TECHNISCHE UNIVERSITÄT DARMSTADT

THE ROLE OF INDUCIBLE NITRIC OXIDE SYNTHASE (INOS) IN HUMAN MYOCARDIAL INFARCTION

Vom Fachbereich Biologie der Technischen Universität Darmstadt

zur Erlangung des akademischen Grades

eines Doctor rerum naturalium (Dr. rer. nat.)

genehmigte Dissertation von

Verena Wilmes, M.Sc.

aus Bad Soden a. Ts

Erstgutachter: Herr Prof. Dr. Gerhard Thiel

Zweitgutachterin: Frau Prof. Dr. Silke Kauferstein

Darmstadt 2023

Diese Arbeit ist Kornelia Weber gewidmet,

ohne die ich kein biologisches Studium begonnen hätte.

TABLE OF CONTENTS

INTRODUCTION

1.1 Myocardial Infarction

According to the World Health Organization (WHO), cardiovascular diseases (CVD) are the leading cause of death globally. CVDs include disorders of the heart and its blood vessels, such as myocardial infarction (MI). MI is the result of insufficient blood supply to areas of the myocardium [1,2] and causes necrosis of myocardial cells. Death of cardiac cells is followed by inflammation, which leads to fibrosis [3]. Adverse remodeling and cardiac scarring negatively impact myocardial healing and may lead to further disease states, such as hypertension, heart failure, and death [1] [3]. Sudden cardiac death (SCD) after MI accounts for 50% of overall mortality [4] and is attributed to recurrent MI, cardiac rupture or pump failure within one month of index MI. SCD that occurs after three months of index MI is attributed to arrhythmia [5].

Most patients with MI present with typical chest pain/chest discomfort, however compared with men, women are less likely to manifest central chest pain. Women with MI suffer more frequently from pain in the upper back, arm, neck and jaw, as well as from nausea/vomiting, palpitations and an unsual fatigue [5].

Elevated levels of cardiac-specific troponins (cTnT and cTnI) in peripheral blood, as well as the cardiac specific creatine kinase (CK-MB) are central biomarkers for the diagnosis of MI [6,7]. As biomarkers for adverse cardiac remodelling after MI with subsequent heart failure, interleukins, natriuretic peptides, matrix metallopeptidases (MMPs) and noncoding RNAs, such as microRNAs (miRNAs) are discussed [7].

Since MI has a high mortality rate and leads to further fatal disease states, a thorough understanding of the molecular processes during MI is important for developing improved therapy options and biomarkers for further disease states.

1.2 Nitric oxide synthase enzymes (NOS)

Neuronal nitric oxide synthase (nNOS), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) are the three NOS isoforms that produce nitric oxide (NO) [8]. nNOS and eNOS are calcium-dependent enzymes and produce nanomolar concentrations of NO for seconds or minutes [8,9], whereas iNOS is a calcium-independent enzyme and generates micromolar amounts of NO for hours or days upon stimulation [8]. eNOS is localized within the caveolae and expressed in numerous cell types, such as vascular smooth muscle cells, where it functions as a homeostatic controller of cardiovascular functions. eNOS is thought to protect against CVD [10–12]. nNOS localization is described at the mitochondria, the sarcolemma and the sarcoplasmatic reticulum. In resting cardiac myocytes nNOS is found at the sarcoplasmatic reticulum, while in disease states it is

translocated to the mitochondria and sarcolemma. It is thought to have a cardioprotective role in different CVD [13].

While eNOS and nNOS are constitutively expressed, iNOS is absent in healthy tissues/states and expressed upon stimuli such as hypoxia or inflammation, which leads to the generation of high amounts of NO by iNOS [8,14,15].

1.3 iNOS and oxidative nitrosative stress

The iNOS gene (*NOS2*) is located on human chromosome 17q11.2-12. It comprises 26 exons and 25 introns and is regulated at different levels [16,17]. The gene product catalyzes the oxidation of L-arginine and O_2 into L-citrulline and NO where NO is produced from the terminal guanido nitrogen of L-arginine [18–20].

NO functions as an antimicrobial agent in the immune system [18,21] and is thought to have a protective role against severe malaria [22]. However, iNOS derived NO is also associated with pathologies, such as cancer [18,23,24], COPD [25] and CVD [14] where it is thought to have dual roles.

At low concentrations NO produced from iNOS plays crucial roles in vasorelaxation and blood flow regulation, platelet activity, cell survival and proliferation, as well as in the immune and neurological system [8,19,21]. At high concentrations NO can be cytotoxic and apoptosis inducing [8,26]. In cancer low NO concentrations seem to favour tumorigenesis and progression, while high concentrations produce antitumor activity, still NO produced from iNOS has pro- and anti-tumoral effects, depending on dose-, time- and compartment [8,18,23,24]. High levels of iNOS in human breast cancer tissues predict increased tumor progression and a poor outcome of survival [18]. Hence, the dual role of iNOS in cancer.

Further, NO has indirect effects via NO-derived species, which are generated by reactions of NO with O_2 or superoxide anion (O_2^-). The reaction of NO with O_2^- leads to reactive intermediates that can nitrate, nitrosate or oxidize their biological environment. This oxidative nitrosative stress promotes DNA damage, suppresion of DNA-repair enzymes and post-translational modifications of proteins. Further, NO influences several epigenetic regulators, therefor enabling dysregulation of DNA methylation and acetylation, which promotes inflammation, genomic instability and carcinogenesis. Genes controlling cell growth and DNA repair are prime targets for NO and oxidative nitrosative stress [18,19,24]. While induced oxidative stress at high concentrations by iNOS and NO has the potential to diminish tumor cells via cytotoxic conditions it poses a threat to healthy tissues [23].

1.4 iNOS in CVD

Increased iNOS expression is suggested to play a rather harmful role in several CVDs. In experimental models of sepsis, iNOS inhibition has been implicated in the restoration of blood pressure [27,28] and

overexpression of iNOS in cardiomyocytes of transgenic mice has been associated with and suggested to cause bradyarrythmia, cardiomyopathy and SCD [29]. In atherosclerotic lesions iNOS is expressed, leading to the reaction of NO with O_2^- and thereby causing oxidative nitrosative stress which leads to cellular damage and inflammation [14,18]. Further excessive production of NO contributes to contractile dysfunction, which plays a role in chronic heart failure [30]. In patients with end-stage heart failure, cardiomyopathy and ischemic heart disease increased expression of iNOS and increased oxidative nitrosative stress has been found in the heart [31].

In murine and rabbit models of MI increased iNOS expression has been described and is suggested to contribute to left ventricular dysfunction, heart failure progression, myocardial injury and extent of infarct size, even late after ischemia and reperfusion [31–35]. Further survival after MI in $iNOS^{-/-}$ mutant mice was increased compared to wildtype mice [9]. Li et al. [36] showed that loss of transplanted rat stem cells in MI was partially due to increased apoptosis and iNOS overexpression and early iNOS inhibition in MI may increase cell survival. Increased iNOS expression after MI in murine models was also described in the non-infarcted myocardium [9,36].

Despite this dogma, some researchers suggest iNOS having a cardioprotective mechanism in pathological conditions [14]. During ischemic preconditioning (IPC) where tolerance of the heart is increased by a milder stressor, iNOS has been suggested of having a cardioprotective role [37]. Likewise, several studies found that the absence or inhibition of iNOS does not improve pathology in CVD and in some cases even worsens it [38,39].

In human MI little to no research has been conducted to clarify the role of iNOS.

1.5 Aim of this work

iNOS is discussed as a potentially detrimental enzyme in several diseases, including CVD and more specifically MI. However, most studies identifying the role of iNOS in MI have been conducted in murine models, where coronary artery ligation was performed and iNOS expression was monitored in the days following MI. Hence, in human MI no data are available for iNOS expression, the cell expression pattern of iNOS or regulation of iNOS expression, besides transcription factors. Further, in murine models of MI, the heterogeneity of human MI, such as acute infarction, re-infarction and old infarction, cannot be addressed. This leads to the question, whether the findings of iNOS involved in molecular processes during murine MI is equally transferable to human MI. Since iNOS may be a potential target for therapy options, may lead to a more thorough understanding of the pathophysiology of MI and to development of biomarkers for the progression of heart failure after MI, examining the role of iNOS in human MI is important.

Therefore, the aim of this work was to examine the iNOS expression in heart tissue of human MI and the cell expression pattern of iNOS. Further, oxidative nitrosative stress caused by increased iNOS expression was measured in human MI tissue. Additionally, regulators of iNOS gene expression, such

as miRNAs and genetic polymorphisms in the *NOS2* gene were analyzed, and potential markers of an increased iNOS expression in serum and urine were examined.

The results of this work may contribute to a better understanding of the role of iNOS in human MI and may lead to further studies and improved therapy options for the leading cause of death globally.

PART 1 INOS MRNA EXPRESSION AND CELL EXPRESSION PATTERN IN HUMAN MYOCARDIAL INFARCTION

Parts of this chapter have been published in Frontiers in Cardiovascular Medicine (Wilmes et al., 2023 [40]).

2.1 Abstract

Inducible nitric oxide synthase (iNOS) produces micromolar amounts of nitric oxide (NO) upon the right stimuli, whose further reactions can lead to oxidative stress. In murine models of myocardial infarction (MI), iNOS is known to be expressed in infiltrating macrophages, which at early onset enter the infarcted zone and are associated with inflammation. In contrast cardiac tissue resident macrophages are thought to enhance regeneration of tissue injury and re-establish homeostasis. Both detrimental and beneficial effects of iNOS have been described, still the role of iNOS in MI is not fully understood. Our aim was to examine cell expression patterns of iNOS and nitrotyrosine (NT) production in human MI.

We examined in postmortem human MI hearts the iNOS mRNA expression by means of qPCR. Further we performed immunohistochemical stainings for cell type identification. Afterwards a distance analysis between iNOS and NT was carried out to determine causality between iNOS and NT production.

iNOS mRNA expression was significantly increased in infarcted regions of human MI hearts and iNOS protein was detected in resident macrophages in infarcted human hearts as well as in controls hearts, being higher in resident macrophages in MI hearts compared to control. Furthermore, in MI and in healthy human hearts the number of cells showing signs of NT production peaked within 10-15 µm proximity of iNOS+ cells.

These results indicate that, unexpectedly, resident macrophages are the main source of iNOS expression in postmortem human MI hearts. The peak of NT positive cells within 10-15 µm of iNOS+ cells suggest an iNOS dependent level of NT and therefore iNOS dependent oxidative stress.

Our results contribute to understanding the role of iNOS in human MI.

2.2 Introduction

So far the role of iNOS in MI has been exclusively studied in murine and rabbit models of MI [31–35]. Even though, iNOS is discussed as having dual roles in CVD, the detrimental effects of iNOS derived oxidative nitrosative stress are well known [18,19,24,37–39]. Specifically, peroxynitrite ($ONOO^-$) is formed by the reaction of NO with O_2^-. Peroxynitrite is a strong oxidant causing oxidative nitrosative stress by lipid peroxidation and nitrosylation of proteins, which has detrimental effects, e.g. to

cardiomyocytes [8,14,19,31,41]. Due to the burst of NO produced by iNOS, NO outcompetes the reaction of superoxide dismutase for O_2^-, hence NO is a key regulator of peroxynitrite formation [42]. In lung tissue iNOS derived NO and peroxynitrite formation may promote alveolar destruction and emphysema [43]. Further peroxynitrite inactivates anti-proteases and activates matrix metallopeptidases (MMPs), which are known for degrading and remodeling extracellular matrix under physiological and pathological conditions [44,45]. After MI, the loss of myocardial extracellular matrix, driven by MMPs, is one of the determinants of left ventricular remodeling [46].

In human MI no data are available regarding iNOS derived oxidative nitrosative stress. However, Turillazzi et al. [47] found an increase of iNOS protein and nitrotyrosine (NT) the fingerprint of peroxynitrite in tissues, and subsequent oxidative nitrosative stress in human hearts of cocaine bodypackers, who died following the consumption of lethal doses of cocaine. Implicating an additional mechanism of acute cardiac injury by iNOS and subsequent oxidative nitrosative stress. Further, Frustaci et al. [48] reported increased iNOS and NT expression in endomyocardial tissue of cocaine-related cardiomyopathy in humans, compared to dilated cardiomyopathy and normal controls, suggesting oxidative nitrosative stress as a major mechanism of myocardial damage.

Further the cell expression pattern of iNOS has been predominantly studied in murine and rabbit models of MI and in these models, iNOS seems to be expressed in cardiomyocytes and macrophages [9,26,31,36,49]. In the human heart different subsets of macrophages are present [50–53], which are roughly divided into infiltrating “M1” (CD68+, CD206-, CD163-) and resident “M2” macrophages (CD68+, CD206+, CD163+), with CD68 being an antigen expressed by both subsets. Prenatally yolk-sac-derived tissue-resident macrophages regenerate themselves locally without contribution of blood monocytes. These resident cardiac macrophages express a large amount of the “M2-designated” markers [52]. The functions of those tissue-resident macrophages differ and depend mainly on their microenvironment. However, it is known that they enhance regeneration after tissue injury and re-establish homeostasis [54].

“M1” infiltrating macrophages, which comprise blood derived monocytes, express numerous pro-inflammatory markers [52] and are known to express iNOS in mice [50]. Infiltrating macrophages enter the infarcted zone in the initial stage of MI for clearance of dead cells and matrix debris and cause a gradual enlargement of the infarcted zone of MI hearts [52]. Even though inflammation is required for clearance of dead cells and tissue regeneration, an exaggerated inflammatory reaction can delay the healing process of the myocardium [52]. iNOS is classically viewed as a marker of infiltrating macrophages [50,51], however, the limitations of this nomenclature are described [25,51,55].

In human MI hearts Shimojo et al. [56] approached to describe the cellular expression pattern of iNOS, but except for this attempt no detailed examination of the iNOS cell expression pattern in human MI hearts, has been conducted.

