injury will help us identify if the reason behind the higher tissue survival of *Ddah2*^{-/-} hearts is due to the fact that *Ddah2*^{-/-} cardiomyocytes do not sense hypoxia or they can survive in low amounts of oxygen without initiating a hypoxic response.

Isolated Cardiomyocytes

Whole Heart

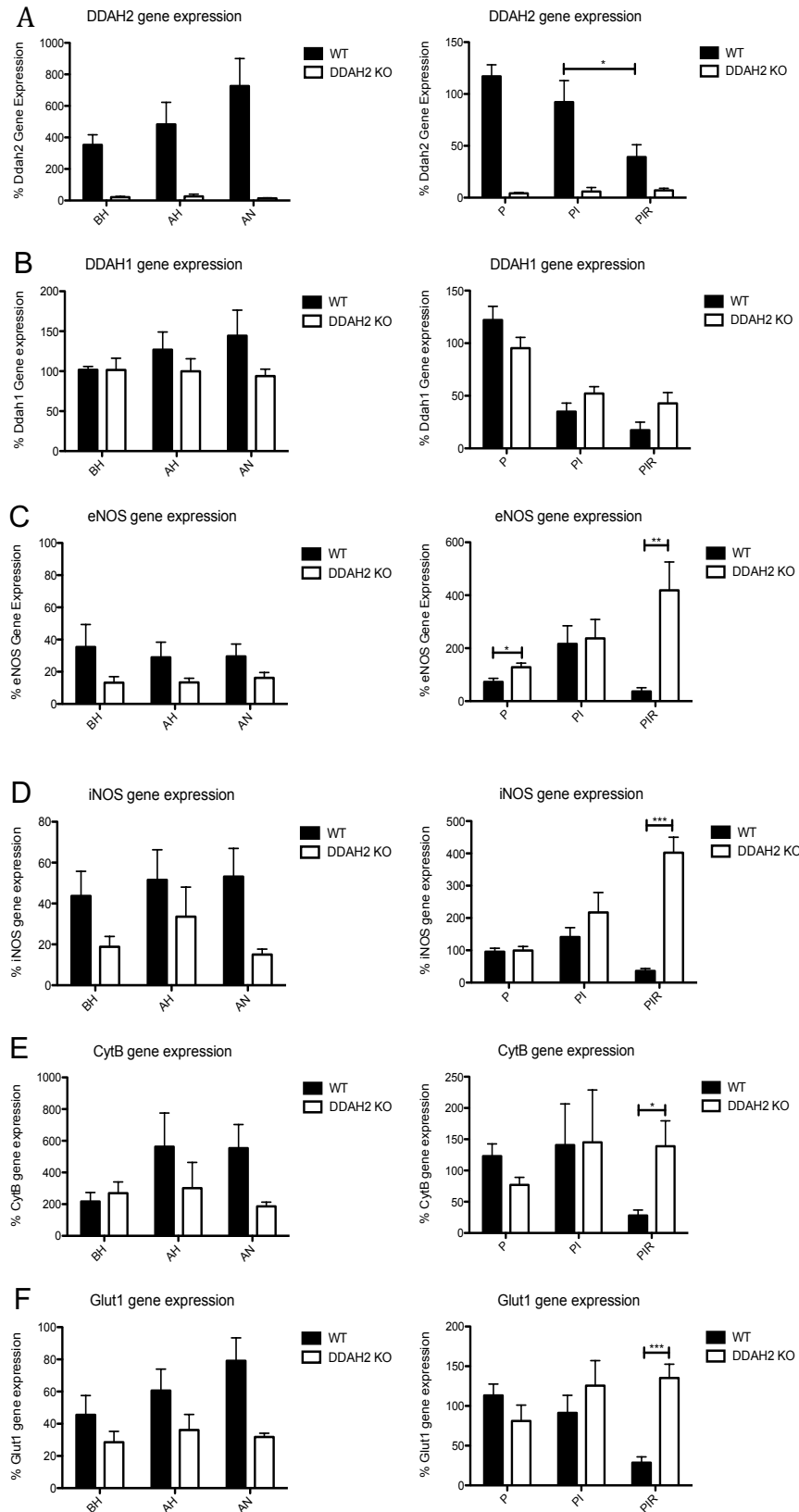


Figure 66. qPCR analysis of various markers in WT and *Ddah2*^{-/-} whole hearts (right column) and isolated cardiomyocytes (left column) during an ischemia/reperfusion injury using an *ex vivo* (whole heart) and an *in vitro* (isolated cardiomyocytes) model. A. Ddah2 gene expression analysis B. Ddah1 gene mRNA levels C. eNOS mRNA analysis D. iNOS gene expression E. CytB mRNA levels and F. Glut1 expression pattern. Whole Heart n=8, Isolated Cardiomyocytes n=6. Two-way ANOVA, * p<0.05, ** p<0.01, *** p<0.001