In the present work, the iNOS gene expression in infarcted myocardium, non-infarcted myocardium and control hearts was examined. Furthermore, detection of NT as the fingerprint of peroxynitrite in tissue, was evaluated and a distance analysis between iNOS and NT performed, to gain an understanding of the role of iNOS derived oxidative nitrosative stress in human myocardial infarction.

2.3 Material and Methods

2.3.1 Samples

Cardiac tissue samples from 39 deceased individuals were collected during court ordered autopsies at the Institute of Legal Medicine, University Hospital, Goethe University Frankfurt, Germany. The control group consisted of 12 microscopically healthy, unharmed hearts from overall healthy individuals who died from accidents or committed suicide. Therefore, only individuals who died a non-natural death were included. The MI group consisted of hearts with macroscopically visible signs of cardiac infarction. All samples were histologically revised by an experienced pathologist and RNA was extracted. From the infarction zone of the MI hearts, two replicates were taken, either from posterior or anterior wall, depending on the infarct localization. Additionally, two samples from the macroscopically unaffected wall of the MI hearts and two from the anterior and posterior wall of control hearts were taken. Variations within myocardial tissue and the heterogeneity of the infarctions were considered by taking more than one tissue sample. All samples were obtained from the left ventricle.

The MI and the control group are shown in Table 1 and 2. The MI group was further divided into acute infarction, old infarction (months or years after index MI), re-infarction and subacute infarction (days or weeks after index MI), depending on the age of infarction.

Table 1: Gender, mean Age (years) and mean postmortem interval (PMI, days) of the myocardial infarction group.

Gender **Male/Female**	**Age**	**PMI**
29/10	68	7

Table 2: Gender, mean Age (years) and mean postmortem interval (PMI) of the control group.

Gender **Male/Female**	**Age**	**PMI**
9/3	43	5

2.3.2 Immunhistological (IHC) examinations of iNOS, NT and CD68

Tissue samples were fixed in 4.5 % buffered formalin and embedded in paraffin. Sections of 5 µm were deparaffinized with xylol and graded alcohol after heating for 30 minutes at 60°C. Blocking of endogenous peroxidase was applied by incubation with 3% hydrogen peroxidase for 15 minutes. For antigen retrieval and to increase cell permeability for the antibody, pre-treatment was applied. Therefore, the samples were boiled in citratebuffer (0,1 M, pH = 6) three times for 1 minute with 5 minutes breaks in between. Subsequently slides were incubated with 20% goat normal serum for 20 minutes at room temperature for blocking. Incubation with primary antibody anti-iNOS (NOS2 mouse monoclonal, Santa Cruz, CA, USA) diluted 1:50 in phosphate buffered saline (PBS) was applied over night at 4-8°C. Afterwards, the samples were incubated at room temperature for 30 minutes and washed twice with PBS. The utilized detection system was the LSAB2 system-HRP kit (Dako, Copenhagen, Denmark) an avidin-biotin technique in which the biotinylated secondary antibody reacts with several peroxidase-conjugated streptavidin molecules. The biotinylated secondary antibody was applied for 30 min at room temperature. After washing wish PBS twice, the samples were incubated with horseradish peroxidase (HRP) for 30 min at room temperature und washed twice again with PBS. Afterwards slides were incubated with AEC (Dako, Copenhagen, Denmark) for 15 minutes and washed with PBS. The samples were counterstained with haematoxylin for 3 minutes, dehydrated, cover slipped and examined with a transmission light microscope (Zeiss, Jena, Germany).

For NT staining, samples were deparaffinized at 60°C for 30 minutes and for antigen retrieval boiled for 40 minutes in EnVisionTM Flex Target Retrieval Solution Low pH (Dako, Copenhagen, Denmark). The primary antibody anti-nitrotyrosine (nitrotyrosine mouse monoclonal, Santa Cruz, CA, USA) was diluted 1:400 in antibody diluent (Dako, Copenhagen, Denmark) and applied on the samples for 30 minutes. Slides were washed with tris buffered saline (TBS) and the subsequently utilized detection system was the EnVisionTM Flex (Dako, Copenhagen, Denmark). Samples were incubated with hydrogen peroxidase for 5 minutes and washed with TBS. HRP was applied for 20 minutes and samples were washed in TBS again. Incubation with DAB (Dako, Copenhagen, Denmark), followed for 10 minutes. Every incubation step was applied at room temperature. After counterstaining with haematoxylin, samples were dehydrated, cover slipped and examined with a transmission light microscope (Zeiss, Jena, Germany).

For staining of CD68, samples were deparaffinized in a two-phase dewaxing procedure using ClearifyTM Clearing Agent (Dako, Copenhagen, Denmark). Antigen retrieval was applied using EnVisionTM Flex Target Retrieval Solution High pH at 97°C for 20 minutes. After incubation with washing buffer of the EnVision FlexTM Kit (Dako, Copenhagen, Denmark) for 2 minutes, the primary antibody CD68 KP1 (EnVision FlexTM mouse monoclonal, Dako, Copenhagen, Denmark) was applied for 20 minutes. After another washing step for 2 minutes, slides were incubated with

peroxidase for 3 minutes. Subsequently samples were washed again for 2 minutes and incubated for 20 minutes with HRP. After several rounds of washing, samples were incubated 5 minutes with DAB (Dako, Copenhagen, Denmark). After counterstaining with haematoxylin, samples were dehydrated, cover slipped and examined with a transmission light microscope (Zeiss, Jena, Germany).

For semiquantitative analysis of iNOS, NT and CD68 staining, samples were scored in a double-blind manner. The intensity of immunopositive signals was assessed on a scale of 0-3 as follows: only perivascular staining (1), perivascular and isolated tissue staining (2) and pronounced perivascular and tissue staining (3). Quantity of staining was indicated by: + (weak), normal (++) and strong (+++). For analysis of nitrotyrosine, staining intensity was evaluated only as quantity.

2.3.3 Tissue preparation and immunostaining with BOND-RX Multiplex IHC Stainer

Tissue sections were deparaffinized by 1 hour incubation at 60°C. Tissue sections (5 µm) were stained with Opal 7-Color Automation IHC Kits (Akoya Bioscience) in the BOND-RX Multiplex IHC Stainer (Leica). The first step of the automated IHC included a fixation step with 4% Formalin (Roth, P087.5) in PBS. Each section was put through 6 sequential rounds of staining, which included blocking in 5% BSA, followed by incubation with primary antibodies of the following panel (NT, Santa Cruz, sc-32757; NOS2, Santa Cruz, sc-7271; CD163, Abcam, ab182422; CD206, Cell signaling, 91992S; CD68, DAKO, M0876), corresponding secondary HRP-conjugated antibodies and Opal fluorophores as described. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) contained in the Opal 7-Color Automation IHC Kits, and slides were mounted with Fluoromount-G (SouthernBiotech).

2.3.4 Microscopy and image analysis

Imaging was performed with the VectraPolaris imaging system (Akoya Bioscience), and images were analyzed by using the phenotyping application of the inForm software V2.4.10 (Akoya Bioscience). Phenotype classification was performed automatically by the written algorithm using inform Software. Multispectral image analysis was performed with Phenochart, InForm Image analysis software (Akoya Biosciences Inc.), and HALO software (Indica labs). For distance analysis in HALO software 5 slides of the infarcted regions, 5 slides of the non-infarcted regions and 5 healthy controls were examined, calculating proximity distances from one population to the other and vice-versa in a delimited radius range.

2.3.5 Reverse transcription quantitative polymerase chain reaction

RNA extraction, cDNA synthesis and quantitative real-time PCR (qPCR) was carried out as previously described [57]. One hundred milligrams of cardiac tissue were mechanically homogenized in 1 mL Trizol. Genomic DNA digestion was performed and subsequently extracted RNA samples

were purified. For synthesizing single stranded cDNA random the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) was used according to the manufactures protocol. The priming conditions were random. The Thermocycler program is described in table 3.

Table 3: Thermocycler program for cDNA synthesis.

Step	Temperatur (°C)	Duration (h)
1	25	00:10
2	37	02:00
3	85	00:05
4	4	Stop

qPCR was performed using the following Taq Man® Gene Expression Assays: inducible nitric oxide synthase (NOS2, amplicon size 67 bp, assay ID: Hs01075529_m1, refseq.-nr.: NM_000625.4). Peptidylprolyl isomerase A (PPIA, amplicon size 98 bp, assay ID: Hs99999904_m1, refseq.-nr.: NM_021130.4), TATA-box binding protein (TBP, amplicon size 91 bp, assay ID: Hs00427620_m1, refseq.-nr.: NM_003194.4) and tumor protein translationally controlled 1 (TPT1, amplicon size 131 bp, assay ID: Hs02621289_g1, refseq.-nr.: NM_003295.3) (Applied Biosystems, Darmstadt, Germany) were used as endogenous reference genes due to their postmortem stability and their stable expression in cardiac tissue, as previously described [58,59]. For each sample triplicates were applied as well as no template negative controls for each assay. The qPCR reaction mix as well as the cycler program are described in tables 4 and 5.

Table 4: Reaction mix in the qPCR per each well.

Reagent	Volume in µl
Maxima Probe/ ROX qPCR Master Mix	5
TaqMan-Gene Expression Assay	0,5
cDNA	1-4,5 (200ng)
Nuclease free water	Ad 10

Table 5: Program of the qPCR.

Step	Temperature (°C)	Duration (min)
1	50	02:00
2	95	10:00
3	85	00:15
4	60	01:00 (3-4 40x)

Analysing the qPCR efficiency was performed by using LinReg PCR Software [60]. The qPCR was efficient when the values were between 1,8 and 2,0. Values lower than 1,8 indicate an inadequate efficacy and were excluded. Efficacy values per heart assay were determined and used to correct the corresponding Cq values.
For qPCR 200 ng cDNA were used. After reverse transcription cDNA concentrations were not measured, since the reverse transcription ratio of RNA:cDNA is 1:1.

2.3.6 Data analysis

qPCR was carried out by using the Software 7500, version 2.0.6 and Data Assist v3.01 served for analyzing the qPCR raw data. Calculation of fold change was carried out using the ΔΔCq method [61]. The resulting fold change is the relative gene expression in comparison to a calibrator sample. As calibrator sample a control heart was chosen, reflecting most closely the mean age (43 years) of the control group and the dominant gender (male) in control and infarction group. All samples of infarction and control group were normalized against this calibrator sample.

ΔCq = average Cq of the triplicates of the gene of interest – normalization factor of the endogenous references.
ΔΔCq = ΔCq of the gene of interest – ΔCq of the calibrator sample
2^-ΔΔCq = 2^-(ΔCq of gene of interest – ΔCq of the calibrator sample)

The software R version 3.5.1 was used for statistical analysis. Linear mixed effects model and pairwise post hoc comparisons were applied and significance was adjusted, by using Tukey's multiple comparisons test. Two individual measurements of the unaffected and the affected regions of MI hearts were included in the statistical analysis to detect local differences in the gene expression per heart and two of the anterior and posterior wall for each control heart.
For statistical evaluation of different macrophage populations Mann-Whitney tests were applied.

2.4 Results

2.4.1 Sample Selection

Histological examinations confirmed the macroscopic pathological findings of MI. Acute, subacute infarctions or scar areas were present, either focal or diffuse. The non-infarcted areas revealed no cellular changes and no obvious cardiomyocyte injuries. In some cases, mild diffuse or localized scaring after older myocardial hypoxia was observed. The control group did not show any histopathological cardiac changes.

2.4.2 Increased iNOS mRNA expression in MI hearts

iNOS mRNA expression in control hearts, as well as in non-affected and affected regions of MI hearts is shown in Figure 1. In infarcted regions (p = 0,001) and in non-infarcted regions (p = 0,02) the iNOS expression was significantly increased in comparison to the control group.

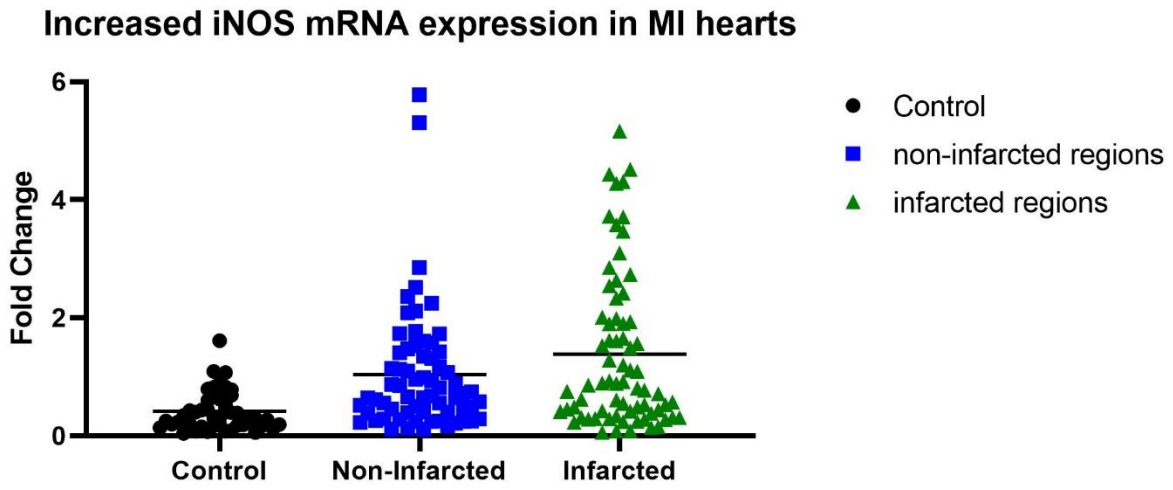


Figure 1: iNOS mRNA expression in controls, non-infarcted and infarcted regions. iNOS mRNA expression level in control hearts (n=12), in non-infarcted (n=33) and in infarcted (n=37) regions of MI hearts were determined by qPCR. Individual values were grouped and plotted and show significant upregulation of iNOS expression in non-infarcted (p = 0,02) and infarcted regions (p = 0,001) in comparison to controls. Horizontal bars display the mean Fold Change in each group. Linear mixed effects model was applied and significance was adjusted after normality by using Tukey's multiple comparisons test.