7.5 Chapter 7 Key Findings

- Isolated adult mouse cardiomyocytes express only the DDAH2 isoform. DDAH1 levels were below detectable levels by Western Blot.
- During a Hypoxia-Reoxygenation challenge, WT isolated adult mouse cardiomyocytes fail to upregulate eNOS and iNOS expression when compared to *Ddah2*^{-/-} cardiomyocytes.
- During a Hypoxia-Reoxygenation challenge, WT isolated adult mouse cardiomyocytes follow a trend to downregulate Glut1 expression, a hypoxia marker, in comparison to *Ddah2*^{-/-} cardiomyocytes that successfully upregulate Glut1 expression.

8. General Discussion

Nitric oxide (NO) signaling has been shown to be involved in regulation of a wide variety of physiological and pathophysiological processes of the cardiovascular system.

During development, NO has been shown to play an important role in cardiac differentiation of embryonic stem cells (Mummery et al., 2003). Our data verified those findings and also suggest that NO level manipulation via ADMA treatment, an endogenous NOS inhibitor, can affect the differentiation fate of mesoderm cells. More specific, high levels of NO during differentiation promote cardiomyocyte differentiation when lower levels of NO promote hematopoietic differentiation and also decrease endothelial and cardiac differentiation.

These results can have a positive impact on both research and clinical applications. In research, a better understanding of mesoderm differentiation could lead to more precise and effective protocols for differentiation of embryonic stem cells. More specifically, in our study we showed that low levels of NO during differentiation affect the levels of GATA4 protein surprisingly though without affecting the transcription levels of the GATA4 gene. This finding will further need to be validated by immunocytochemistry but is a good indicator of a potential role of NO in post-transcriptional regulation of the GATA4 gene either by directly modifying proteins involved in translation or by affecting other pathways that could have an effect on post-translational mechanisms and as a result affecting GATA4 protein levels. Since GATA4 protein is a master regulator and as such is involved in a high number of cellular mechanisms and processes especially during embryonic cardiac development (Durocher et al., 1997; Molkenstein et al., 1997), a better understanding of the effect of NO on post-transcriptional control of GATA4 expression would lead to the discovery of novel pathways involved in cardiac differentiation and as a result better manipulation of those pathways in research (differentiation protocols) but also possible implications of these role in congenital heart diseases in the clinic (Misra et al., 2012; E. Wang et al., 2013). An improvement in differentiation protocols will further improve our understanding about the complexity of the differentiation process and also help us achieve higher numbers of differentiated stem cells for clinical applications such as cardiac regeneration. Finally, our findings could have a clinical application on diseases associated

with the endothelium or the hematopoietic system. Since we identified a potential role of inhibition of NOS enzymes by ADMA and low NO levels in endothelial cell differentiation, this could be beneficial not only in conditions associated with the endothelium but also in identifying mechanisms that promote endothelial cell production. These mechanisms could be implicated in vasculogenesis (Ziche & Morbidelli, 2000), tumor growth in cancer (Chhatwal, Moochhala, Chan, & Ngoi, 1996) and cardiac remodeling following a myocardial infarction (Y.-H. Liu et al., 2005). Finally, supplementation of a NOS inhibitor or an increase in *Ddah1/2* activity by a specific enzyme activator could have a beneficial effect on diseases associated with low hematopoietic potential or damage of the endothelium.

In the adult heart, regulation of NO during an ischemia/reperfusion injury is critical for the protection of the myocardium (Bolli, 2001; Farah & Reboul, 2015; Zaharchuk et al., 1997). Our results show that a treatment with synthetic NOS inhibitors like L-NAME before, during and after an ischemic injury significantly reduces tissue survival of the myocardium. Endogenous NOS inhibitors do not seem to have an effect on tissue survival but can affect coronary circulation. We also showed that *Ddah2* is the only isoform that is expressed in adult mouse cardiomyocytes and we further tested its involvement in cardiac protection during an ischemia/reperfusion injury. Our findings in an *ex vivo* cardiac model using a Langendorff apparatus suggest a cardioprotective role of *Ddah2* deletion with the main effect of the genotype happening during reperfusion. Further analysis using isolated adult cardiomyocytes, showed that *Ddah2*^{-/-} adult cardiomyocytes do not upregulate Glut1, a hypoxia regulated gene, in comparison to WT cells.