When separating the infarction group according to the age of infarction, the strongest iNOS mRNA expression was found in acute MI hearts (data not shown). The ratio of male infarction and control hearts to female infarction and control hearts was approximately 3:1. No difference in iNOS mRNA expression was found between male and female hearts (data not shown).

2.4.3 Increased protein levels of iNOS, Nitrotyrosine (NT) and CD68+ macrophages in MI hearts

In Table 6 the mean IHC staining intensities for iNOS, CD68 and NT in each group are shown. The iNOS protein level was strongly increased in non-infarcted and infarcted areas in comparison to healthy control myocardium. NT was also strongly visible in non-infarcted and infarcted areas in comparison to control tissue. No difference in NT staining was found between acute and older infarctions.

Table 6: Mean immunhistological (IHC) staining intensities for iNOS, CD68 and NT in the controls, non-infarcted and infarcted regions. The intensity of immunopositivity was assessed on a scale of 0-3 as follows:

only perivascular staining (1), perivascular and isolated tissue staining (2) and pronounced perivascular and tissue staining (3). Quantity of staining was indicated by: + (weak), normal (++) and strong (+++).

	Control group	Non-infarcted areas	Infarction areas
iNOS	1++	2+	2++
CD68	1++	2+++	2+++
NT	+	++	++

2.4.4 CD68+ macrophages produce iNOS in human MI hearts

Next, the cellular expression pattern of iNOS was evaluated by comparison of CD68+ and iNOS expressing cells. In control hearts iNOS protein and CD68+ macrophages were restricted to the perivascular tissue and only rarely visible within the myocardium. In the infarcted and non-infarcted regions, an upregulation of iNOS protein expression and an increase of CD68+ macrophages in comparison to healthy control tissue was observed, indicating an accumulation of both in infarcted hearts (Table 6). Furthermore, the CD68+ macrophages were detected as the main iNOS expressing cells in human MI hearts, in the infarcted, as well as the non-infarcted areas (Figure 2). However, more CD68+ cells than iNOS expressing cells were observed (Table 6).

Of note, in a few non-infarcted regions, where microscopical examinations revealed no cellular changes, only acute hypoxia or smallest fibrotic changes, a strong increase in iNOS and CD68 protein levels was observed (Table 6).

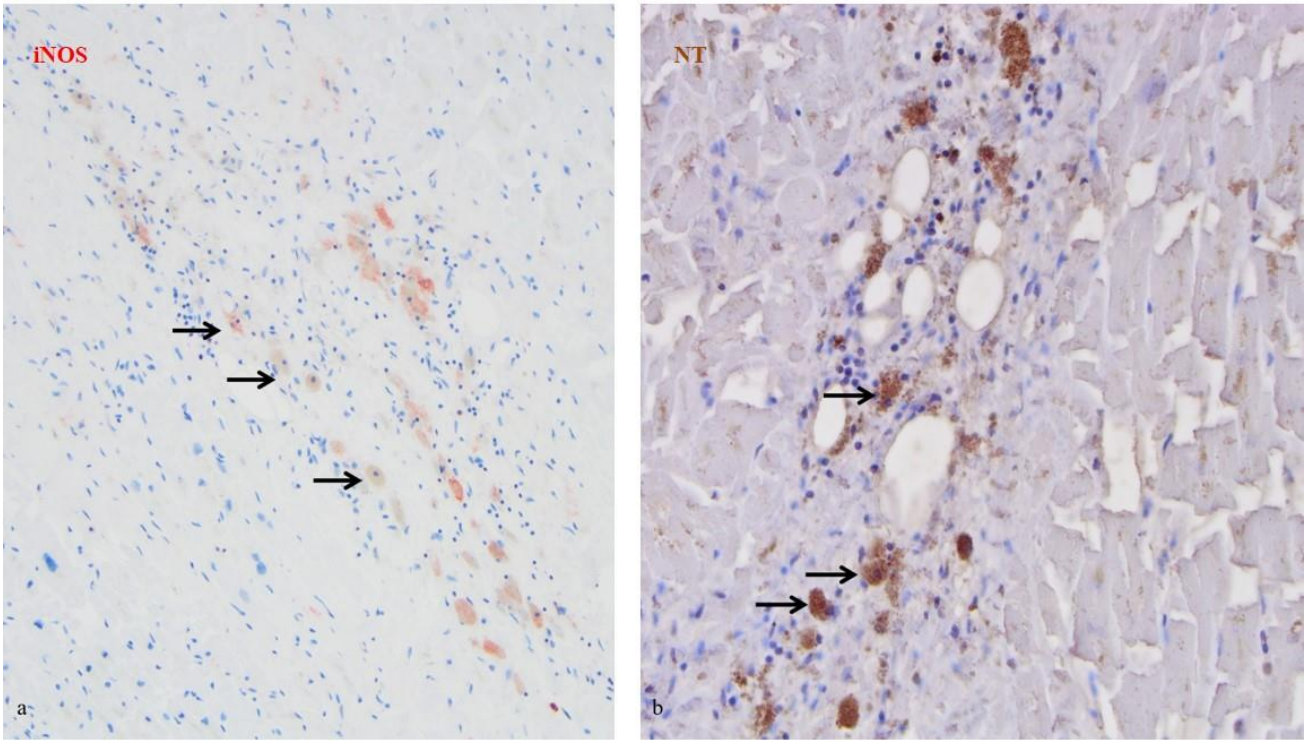


Figure 2: iNOS and NT localization by CD68+ macrophages in infarcted regions. a) iNOS expression by macrophages in the acutely infarcted tissue, as indicated by the arrows (red, 20x). b) NT expression by macrophages in the acutely infarcted tissue, as indicated by the arrows (brown, 20x). Cell nuclei were counterstained with haematoxylin.

2.4.5 The presence of iNOS and NT in macrophages indicates oxidative stress in MI hearts

In the infarcted and non-infarcted areas, a strong NT staining was detected, while in control hearts no or only weak staining of NT was observed (Table 3). NT was mainly found in cardiomyocytes and to a lesser extent in CD68+ macrophages, indicating co-staining of iNOS and NT (Figure 2).

In comparison to acute and older infarctions, a reduced iNOS protein staining was detected in older infarctions. Yet, the intensity of the NT staining showed no difference between older and acute infarctions (data not shown).

2.4.6 Infiltrating macrophages express less iNOS compared to resident macrophages in human hearts

For further exploration of iNOS expressing macrophages in human MI hearts and healthy control hearts the BOND-RX Multiplex Stainer system was used, with antibodies against resident versus infiltrating macrophage markers. The results showed iNOS expression mainly in CD206+ and CD163+ macrophages, which are mostly resident macrophages, and to a lesser extent in CD68+ macrophages, indicating that infiltrating macrophages did not express significantly higher iNOS levels (Figure 3 and 4). Staining was performed in the infarcted regions, the non-infarcted regions and the control hearts.

Even though most macrophages in the heart were CD68+, the percentage of all iNOS expressing CD68+ macrophages did only slightly increase in MI hearts in comparison to control hearts. However, the amount of iNOS expression specifically in CD206+ and CD163+ macrophages did markedly increase in MI hearts, in comparison to control hearts. Therefore, the increase in iNOS protein expression in human MI hearts is due to an increase of iNOS production in CD206+ and CD163+ macrophages. Interestingly, the amount of CD206+ and CD163+ macrophages did not or only slightly increase in the MI hearts in comparison to control hearts, supporting the notion that these cells comprise the resident subset. However, there was an increase of all CD68+ macrophages in MI hearts in comparison to healthy hearts, indicating an influx of newly recruited monocytes/macrophages as expected upon myocardial infarction.

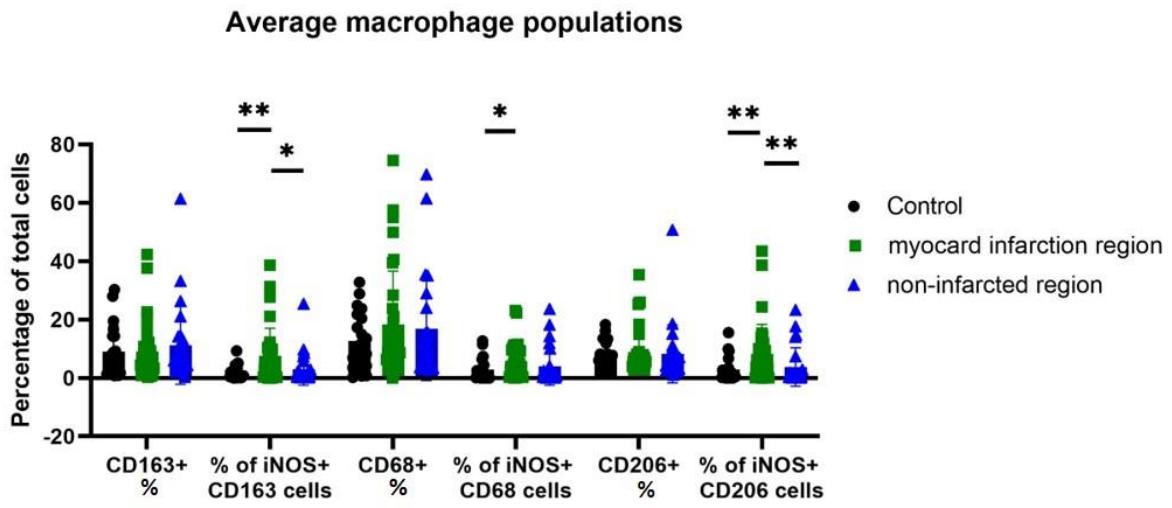


Figure 3: Percentage of iNOS-producing macrophage subpopulation in controls (n=10), non-infarcted (=23) and infarcted regions (n=25). Individual values were grouped and plotted. Statistical significance was indicated after normality and Mann-Whitney tests. Significance from left to right: % of iNOS+ CD163 cells (p = 0,0069 and 0,0116); % of iNOS+ CD68 cells (p = 0,0284) and % of iNOS+ CD206 cells (p = 0,0045 and 0,0060). In CD163+ and CD206+ cells iNOS expression is more strongly increased than in CD68+ cells. Whisker bars indicate the mean percentage of cells in each group and the standard deviation of the mean.

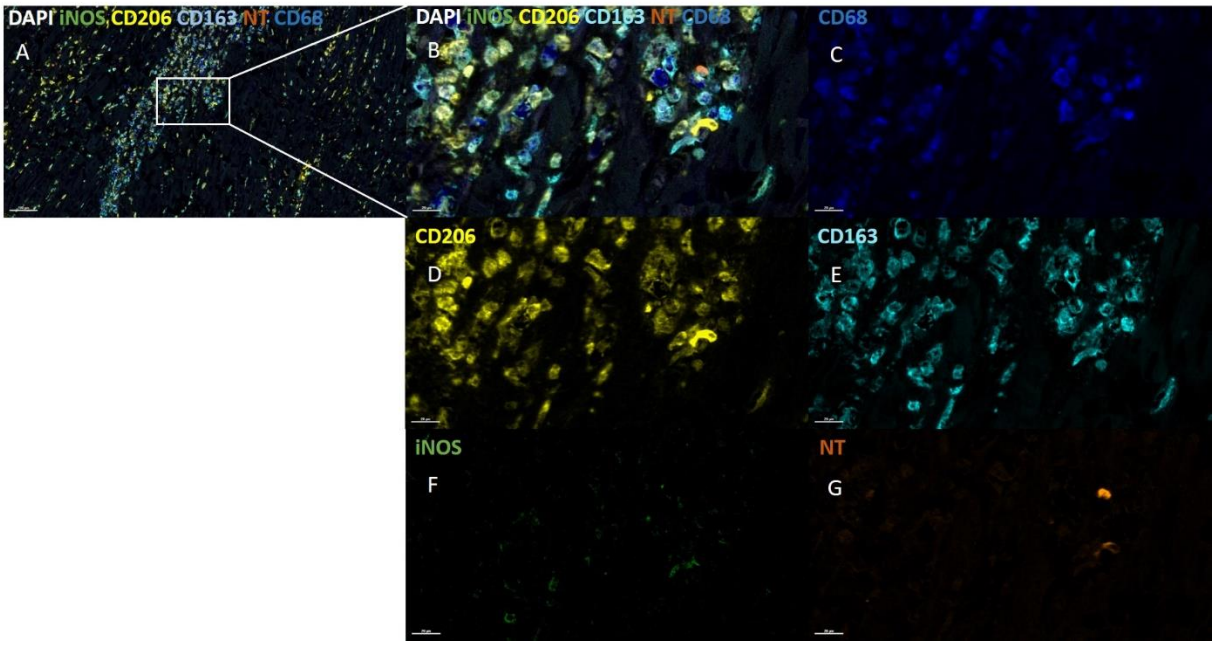


Figure 4: iNOS expression and NT production in different macrophage populations in infarcted regions. Infarcted tissue section, low magnification (A) and high magnification (B) stained using the following markers: CD68 (dark blue, C), CD206 (yellow, D), CD163 (light blue, E), iNOS (green, F) and NT (orange, G). iNOS is

visible within the CD163+ and CD206+ macrophage. Cell counterstain was performed with DAPI (white, not separately shown). Scale bars are shown in the images and indicate size.

2.4.7 NT production occurs in the vicinity of iNOS+ cells

The number of NT producing cells increased in the infarcted and non-infarcted regions in comparison to healthy controls. Since iNOS is a known contributor to peroxynitrite emergence and subsequent NT production, a distance analysis was applied, to examine potential causality between iNOS expression and NT production. In five infarcted regions, five non-infarcted regions and five healthy controls, the distance of iNOS+ and NT+ cells was measured in a radius of 100 µm (Figure 5). In all groups there was a peak of cells within 10-15 µm of iNOS+ and NT+ cells, demonstrating the close proximity between NT and iNOS expressing cells. When the analysis was performed from iNOS to NT, we observed a higher number of cells expressing iNOS towards cells producing NT within 10-15 µm. Not the total amount of iNOS+ and NT+ cells is depicted here, since distance measurements were only performed in a demilited radius range. Thus, explaining the high numbers in the control group (Figure 5).

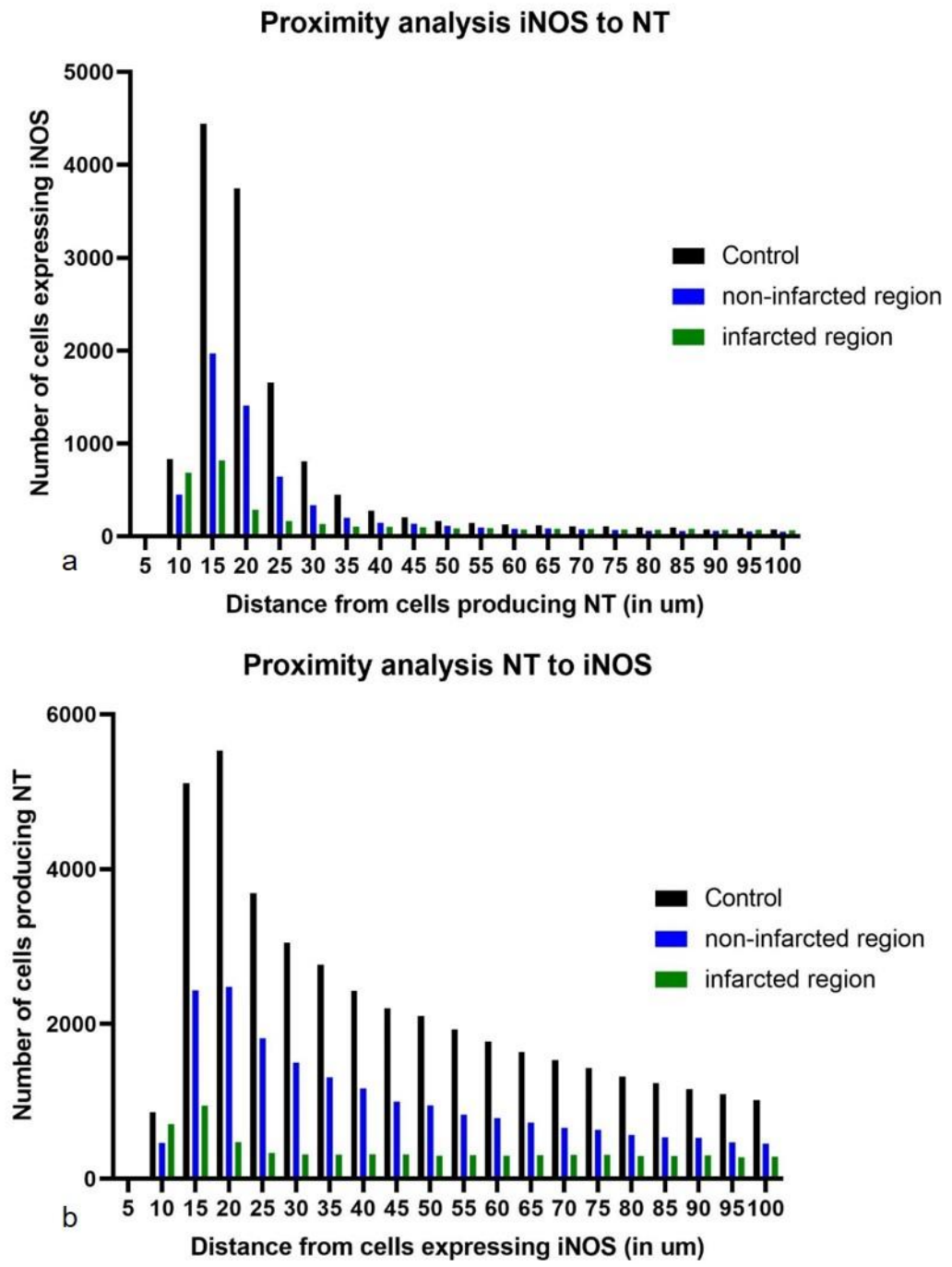


Figure 5: Distance between iNOS+ and nitrotyrosine+ (NT+) cells in controls, non-infarcted and infarcted regions. a) Number of iNOS+ cells within 100 µm of NT+cells in control and MI hearts. iNOS+ cells peak within a distance of 10-15µm of NT. b) Number of NT+ cells within 100 µm of iNOS+ cells in control and MI hearts. NT+ cells peak within a distance of 10-15µm of iNOS.

2.5 Discussion

2.5.1 Resident macrophages are the main source of iNOS in human MI hearts

A significant increase in iNOS mRNA expression in MI hearts was found, together with a strong increase of iNOS protein levels in infarcted and non-infarcted regions compared to control hearts.

Our results indicate that resident CD206+ and CD163+ macrophages, expressing classical markers of "M2" macrophages [52], are the main source of iNOS expression in human MI hearts. The findings of Gredic et al. [25] of CD206+ and iNOS+ macrophages in close proximity of remodeled vessels in lungs of human COPD patients support our observations. This is crucial since iNOS is considered a marker of pro-inflammatory, infiltrating macrophages [50] and resident macrophages are thought to enhance tissue regeneration and re-establish homeostasis [54].

Moreover, Gredic et al. [25] described an increase in the number of CD206+ resident macrophages, when wild type mice expressing iNOS were exposed to tobacco-smoke. This increase was abolished in iNOS knockout mice. Even more interesting they found evidence for an iNOS-dependent crosstalk between resident macrophages and pulmonary artery smooth muscle cells (PASMCs) which drives proliferation of these vascular cells and therefore, pulmonary vascular remodeling upon smoke-exposure, a pathway that can be prevented by deletion of resident macrophage derived iNOS. They hypothesized, that this regulatory role of iNOS is unique for specific conditions, including smoke-exposure and communication between macrophages and PASMCs. These results may apply to the current study in human MI hearts, since smoke exposure resembles hypoxic injury in MI, and we also found increased iNOS production in CD206+ and CD163+ macrophages. Although in the current study we did not examine crosstalk between cardiac macrophages and other cardiac cells, this is a known phenomenon. Ramanujam et al. [62] described miR-21 dependent crosstalk between inflammatory, infiltrating macrophages and fibroblasts in a murine pressure overload heart model, promoting the transition from quiescent fibroblasts to myofibroblasts. An iNOS dependent remodeling in the MI heart via peroxynitrite production and subsequent activation of matrix metalloproteinases may be possible.

However, to the best of our knowledge, our study is the first to describe iNOS production in CD206+ and CD163+ macrophages in human MI and healthy control hearts. Crucially, the percentage of CD206+ and CD163+ macrophages expressing iNOS exceeds the percentage of CD68+ macrophages expressing iNOS in the infarcted regions. This presents an interesting extension on the roles of recruited inflammatory versus resident macrophages in human MI in an iNOS dependent manner.

Additionally, we found increased iNOS mRNA and protein expression in the non-infarcted regions of human MI hearts. Since the supply areas of coronary arteries are not limited to either anterior or posterior wall we assume that an occluded artery causing MI in the anterior wall may also lead to microvascular ischemia in the posterior wall. We believe, this is a likely scenario explaining increased

iNOS mRNA and protein expression in the non-infarcted regions, especially in cases with long standing history of coronary artery disease. In a rat infarction model, Takimoto et al. [36] also found iNOS mRNA and protein levels upregulated in non-infarcted regions. He described this upregulation in a period of 56 days post infarction, suggesting a long-lasting inflammatory reaction in those non-infarcted regions. These results are in line with the findings from other groups, who reported an increase in macrophage numbers in the non-infarcted area of MI hearts [51,53]. This increase is described as slower but longer lasting than in the infarcted area. Local macrophage renewal additional to blood monocyte recruitment, as well as a secondary inflammation reaction and functions of macrophages in heart remodeling are discussed as reasons [51,53].

In the current study, not only CD68+, but also CD163+ and CD206+ macrophage abundance was increased in the non-infarcted myocardium accompanied by an increased iNOS expression in those macrophages. Besides a distant inflammation following infarction or heart remodeling, as a reason, macrophages and iNOS are known responders to hypoxia [51]. However, Takimoto et al. [36] did not report iNOS protein in control hearts. Furthermore, they reported that cardiomyocytes are the major cells expressing iNOS, especially in the non-infarcted regions. We did not detect any iNOS expression in cardiomyocytes. This may be due to species differences and differences between models of myocardial infarction.

2.5.2 iNOS dependent oxidative stress in human MI hearts

The function of iNOS is widely described as producing NO, which reacts with superoxidanion causing oxidative and nitrosative stress in the myocardium via production of peroxynitrite [42], which can be narrowed down by NT stainings, the fingerprint of peroxynitrite in tissue. Still, the extent of iNOS dependent NO production to peroxynitrite in the heart remains unclear, since iNOS is not the only contributor to peroxynitrite production and oxidative stress in the heart [9,47].

In the current study, strong NT staining was found in MI hearts, in infarcted as well as in non-infarcted areas. These results are in accordance with the results of other studies, which examined the immunohistochemical correlation between iNOS and NT in pathological conditions, such as cardiomyopathies, where NT was described in the cardiomyocytes [47,48]. In our study, NT was mainly detected in the cardiomyocytes, however, we found macrophages being positive for NT. Turillazi et al. [47] suggested an additional mechanism of acute cardiac injury triggered by cocaine, by the increase of iNOS and NT and subsequent oxidative stress in the myocardium of cocaine bodypackers. Feng et al. [9], detected NT staining in the non-infarcted myocardium of mice, which increased moderately with NO production by iNOS. Hence, iNOS increases NT but is not the only contributing/modulating factor. Further, they measured nitrate and nitrite in plasma of MI mice and found a consistent increase with myocardial iNOS expression. In comparison with $iNOS^{-/-}$ mice this increase was significant. This suggests iNOS dependent oxidative stress in MI.

To the best of our knowledge, no distance analysis between iNOS+ cells and NT+ cells in human MI and healthy control hearts has been conducted so far. The results of our study confirm that NT+ cells peak within 10-15 µm of iNOS, suggesting a dependence and a range of activity by iNOS produced NO. Within that perimeter, oxidative damage, such as extracellular matrix remodeling, may appear [45], which may lead to left ventricular remodeling after MI [46].

Shimojo et al. [56] found co-expression of NT and iNOS in cardiomyocytes in postmortem human myocardial infarction hearts, which is not confirmed by our study. Since they did not use any macrophage markers the immunohistochemical results may have not been as enlightening as the results in the current study.

Further, the results of our study indicate that the whole heart is affected by oxidative stress, as hinted by NT expression, and this seems long lasting. Therefore, an additional mechanism of cardiac injury driven by iNOS and NT production in MI hearts is occuring.

2.6 Conclusion

The results of the current study revealed that macrophages are the main source of iNOS expression in postmortem human MI hearts. However, iNOS production was predominantly detectable in resident CD163+ and CD206+ macrophages, which express typical “M2” macrophage markers. These findings are unexpected given that iNOS is widely considered a marker of inflammatory “M1” macrophages, at least in mice. It also confirms the limitations of the “M1” and “M2” nomenclature used to describe cardiac macrophages.

Furthermore, we found that NT+ cells peak within 10-15 µm distance of iNOS+ cells, indicating that iNOS expression influences the level of NT in human MI hearts. The oxidative stress in infarction hearts may be responsible for further damage in the myocardium and an impaired prognosis after MI.

Further studies are needed to describe in detail the role of iNOS expression in cardiac macrophages in human MI and how a prolonged inflammatory reaction involving iNOS expressing macrophages impairs the prognosis after MI.

PART 2 REGULATION OF INOS EXPRESSION IN HUMAN MYOCARDIAL INFARCTION

3.1 Abstract

MicroRNAs (miRNAs) are important post-transcriptional regulators in several diseases, including cancer, immunologic and cardiovascular diseases. A growing list of miRNAs are dysregulated in arrhythmias, contractility diseases, myocardial infarction (MI), sudden cardiac death (SCD), chronic heart failure and hypertrophy. However, the exact regulatory pathways, through which miRNAs exert their effects are often unclear.

In this study, we measured the expression patterns of miR-21, miR-939 and miR-30e in postmortem human MI, since in different studies these miRNAs were shown to be dysregulated in cardiovascular diseases. Our aim was to examine the influence of these miRNAs on cardiac iNOS mRNA and protein levels, as well as cardiac macrophage polarization and nitrotyrosine staining.

We measured miRNA expression patterns by means of qPCR. Further we used correlation analyses to determine causality between miRNA expression and cardiac iNOS, macrophage and nitrotyrosine levels.

miR-21, miR-939 and miR-30e were significantly upregulated in infarcted and non-infarcted regions of postmortem human MI hearts in comparison to healthy controls. While miR-21 and miR-939 showed their strongest expression in infarcted regions, miR-30e peaked in the non-infarcted myocardium. Further, we found a significant correlation between miR-939 and iNOS expression levels in controls and infarcted regions.

The results indicate, that miR-939 is a regulator of cardiac iNOS expression. However, a massive iNOS activation might exceed the capability of miR-939 to keep its expression in balance. miR-21 and miR-30e do not seem to influence cardiac iNOS levels in MI.

Further studies are needed to evaluate downstream targets of these miRNAs and their signaling pathways to clarify their role in human MI.