Our findings can have a clinical beneficial effect in patients suffering from an ischemia/reperfusion injury. *Ddah2* can be used as a pharmacological target for the development of inhibitors for the protection of the myocardium during an ischemia/reperfusion type of injury. Since we suggest that the major cardioprotective effect of *Ddah2* deletion occurs during the reperfusion phase and we have specifically identified that the majority of cellular damage seems to happen within 20 minutes of reperfusion, it makes a perfect pharmacological candidate for the development of DDAH2 inhibitors that could be administered during the treatment of an ischemia/reperfusion injury at the hospital by specialized staff. More specifically, a potential inhibitor could be administered

prior to recanalization of the obstructed coronary artery minimizing the potential damage to the myocardium caused by reperfusion. This time frame shows a potential therapeutic use since administering a DDAH2 inhibitor to the general population prior to a myocardial infarction is not a viable option.

9. General Conclusions

The aim of this project was to identify the role of NO signaling during cardiac development, function and failure.

Using an embryonic stem cell model treated with ADMA, an endogenous NOS inhibitor, we managed to identify:

1. NO levels can manipulate the differentiation fate of embryonic stem cells.
2. Low levels of NO during differentiation reduce the number of cells that differentiate towards cardiomyocytes.
3. Low levels of NO also reduce endothelial cell differentiation and increase hematopoietic stem cell differentiation.

In adult hearts, deletion of DDAH2 appears to have a cardioprotective role during an ischemia/reperfusion injury.

1. Heart tissue of DDAH2 knockout mice shows better survival after an ischemic injury when compared to WT controls.
2. In the cellular level, DDAH2 KO cardiomyocytes do not upregulate Glut1, a hypoxia marker, suggesting that DDAH2 KO cardiomyocytes do not sense hypoxia or do not need to initiate a hypoxic response in low oxygen levels.

This new evidence further highlight the importance of NO signaling during the development of heart and the hematopoietic system but also during an ischemia/reperfusion injury of the adult heart.

10. Future Experiments

In our experiments, we managed to identify new potential roles for NO signaling during development but also during an ischemia/reperfusion injury of the adult heart. Future experiments, will help us further characterize and understand those roles in order to develop them as pharmacological targets.

Our observations suggest that low NO synthesis limits cardiomyocyte differentiation and affects the differentiation of the hemangioblast. Further experiments, with guided differentiation protocols for cardiomyocyte, hematopoietic and endothelial cell formation will further verify our results and provide more information on how NO signaling is involved in these developmental processes. Also, treatment at specific timepoints will shed light on the developmental point that NO is involved. Finally, treatment with NO donors during differentiation will help us verify the role of NO in development.

At the end of this project, we isolated *Ddah2*^{-/-} embryonic stem cells with their equivalent control, an important tool for the identification of the role of *Ddah2* in development. Differentiation of *Ddah2*^{-/-} embryonic stem cells using protocols of directed differentiation to specific cell types such as cardiomyocytes, macrophages and endothelial cells could give us an insight to the role of *Ddah2* in the development of those cell types.

Since NO has been shown to have a time dependent effect during an ischemia/reperfusion injury (Farah & Reboul, 2015), it will be particularly helpful to explore the role of inhibition of the different NOS enzymes before and after an ischemic insult. Perfusion of adult mouse hearts with NOS inhibitors and possible NO-donors before and after ischemia, could verify which of the NOS isoforms are involved in cardioprotection, via which pathway and at which time-point of the injury.

In our experiments, for the first time we demonstrated a cardioprotective effect of *Ddah2* deletion during an ischemia/reperfusion injury and further experimental work will help us verify our results and establish the pathway of action. First of all, it will be essential in our understanding of the mechanism to analyze the P-Akt levels as it will help us relate our findings to other already established cardioprotective mechanisms. Furthermore, in order to identify novel mechanisms that might be involved in the propagation of the *Ddah2* deletion in cardioprotection it will be useful to perform an RNA-sequencing

experiment on WT and *Ddah2*^{-/-} hearts that have been undergone an ischemia/reperfusion injury.

Finally, it will be particularly beneficial since it is the first time that *Ddah2* inhibition during an ischemia/reperfusion challenge is proposed as a potential pharmacological target to identify DDAH2 inhibitors to be used during reperfusion. In our lab, we have already identified a DDAH1 specific inhibitor, L-257, and we have an array of structure-based inhibitors that could be used as DDAH2 specific inhibitors. WT heart perfusions with the stronger candidates will help us identify a beneficial effect on WT hearts during an ischemia/reperfusion injury and a potential pharmacological agent.

11. References

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