3.2 Introduction

MicroRNAs (miRNAs) are small noncoding RNAs, approximately 22 nucleotides long and single-stranded, which regulate post-transcriptional gene expression through imperfect base-pairing with complementary sequences in the 3’ untranslated region (3’UTR) of their target mRNA [63–67]. Thereby they inhibit mRNA translation or promote mRNA degradation [65,68]. Since targeting of a mRNA occurs by imperfect base-pairing, a single miRNA can regulate multiple mRNAs involved in different biological processes, and vice versa [64]. Therefore, circulating and tissue miRNAs are

important regulators in a growing list of diseases, including cancer, immunologic diseases and cardiovascular diseases [64,69].

miRNAs can be isolated from several biological materials including formalin-fixed, paraffin-embedded (FFPE) samples [66], which make them suitable as biomarkers. However, therapeutically targeting miRNAs remains difficult, since they influence many target genes and possibly different pathways in the organisms. Still, they are thoroughly investigated as promising diagnostic tools.

The dysregulation of several miRNAs in different cardiac diseases, such as arrhythmias, contractility diseases, MI, chronic heart failure and hypertrophy has been described, as well as specific functions for different miRNAs in apoptosis, necroptosis and autophagy of cardiac cells in MI [64,65]. Additionally circulating miRNAs are abundantly studied to investigate their diagnostic potential in MI, as well as in arrhythmia and inherited cardiomyopathies [64]. Further, in a study of Keller et al. [70] a panel of selected circulating miRNAs was evaluated to predict the outcome in several cardiovascular pathologies. Pinchi et al. [71] examined the diagnostic power of cardiac specific miRNAs to differentiate between MI and sudden cardiac death (SCD) in humans.

Additionally, several miRNAs are documented to be oxidative stress-responsive miRNAs, since they are considered as targets and modulators of oxidative-stress-related pathways. In a rat model of cardiac ischemia/reperfusion injury, which leads to tissue damage through generation of reactive oxygen species, changed expression levels of miRNA-144 were observed. miRNA-144 directly regulates a transcription factor that controls *NOS2* expression [66].

Guo et al. [72] found that in primary human hepatocytes, miR-939 decreases cytokine induced iNOS protein expression and NO synthesis, but it does not decreases mRNA expression or stability. Further they detected two functional miR-939 binding sites within the iNOS 3'UTR, which are critical for translational blockade via miR-939. Additionally, they reported that miR-939 expression is induced by the same cytokines that induce iNOS expression, suggesting a check-and-balance system to protect the organism against prolonged iNOS overexpression.

miR-21 is one of the strongest expressed miRNAs in various cardiac cell types in mice, with the strongest expression in cardiac macrophages [62,69]. The function of miR-21 in fibroblasts is well characterized as an important regulator for fibroblast proliferation and fibrosis. Likewise, inhibition or genetic deletion of miR-21 attenuated pulmonary, kidney, liver and skeletal muscle fibrosis [68,69]. Since excessive fibrosis leads to ventricular dilation, infarct expansion and heart failure after MI [73,74] examining the role of miR-21 after MI in humans is important. Especially, since the precise molecular mechanisms through which miR-21 exerts its effects is unclear. The targets probably range between tens to hundreds [69].

In a study of Chen et al. [75] miR-30e is described as downregulated in hypoxic cardiomyocytes and MI tissue of rats. They suggested that miR-30e protects the myocardium via enhancing cardiomyocyte viability and inhibiting apoptosis. By targeting phosphatase and tensin homologue (PTEN) expression at mRNA and protein levels, it further improved cardiac function.

These miRNAs along with others may be potential targets in the treatment of MI. However, little to no research has been conducted in human MI tissue regarding the dysregulation of miR-939, miR-21 and miR-30e and their potential targets. In the present work, the expression levels of miR-939, miR-21 and miR-30e were evaluated in human MI hearts and control hearts and their expression was correlated with the iNOS mRNA and protein expression, as well as with levels of CD163+ and CD206+ macrophages to examine their potential as regulators in remodeling and heart failure after MI in humans.

3.3 Material and Methods

3.3.1 Sample Selection

Formalin-fixed, paraffin embedded (FFPE) cardiac samples from 38 deceased individuals with MI were collected during court ordered autopsies at the Institute of Legal Medicine, University Hospital, Goethe University Frankfurt. The control group consisted of 11 FFPE cardiac samples from overall healthy individuals who died from accidents or committed suicide. All samples were histologically revised by an experienced pathologist. The control hearts showed no signs of myocardial injury in microscopical examinations. From the infarction zone of the MI hearts and the unaffected wall of the MI hearts 10 µm thick slices were used for miRNA extraction. Additionally, samples from the anterior and posterior wall of each control heart were taken. All samples were obtained from the left ventricle.
The MI and the control group are shown in Table 7 and 8. The MI group was further divided after histological examinations, into acute infarction, old infarction (months or years after index MI), re-infarction and subacute infarction (days or weeks after index MI), depending on the age of infarction.

Table 7: Gender, mean Age (years) and mean postmortem interval (PMI, days) of the myocardial infarction group.

Gender **Male/Female**	**Age**	**PMI**
28/10	68	7

Table 8: Gender, mean Age (years) and mean postmortem interval (PMI, days) of the control group.

Gender **Male/Female**	**Age**	**PMI**
8/3	40	5

3.3.2 Reverse transcription polymerase chain reaction

miRNA extraction from FFPE tissue was performed with xylene to remove paraffin and ethanol in decreasing concentrations (absolute, 96% and 75%) for rehydration. Subsequently, extraction was finalized according to the High Pure miRNA Extraction Kit (Roche, Mannheim).

For quantification of the miRNAs single stranded cDNA synthesis and qPCR were performed using the following TaqMan® MicroRNA Assays (Applied Biosystems, Darmstadt): hsa-miR-21-5p (MIMAT0000076, assay ID: 000397), hsa-miR-939-5p (MIMAT0004982, assay ID: 002182) and hsa-miR-30e-5p (MIMAT0000692, assay ID: 002223). As stable non-coding reference RNAs for analyses in cardiac tissue RNU44 (refseq.-nr.: NR_002750, assay ID: 001094), RNU48 (refseq.-nr.: NR_002745, assay ID: 001006) and snRNA U6 (refseq.-nr.: NR_004394, assay ID: 001973) are described [71,76] and were used for normalization. cDNA synthesis was carried out using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt), according to the manufacturer's protocol. The Thermocycler program is described in Table 9.

Table 9: Thermocycler program for cDNA synthesis.

Step	Temperatur (°C)	Duration (h)
1	16	00:30
2	42	00:30
3	85	00:05
4	4	Stop

The qPCR reaction mix is described in Table 10.

Table 10: Reaction mix in the qPCR per each well.

Reagent	Volume in µl
Maxima Probe/ ROX qPCR Master Mix	5
TaqMan-Gene Expression Assay	0,5
cDNA	1
Nuclease free water	3,50

qPCR Program was performed as described in Part 1. For each sample triplicates were applied in the quantitative real time PCR (qPCR), as well as no template negative controls for each assay.

Analysing the qPCR efficiency was performed by using LinReg PCR Software [60]. The qPCR was efficient when the values were between 1,8 and 2,0. Values lower than 1,8 indicate an inadequate efficacy and were excluded. Efficacy values per heart assay were determined and used to correct the corresponding Cq values.

10ng of cDNA were used for qPCR.

3.3.3 Data analysis

qPCR was carried out by using the Software 7500, version 2.0.6 and Data Assist v3.01 served for analyzing the qPCR raw data. As calibrator sample a control heart was chosen, reflecting most closely the mean age (40 years) of the control group and the dominant gender (male) in control and infarction group. Calculation of fold change was carried out using the ΔΔCq method, as described in Part 1 [61]. The software R version 3.5.1 was used for statistical analysis. Linear mixed effects model and pairwise post hoc comparisons were applied, and significance was adjusted, by using Tukey's multiple comparisons test. Furthermore, after the Mudholkar test of bivariate Gauß distribution, Pearson and Spearman correlations were applied.

3.4 Results

Sample Selection has been described in Part 1.

3.4.1 miRNA expression in MI hearts

The expression levels of miR-21, miR-939 and miR-30e in MI hearts and control hearts were analyzed. miR-21 ($p = 0{,}001$ and $0{,}0024$), miR-939 ($p = 0{,}001$ and $0{,}0023$) and miR-30e ($p = 0{,}0279$ and $0{,}001$) were significantly upregulated in infarcted and non-infarcted regions of MI hearts in comparison to healthy controls. However, miR-30e showed the strongest increase in the non-infarcted regions, while expression of miR-21 and miR-939 were more strongly increased in the infarcted regions (Figure 6).

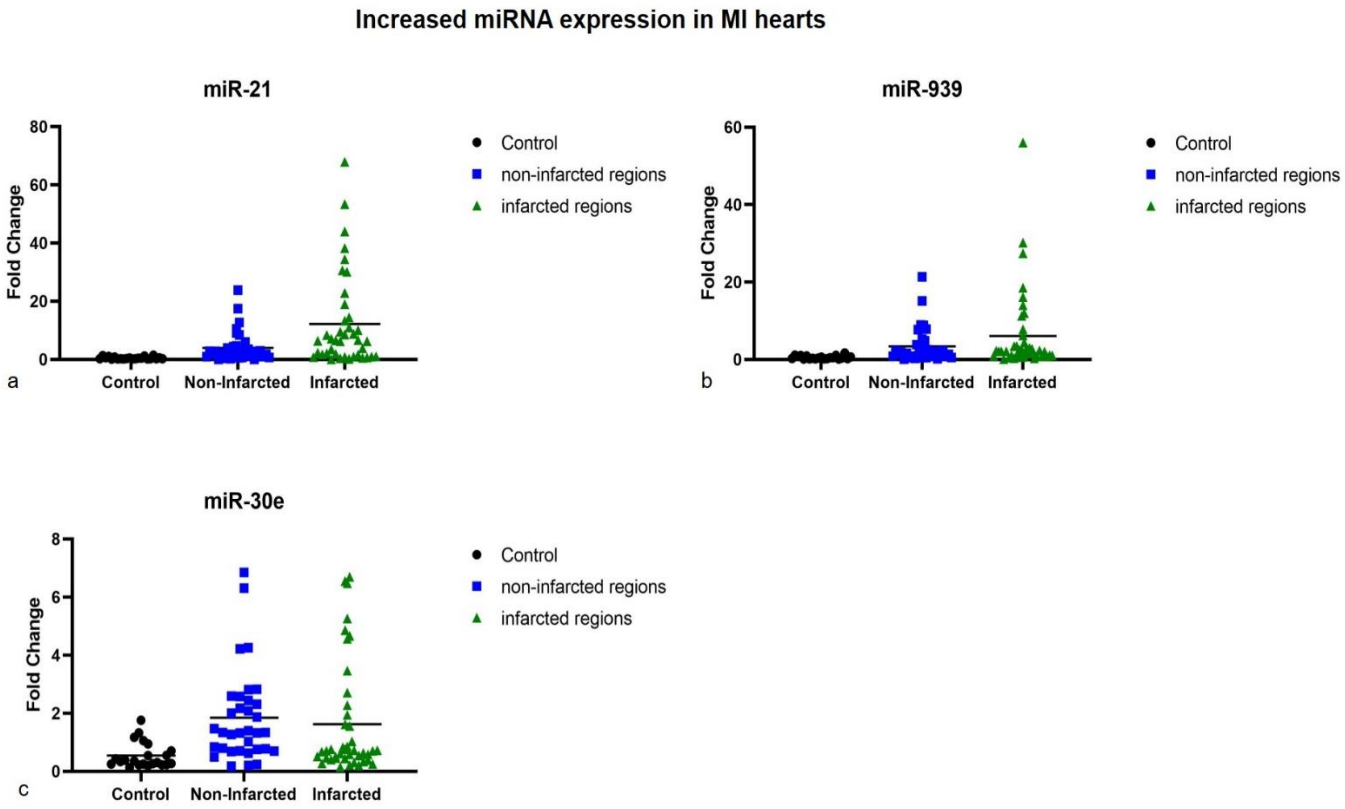


Figure 6: miRNA expression in controls, non-infarcted and infarcted regions. miR-21 (a), miR-939 (b) and miR-30e (c) expression level in control hearts (n=11), in non-infarcted (n=34) and in infarcted (n=37) regions of MI hearts were determined by qPCR. Individual values were grouped and plotted. Linear mixed effects model was applied, and significance was adjusted after normality by using Tukey's multiple comparisons test. miR-21 (p = 0,001 and 0,0024), miR-939 (p = 0,001 and 0,0023) and miR-30e (p = 0,0279 and 0,001) were significantly upregulated in infarcted and non-infarcted regions of human MI hearts, in comparison to the control group. Horizontal bars indicate the mean Fold Change of each group.

3.4.2 miR-21

When separating the infarcted and non-infarcted regions according to the infarction age, differences in the miR-21 expression were observed (Figure 7). Compared to the control group miR-21 expression was significantly increased in acute infarcted regions (p = 0,01), in re-infarcted regions (p = 0,01) and in subacute infarcted regions (p = 0,01). In the old infarcted regions no significant upregulation of miR-21 was found (p = 0,09). The highest increase in miR-21 expression was found in re-infarcted regions.

In the non-infarcted regions only in subacute infarctions a significant upregulation of miR-21 was observed (p = 0,05).

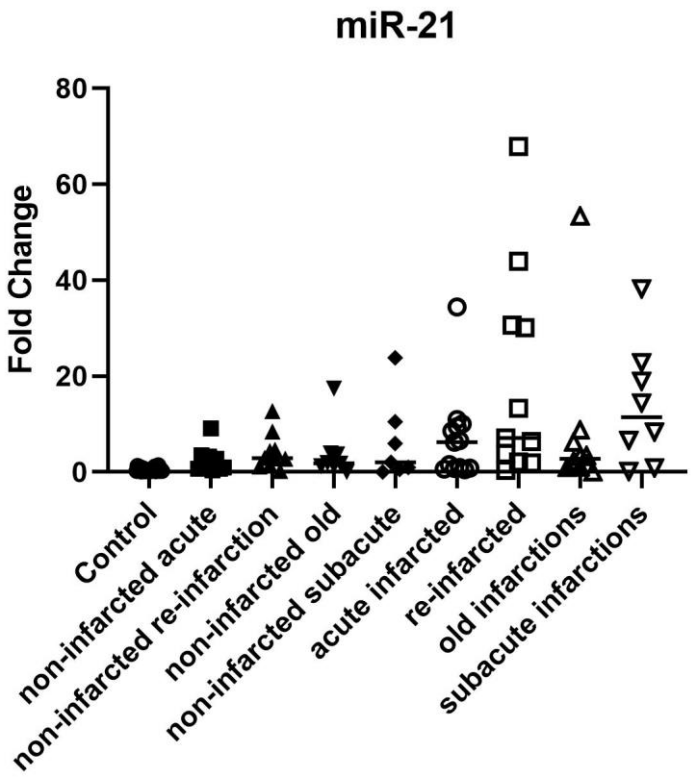


Figure 7: miR-21 expression in different MI ages. miR-21 expression levels in control hearts (n=11) in acute infarctions (n=11), in re-infarctions (n=10), in old infarctions (n=9) and in subacute infarctions (n=8) were determined by qPCR. Individual values were grouped and plotted. Linear mixed effects model was applied, and significance was adjusted after normality by using Tukey's multiple comparisons test. miR-21 expression was significantly increased in acute (p = 0,01), re-infarcted (p = 0,01) and subacute infarcted regions (p = 0,01) in comparison to healthy controls. Horizontal bars indicate the mean Fold Change of each group.

For correlation analyses, the mRNA and immunhistological results from Part 1 were taken. Pearson and Spearman correlation analyses showed no significant correlation between the miR-21 expression level and iNOS gene or protein expression, nitrotyrosine staining and the percentage of infiltrating or resident macrophages and their respective iNOS protein expression. Hence, no influence of increased miR-21 expression on iNOS expression or macrophage numbers could be found.

3.4.3 miR-939

When separating the infarcted and non-infarcted regions according to the infarction age, differences in the miR-939 expression were observed (Figure 8). Compared to the control group miR-939 expression was significantly increased in the acute infarcted regions (p = 0,01), in the re-infarcted regions (p = 0,01) and in the subacute infarcted regions (p = 0,01). In the old infarcted regions, no significant

upregulation of miR-939 was found (p = 0,6). Comparable to the miR-21 expression, the highest levels of miR-939 were found in the re-infarcted regions.
In the non-infarcted regions no significant upregulation of miR-939 was observed, when separating the regions according to infarction age.

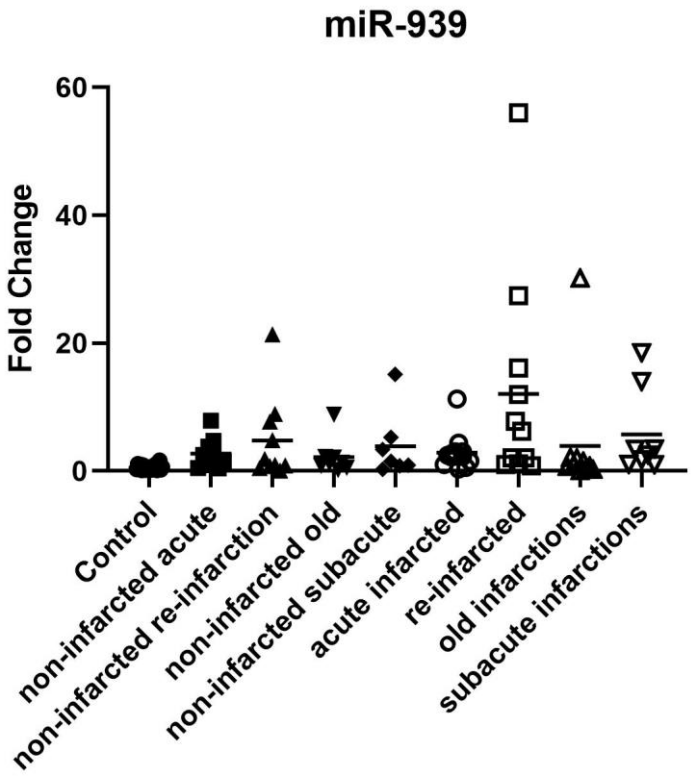


Figure 8: miR-939 expression in different MI ages. miR-939 expression levels in control hearts (n=11) acute infarctions (n=11), in re-infarctions (n=10), in old infarctions (n=9) and in subacute infarctions (n=8) were determined by qPCR. Individual values were grouped and plotted. Linear mixed effects model was applied, and significance was adjusted after normality by using Tukey's multiple comparisons test. miR-939 expression was significantly increased in acute (p = 0,01), re-infarcted (p = 0,01) and subacute infarcted regions (p = 0,01) in comparison to healthy controls. Horizontal bars indicate the mean Fold Change of each group.

Spearman analysis showed a significant correlation between the miR-939 gene expression and the iNOS gene expression in the control group (rho = 0,6739, p = 0,002) and the infarction group (rho = 0,4475 p = 0,002) (Figure 9).

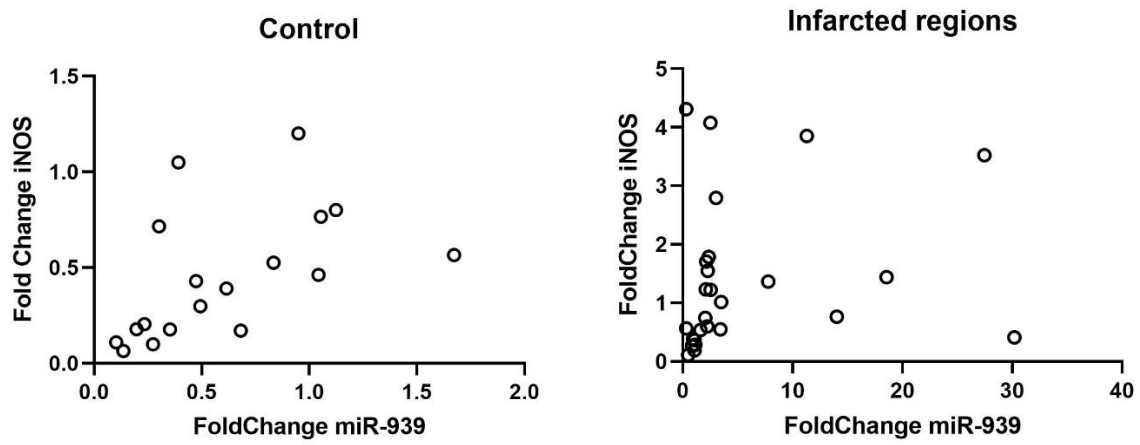


Figure 9: Correlation between miR-939 and iNOS mRNA expression levels in controls and infarcted regions. miR-939 expression levels in control hearts (n=11) and infarcted regions (n=38) were determined by qPCR. Individual values were plotted. Spearman correlations were applied. miR-939 and iNOS mRNA level were significantly correlated in controls (p = 0,002) and infarcted regions (p = 0,002), with a stronger correlation in controls.

3.4.4 miR-30e

When separating the infarcted and non-infarcted regions according to the infarction age differences in the miR-30e expression were observed (Figure 10). Interestingly, only in the non-infarcted regions of re-infarcted hearts a significant upregulation of miR-30e in comparison to healthy controls was observed (p = 0,01). In general, the non-infarcted regions showed higher miR-30e expression than the infarcted regions.

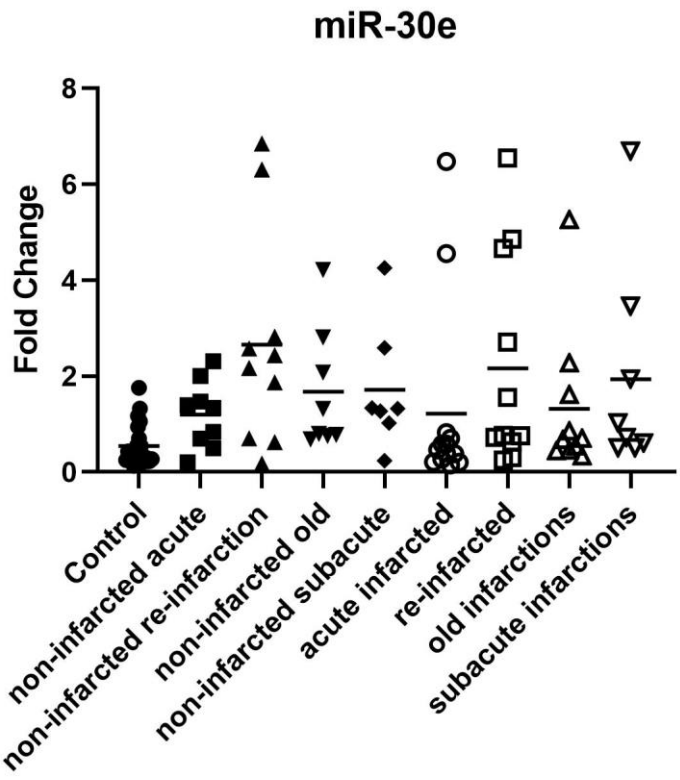


Figure 10: miR-30e expression in different MI ages. miR-30e expression levels in control hearts (n=11) acute infarctions (n=11), in re-infarctions (n=10), in old infarctions (n=9) and in subacute infarctions (n=8) were determined by qPCR. Individual values were grouped and plotted. Linear mixed effects model was applied, and significance was adjusted after normality by using Tukey's multiple comparisons test. miR-30e expression was significantly increased in non-infarcted regions of re-infarcted hearts in comparison to control hearts. Horizontal bars indicate the mean Fold Change of each group.

3.5 Discussion

The results in the present work revealed dysregulated miRNAs in human MI hearts. miR-21, miR-939 and miR-30e were significantly upregulated in infarcted and non-infarcted regions of human MI hearts in comparison to healthy controls, suggesting their involvement in a regulatory mechanism during ischemia. Interestingly, while miR-21 and miR-939 showed the strongest increase in the infarcted regions, miR-30e peaked in the non-infarcted regions in comparison to healthy hearts. To the best of our knowledge, the present study is the first to describe the expression levels of these three miRNAs in human MI hearts.

Our findings regarding miR-21 are in line with animal studies. Yuan et al. [68] detected miR-21 upregulation after MI in mice with the most remarkable expression in the infarcted zone. They

reported that miR-21 levels were associated with the extent of fibrosis via inhibiting Smad7. Inhibiting miR-21 reversed fibrosis after MI. In a pig model of heart failure after MI, expression of miR-21 was upregulated in remote and border-zone regions of infarcted myocardium [69]. After intracoronary delivery of antimiR-21 a significant reduction of miR-21 was detected, alongside with significantly repressed fibroblast proliferation and macrophage infiltration. In conclusion, inhibition of miR-21 led to reshaping of the myocardial transcriptome and attenuating the detrimental role of miR-21 in promoting myocardial remodeling after MI [69]. These results suggest that miR-21 is a crucial regulator in the heart when it comes to fibroblast activation after MI.

Ramanujam et al. [62] investigated the expression of miR-21 in a pressure overload model of the left ventricle in mice and determined a progressive increase in miR-21 in cardiac macrophages 6 and 21 days after initiation of pressure overload. Further, genetic deletion of miR-21 specifically in cardiac macrophages led to an increase in the "M2" or resident macrophage population in the heart and even prevented "M1" polarization of macrophages. Subsequently, they concluded that miR-21 is a crucial molecule for the proinflammatory activation of macrophages. Additionally, loss of miR-21 in cardiac macrophages prevented myofibroblast transformation and myocardial fibrosis. Therefore, miR-21 has a key regulatory role, as it is essential for control of paracrine, profibrotic macrophage signaling toward cardiac fibroblasts and thereby determines cardiac remodeling and function.

In the present study, the detected enhanced miR-21 expression in non-infarcted and infarcted human heart tissue further underlines its key regulatory role in human MI. The observed greater level of miR-21 expression in infarcted regions, compared to non-infarcted regions is further in line with the results of Bejerano et al. [77] in a murine MI model. In the present work, miR-21 expression varied with infarction age, however in acute, subacute and re-infarcted tissues, miR-21 was significantly upregulated. An increase in proinflammatory macrophages and enhanced profibrotic signaling towards myofibroblasts via miR-21 seems plausible. Also, no increase in miR-21 was observed in old infarctions, as no acute myocardial injury was present, making fibroblast activation not necessary. However, no correlation between miR-21 expression and the increase in macrophage subpopulations in human MI and healthy control hearts was found.

The present study is the first to describe significantly increased miR-939 expression in human MI hearts. Li et al. [78] examined serum exosomes from patients with myocardial ischemia, since exosomes are crucial in cell-cell communications. They act as cargos for the delivery of nucleic acids and proteins from surviving cells to nearby or distant cells. They detected that exosomes from myocardial ischemia patients display downregulation of miR-939 in comparison to the control group. Furthermore, those exosomes promoted endothelial cell proliferation and angiogenesis in mouse hind-limb ischemia. In addition, they reported iNOS being a direct target of miR-939 and observed decreased levels of iNOS mRNA, protein and NO synthesis after miR-939 overexpression in endothelial cells. Hence, miR-939 may be an angiogenesis suppressor, while downregulated miR-939

promotes angiogenesis [78]. They further hypothesized that pro-angiogenic exosomes may be secreted by ischemic cardiomyocytes.

Chen et al. [79] investigated elevated miR-939 levels in serum of chronic heart failure patients and also reported iNOS as a direct target of miR-939. They hypothesized induction of miR-939 in the initial stages of heart failure to control iNOS levels. However, with progressing disease and massive iNOS activation and subsequent NO synthesis, miR-939 is not able to inhibit iNOS production. In the present study we found upregulated miR-939 expression levels in non-infarcted and infarcted regions of human MI hearts, especially in acute, subacute and re-infarcted tissues. Our findings are in line with other studies in different biological systems, suggesting an increase in miR-939 levels to inhibit iNOS expression and thereby protect the heart from prolonged iNOS overexpression.

Spearman analyses showed a significant correlation between miR-939 and iNOS mRNA levels in the control and the infarction group. This suggests a regulatory role for miR-939 regarding the iNOS expression in a check-balance-system. However, this system seems to become out of balance in the infarction group, once the iNOS levels increase too strongly.

We further detected increased miR-30e expression in human MI. miR-30e is thought to protect the myocardium after MI by alleviating inflammation and myocardial injury via suppressing PTEN [75]. Chen et al. [75] described miR-30e as downregulated in a rat infarction model, which is not confirmed in the present work. However, the authors of this study investigated rats with acute MI, whereas our study was based on results from human tissue. In our study, the lowest expression of miR-30e was detected in acute human MI and the expression was comparable to the control group. Further studies by Pu et al. [80] and Wang et al. [81] describe miR-30e as strongly downregulated in rat infarction. Pu et al. [80] investigated the effect of miR-30e overexpression on heart failure after MI in rats. Again a protective role of miR-30e was described, as it reduced the level of fibrosis and the proportion of apoptotic cells in MI tissue of rats. The bioinformatic analysis of Wang et al. [81] revealed possible targets of miR-30e, which suggest cardiomyocyte responses to MI. Additionally, Cheng et al. [82] describes a protective effect of miR-30e in a rat model of myocardial ischemia-reperfusion injury, where it suppressed oxidative stress and inflammation and reduced cardiac function damage.

In the present work no possible downstream targets of miR-30e were examined and no statements about a possibly protective role can be made. However, the seen upregulation of miR-30e in subacute, old and re-infarcted regions could be an attempt to decrease the level of fibrosis and subsequently the progression of heart failure after MI, as mentioned by Pu et al. [80] for the rat model.

In general, miR-30e was stronger expressed in the non-infarcted regions than in the infarcted regions. We hypothesize that even in non-infarcted regions microvascular ischemia might be present, since the supply areas of coronary arteries are not limited to only posterior or anterior wall. Therefore, miR-30e expression could be triggered to exert its protective effects.

3.6 Conclusion

The results in the present work revealed significantly upregulated miR-21, miR-939 and miR-30e expression in human MI hearts. While miR-21 and miR-939 showed their strongest expression in infarcted regions, miR-30e peaked in the non-infarcted myocardium.

Further, we showed a significant correlation between miR-939 and iNOS mRNA expression levels in controls and infarcted regions. A massive iNOS activation could have exceeded the capability of miR-939 to keep its expression in balance. iNOS expression levels do not seem to be regulated by miR-21 and miR-30e. However, miR-21 is thought to drive cardiac fibrosis via profibrotic signaling from macrophages to fibroblasts and therefore, might have a regulatory role in MI.

Further studies are needed to evaluate downstream targets of these miRNAs and their signaling pathways to clarify their role in human MI.

PART 3 GENETIC POLYMORPHISMS OF THE *NOS2* GENE AS RISC FACTORS FOR INCREASED INOS EXPRESSION AND NO PRODUCTION IN HUMAN MI

4.1 Abstract

Inducible nitric oxide synthase (iNOS) is encoded by the *NOS2* gene. Polymorphisms in this gene are found in the regulatory and the coding region, affecting the level of gene product and the activity of the gene product, respectively. The altered responsiveness of iNOS is described to play a role in cardiovascular diseases, such as atrial fibrillation and hypertension.

Blood and urine levels of nitrite/nitrate (NO_x) are stable end products of NO production by iNOS and considered as markers for iNOS activity. Malondialdehyde (MDA) is considered as marker for oxidative stress in the heart associated with myocardial infarction (MI), while creatinine is considered as an independent risk factor for and after MI. The aim of our work was to identify risk genotypes of iNOS in human postmortem MI and correlate the genetic findings to serum and urine levels of MDA, creatinine and NO_x.

The *NOS2* gene was genotyped by means of Sanger sequencing to determine the $(CCTTT)_n$ repeat length polymorphism in the promoter region and single nucleotid polymorphisms (SNPs) at -1026bp (rs2779249, G>T) before transcription start site and in exon 16 (rs2297518, C>T). Further serum and urine levels of NO_x, MDA and creatinine were determined by Gas-Chromatography/Mass Spectrometry (GC-MS).

The results revealed an accumulation of *NOS2* risk polymorphisms in the MI group in contrast to healthy controls. We further detected an increase in iNOS protein expression in *NOS2* risk polymorphism groups and increased MDA, creatinine and nitrate serum levels seem to be connected to long $(CCTTT)_n$ repeat polymorphisms and the T allele at -1026bp (rs2779249, G>T).

Further studies are needed to draw conclusions about the relationship between cardiac iNOS expression, altered iNOS activity by *NOS2* polymorphisms and serum markers of iNOS activity in MI.

4.2 Introduction

The human iNOS promoter is one of the largest and most complex promoters, suggesting a strictly controlled gene expression [83]. Polymorphisms in the iNOS gene (*NOS2*) are described in the coding and the regulatory region. While polymorphisms in the promoter region are thought to affect the level of gene product, polymorphisms in the coding region are thought to affect the activity of the gene product [21]. Therefore, the responsiveness of iNOS depends on the genetic profile of an individual [84] and further, allelic frequencies in the *NOS2* promoter region are thought to predispose to several infectious diseases [85].

A single nucleotide polymorphism (SNP) in the promoter region of *NOS2* -1173 basepair (bp) upstream of the transcription start is associated with protection from cerebral malaria and severe malaria anaemia in Tanzanian and Kenyan children [22]. The -1173 C>T polymorphism is thought to increase the iNOS mRNA expression and consequently the protein level, leading to increased NO production, which is described to attenuate the growth of malaria parasites [22]. Hsu et al. [84] examined the association between the number of a $(CCTTT)_n$ pentanucleotide repeat polymorphism in the *NOS2* promoter, which can determine the level of gene transcription, and the iNOS expression in atrial fibrillation in a taiwanese population. Their results showed that patients with atrial fibrillation carried significantly longer $(CCTTT)_n$ repeats than the control group, accompanied with increased oxidative stress in the myocardium of atrial fibrillation patients. Therefore, long repeats of $(CCTTT)_n$ are associated with atrial fibrillation. Among the repeat polymorphisms in *NOS2* promoter region, is the $(TAAA)_n$ repeat polymorphisms, which can occur with 3 or 4 repeats, leading to a strong increase in the iNOS expression [86,87].

Another SNP -1026bp (rs2779249, G>T) upstream of the transcription start site in the *NOS2* promoter region was examined in patients with hypertension, a disease in which iNOS is suggested to play a pathological role. A study in a Finnish population revealed an increased risk for hypertension in those who carried homozygous the rs2779249, G>T allele, already at the age of 35 years [88]. Fu et al. [89] described a five-fold increase in the iNOS transcription activity in homozygous carriers, suggesting an association of this polymorphism and hypertension in a chinese han population.

A SNP in the coding region of *NOS2* (rs2297518, C>T), leading to an amino acid substitution from serine to leucine, is located in exon 16 and associated with a higher activity of the iNOS protein [21,90]. In cardiovascular diseases this SNP in the coding region was examined for its association with hypertension. A haplotype analysis revealed that SNP rs2779249, G>T in the promoter region and SNP rs2297518, C>T in exon 16 occured significantly higher in patients with hypertension than in the control group [88].

Even more interesting, a study of Sowjanya et al. [91] genotyped the *NOS2* gene in women with different cancerogenous lesions of the cervix for rs2779249, G>T , rs2297518, C>T and the $(CCTTT)_n$ repeat polymorphism to reveal different risk genotypes of iNOS. They further correlated the iNOS mRNA expression and combined genetic iNOS variants to plasma nitrite/nitrate levels to reveal risk genotypes in cervix cancer. Blood and urine nitrite/nitrate (NO_x) levels are considered stable end products of NO and an increased iNOS expression [92,93].

Blood markers for MI, such as CK-MB, cTnT and cTnI have been thoroughly investigated [6,7]. Additionally, malondialdehyde (MDA) is the end product of lipid peroxidation and considered as marker for oxidative stress in the heart and for oxidative stress associated with MI [94,95]. Further it is considered as an early marker in serum and plasma of patients with MI within the first 48 hours after symptom onset [96]. Another independent risk factor for and after MI is creatinine [97,98].

The aim of the present work was to identify risk genotypes of the *NOS2* gene in human MI, by correlating the genetic findings to iNOS mRNA and protein levels and the levels of stable end products of NO, as well as MDA and creatinine in serum and urine.

4.3 Material and Methods

4.3.1 Sample Selection

Blood samples of 39 deceased individuals with diagnosed MI were collected during court ordered autopsies at the Institute of Legal Medicine, University Hospital, Goethe University Frankfurt for DNA extraction. The control group consisted of 12 blood samples from overall healthy individuals with microscopically healthy, unharmed hearts, who died from accidents or committed suicide.

Of the 39 MI samples 16 serum and urine samples were further analyzed at the Centre of Pharmacology and Toxicology at Hannover Medical School, Germany. Of the control group 3 serum and urine samples were further analyzed. The small proportion of samples further analyzed in the MI and control group is explained by the amount of serum and urine needed for further analyses. Not in all samples of the MI and control group enough serum and urine were asservated.

The MI and the control group are shown in Table 11 and 12.

Table 11: Gender, mean Age (years) and mean postmortem interval (PMI, days) of the myocardial infarction group for genotyping *NOS2*.

Gender **Male/Female**	**Age**	**PMI**
29/10	68	7

Table 12: Gender, mean Age (years) and mean PMI (days) of the control group for genotyping *NOS2*.

Gender **Male/Female**	**Age**	**PMI**
9/3	43	5

4.3.2 DNA extraction and genetic analysis

DNA extraction of the blood samples was performed using the NucleoSpin Tissue Kit (Macherey Nagel) according to the manufactorer's protocol. Subsequently the extracted DNA was amplified via PCR using specific primers. PCR of the $(CCTTT)_n$ repeat polymorphism was performed using

primers: Forward: 5'acccctggaagcctacaactgcat3' Reverse: 5'gaccatagctctctgcagcc3'. The PCR conditions are shown in Table 13.

Table 13: PCR program for amplification of the $(CCTTT)_n$ repeat polymorphism.

Step	Temperature (°C)	Time (h)
1	94	00:02
2	94	00:01
3	60	00:01
4	72	00:01 (2-4 30x)
5	72	00:04
6	4	Stop

The PCR product spanning the rs2779249, G>T polymorphism and the $(TAAA)_n$ repeat polymorphism was generated using primers: Forward: 5'ggcattataaggaatgaaat3' Reverse: 5'cctacctgccattccaccaaagcttacc3'. The PCR amplifying the rs2297518, C>T polymorphism in exon 16 was performed using primers: Forward: 5'ttattcatcctggcttgagaactctg3' and Reverse: 5'cacaggggtcttctcagaa3'. The PCR conditions are depicted in Table 14.

Table 14: PCR program for amplification of rs2779249, G>T, rs2297518, C>T and $(TAAA)_n$ repeat polymorphism.

Step	Temperature (°C)	Time (h)
1	94	00:02
2	94	00:01
3	65	00:01
4	72	00:01 (2-4 30x)
5	72	00:04
6	4	Stop

For genotyping Sanger sequencing was performed, using the mentioned primers. The PCR program for Sanger Sequencing is described in Table 15.

Table 15: Sanger Sequencing program.

Step	Temperature (°C)	Time (min)
1	96	00:10
2	50	00:05
3	55	04:00 (1-3 30x)
4	8	Stop

The purified samples were then sequenced with ABI prism 3100/3130 Genetic Analyzer (Applied Biosystems).

The $(CCTTT)_n$ repeats were counted. On basis of previous studies [84,91] we identified alleles of <11 repeats as short form (S) and alleles of >12 repeats as long form (L). The individuals were categorized into three groups: S/S, S/L and L/L according to their homozygous or heterozygous repeat length. Longer repeats have higher transcription activity [91].

To determine the combined effects of *NOS2* SNP and length repeat polymorphisms on iNOS expression, individuals were categorized into three groups based on the risk genotypes present across the examined *NOS2* regions: 1) low risk group (individuals carrying 0-1 risk genotype), 2) medium risk group (individuals carrying 2 risk genotypes) and 3) high risk group (individuals carrying 3 or 4 risk genotypes), according to Sowjanya et al. [91].

Further, the genetic findings were correlated with urine and serum levels of nitrate/nitrite (NO_x), MDA and creatinine.

4.3.3 Gas Chromatography/Mass Spectrometry (GC-MS)

Serum and urine samples of 16 MI cases and 4 controls were analyzed at the Centre of Pharmacology and Toxicology at Hannover Medical School, Germany using GC-MS as previously described [99,100]. The serum samples were tested for the levels of NO_x, creatinine and MDA. The urine samples were tested for the levels of NO_x and MDA, which were corrected for creatinine excretion [100].

4.3.4 Data Analysis

The software R version 3.5.1 was used for statistical analysis. Linear mixed effects model and pairwise post hoc comparisons were applied, and significance was adjusted, by using Tukey's multiple comparisons test. Additionally, Fisher's Test was applied. After the Mudholkar test of bivariate Gauß distribution, Pearson and Spearman correlations were applied.

4.4 Results

4.4.1 Genetic polymorphisms in the MI and control group

In tables 16-19 the variations of homozygous and heterozygous polymorphisms, as well as the distributions of the shorter or longer repeats of $(CCTTT)_n$ and of the risk genotypes within MI and control group are shown. In the MI group the majority of cases carried homozygous long $(CCTTT)_n$ repeat polymorphisms, however, no statistical significance was reached. The homozygous risk alleles for rs2779249, G>T and rs2297518, C>T were only found in the MI group, even though they were

found in the minority of cases. When categorizing individuals according to the number of homozygous or heterozygous genotypes present across the examined *NOS2* regions into risk groups, the majority of cases in both groups showed a low risk type. However, in the MI group more than half of the cases showed at least two variations in *NOS2*. No statistical significance was reached.

Table 16: Length of $(CCTTT)_n$ repeat polymorphisms and carriers in the MI and control group.

$(CCTTT)_n$ repeat length	Carriers in MI group	Carriers in Control group
L L	18	3
S L	17	7
S S	4	2

Table 17: Homozygous GG, heterozygous (TG) and homozygous TT carriers of rs2779249, G>T polymorphism in MI and control group. In one individual the genotype in this position was not determinable. Hence, only 38 individuals of the MI group were evaluated.

rs2779249, G>T	Carriers in MI group	Carriers in Control group
G G	16	8
T G	19	4
T T	3	0

Table 18: Homozygous CC, heterozygous (CT) and homozygous TT carriers of the rs2297518, C>T polymorphism in MI and control group.

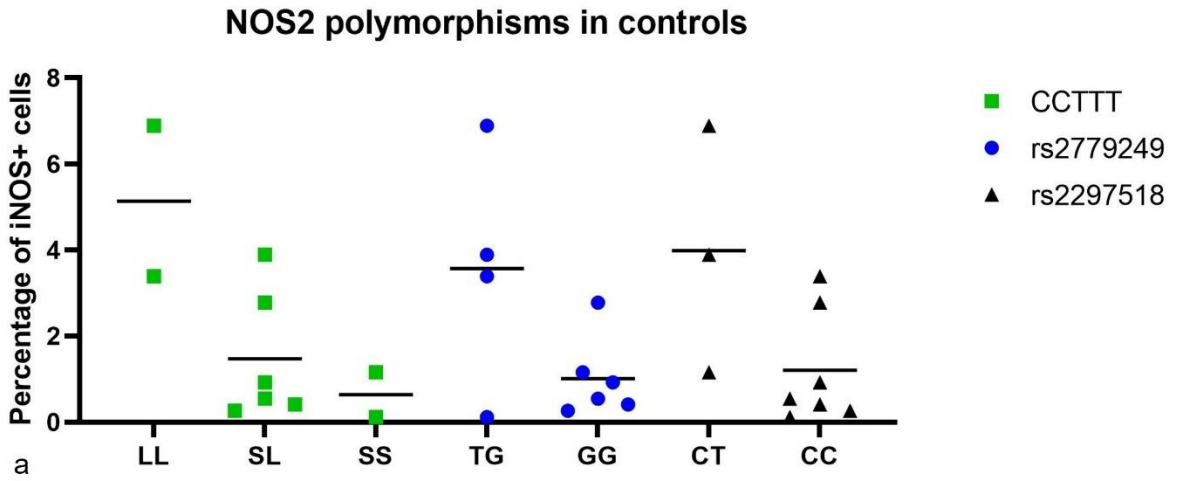
rs2297518, C>T	Carriers in MI group	Carriers in Control group
C C	29	8
C T	8	4
T T	2	0

Table 19: Low, medium and high risk genotype carriers of *NOS2* polymorphisms in MI and control group.

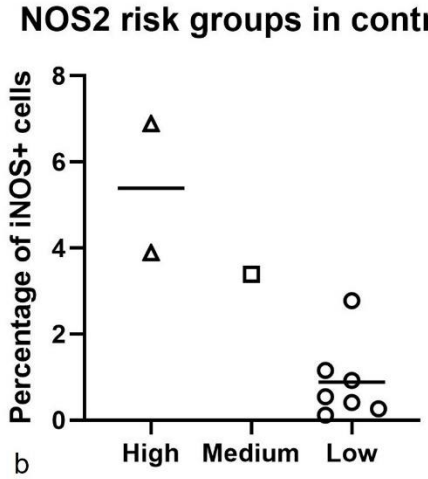
Risk type	Carriers in MI group	Carriers in Control group
High	8	2
Low	16	8
Medium	15	2

When categorizing the iNOS mRNA expression, determined in Part 1 of this thesis, according to *NOS2* polymorphisms within the MI and control group no significant differences were found. Hence, no influence of polymorphisms and risk groups on the iNOS mRNA level was detected (data not shown).

However, when categorizing the iNOS protein levels, determined in Part 1 of this thesis, according to polymorphisms within the control group significant differences were found (figure 11). Individuals carrying homozygous long $(CCTTT)_n$ repeats showed a significant increase of iNOS protein levels compared to heterozygous long repeat carriers ($p = 0{,}0136$) and homozygous short repeat carriers ($p = 0{,}0137$). Further individuals with the T allele on positions rs2779249, G>T and rs2297518, C>T showed significant iNOS protein upregulation in comparison to homozygous carriers of GG and CC ($p = 0{,}03$ and $0{,}0279$, respectively). Additionally, individuals categorized into the high risk group showed highly significant increased iNOS protein levels, in comparison to the low risk group ($p < 0{,}001$).



a



b

Figure 11: Percentage of iNOS+ cells in the control group (n=10) according to *NOS2* polymorphisms. a) Long $(CCTTT)_n$ repeat polymorphisms (p values LL vs SL 0,0136 and LL vs SS 0,0137) and T allele mutations at rs2779249, G>T (p value TG vs GG 0,03) and at rs2297518, C>T (p value CT vs CC 0,02) show significant upregulation of iNOS protein in the control group. b) Three or more combined *NOS2* polymorphisms significantly increased the iNOS protein expression within the control group (p value high vs low <0,001). Since iNOS protein expression was measured in only 10 controls, not the whole control group is depicted here. Horizontal bars depict the mean percentage of iNOS+ cells in each group.

When categorizing the iNOS+ cells according to *NOS2* polymorphisms within the MI group no significant differences were found. However, an increase in iNOS producing cells was detected in individuals carrying homozygous long repeats of $(CCTTT)_n$ and the T alleles on positions rs2779249, G>T and rs2297518, C>T (figure 12).

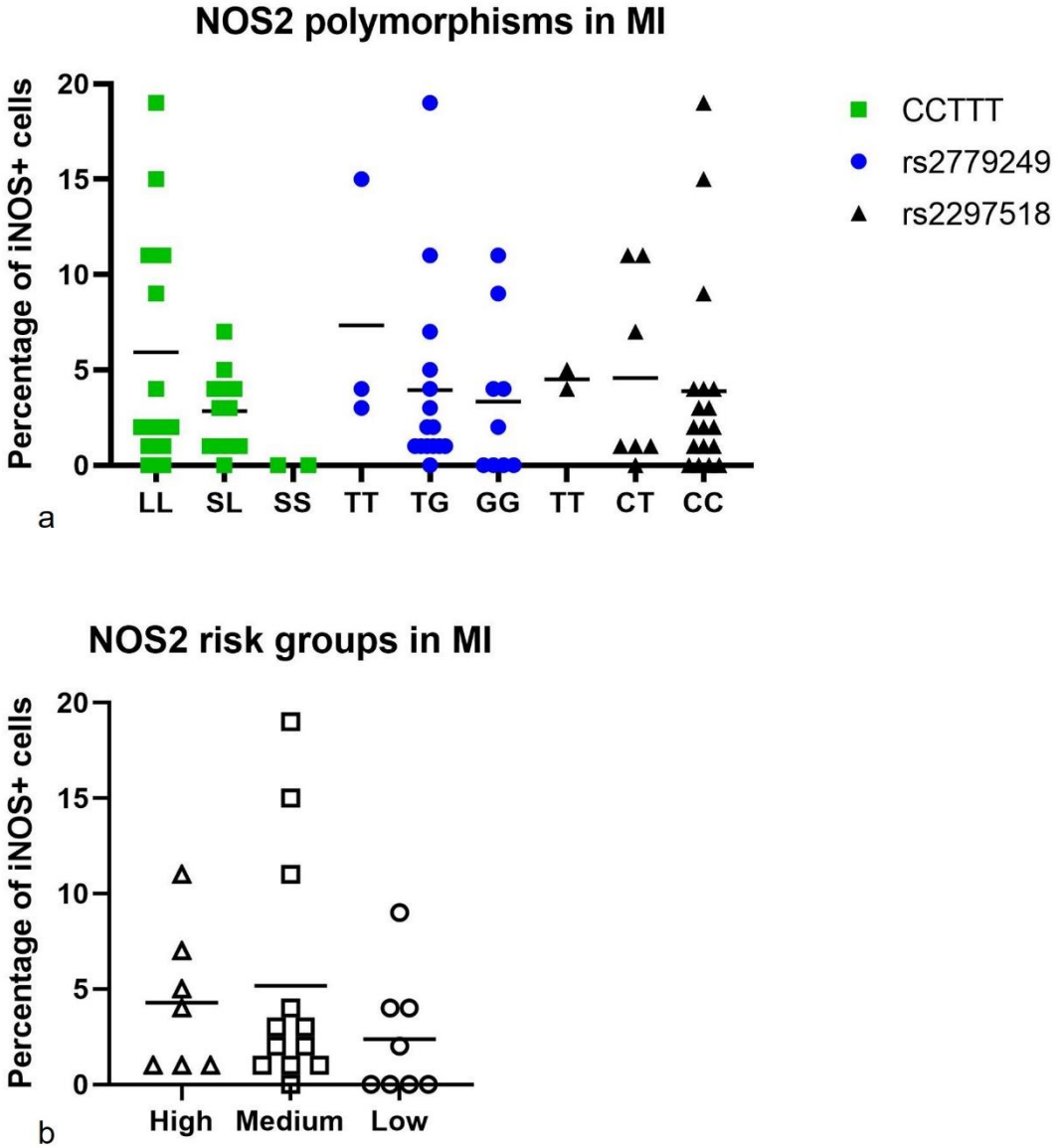


Figure 12: Percentage of iNOS+ cells in infarcted and non-infarcted regions of the MI group (n=27) according to *NOS2* polymorphisms. a) Long $(CCTTT)_n$ repeats and T allele mutations at rs2779249, G>T and at rs2297518, C>T showed increased iNOS producing cells within the MI group. b) Two or more combined *NOS2* polymorphisms increase the iNOS producing cells in comparison to the low risk group. Differences did not reach statistical significance. Since iNOS protein expression was measured in only 27 MI hearts, not the whole MI group is depicted here. Horizontal bars depict the mean percentage of iNOS+ cells in each group.

No influence of *NOS2* polymorphisms on nitrotyrosine staining in MI and control group was detected (data not shown).

4.4.2 Serum and urine NO_x, MDA and creatinine levels

No significant differences in NO_x and MDA levels in urine were found between MI and control group. In serum no significant differences regarding the MDA values between MI and control group were found. Still, the MI group revealed higher MDA levels than the control group. Creatinine values did not differ significantly between MI and control group, but the MI group showed higher creatinine than the control group. NO_x levels did also not differ significantly between MI and control group, however, again the MI group showed higher NO_x levels, than the control group (figure 13). No correlation between iNOS mRNA and protein expression and NO_x, MDA and creatinine levels in urine and serum was found. Therefore, no influence of cardiac iNOS expression and oxidative stress on systemic serum and urine values was found (data not shown).

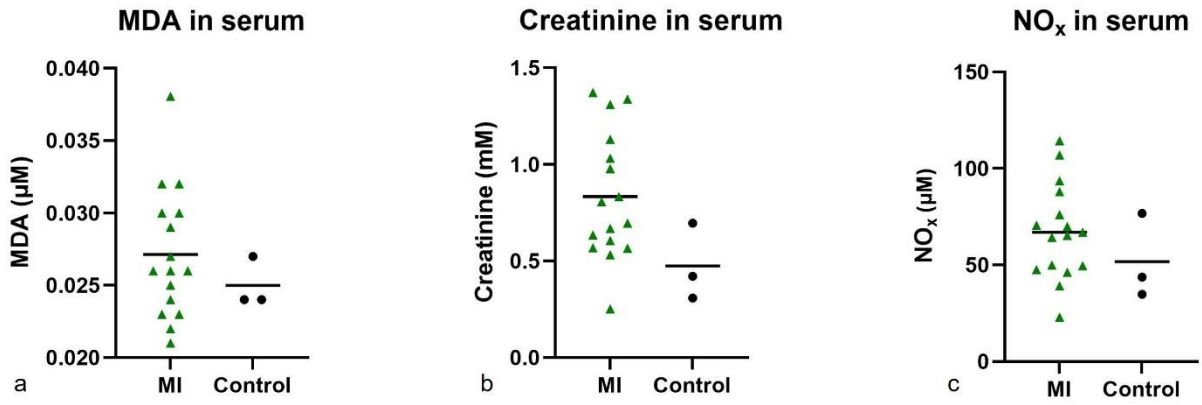


Figure 13: MDA, creatinine and NO_x levels in serum in MI and controls. MDA, creatinine and NO_x levels were increased in serum in the MI group (n = 16) in comparison to healthy controls (n = 3). The increase did not reach statistical significance. Horizontal bars depict the mean serum values in each group.

4.4.3 Influence of genetic polymorphisms on serum and urine NOx, MDA and creatinine levels

When categorizing the MI group according to $(CCTTT)_n$ repeat polymorphisms, within the group of the homozygous long repeats (LL) the highest MDA values were observed, in comparison to the heterozygous long repeats (SL) and the homozygous short repeats (SS). However, this difference was not significant (p = 0,1) (figure 14).

When categorizing the MI group according to the rs2779249, G>T *NOS2* polymorphism homozygote carriers of the T allele showed strongly increased nitrate levels in comparison to homozygous carriers of GG and heterozygote carriers ofTG (p = 0,0874) (figure 14). Additionally, creatinine levels were significantly increased in carriers of the homozygous T allele in comparison to homozygous carriers of GG and heterozygous carriers (figure 14).

In urine no correlation between *NOS2* polymorphisms and NO_x or MDA levels was found.

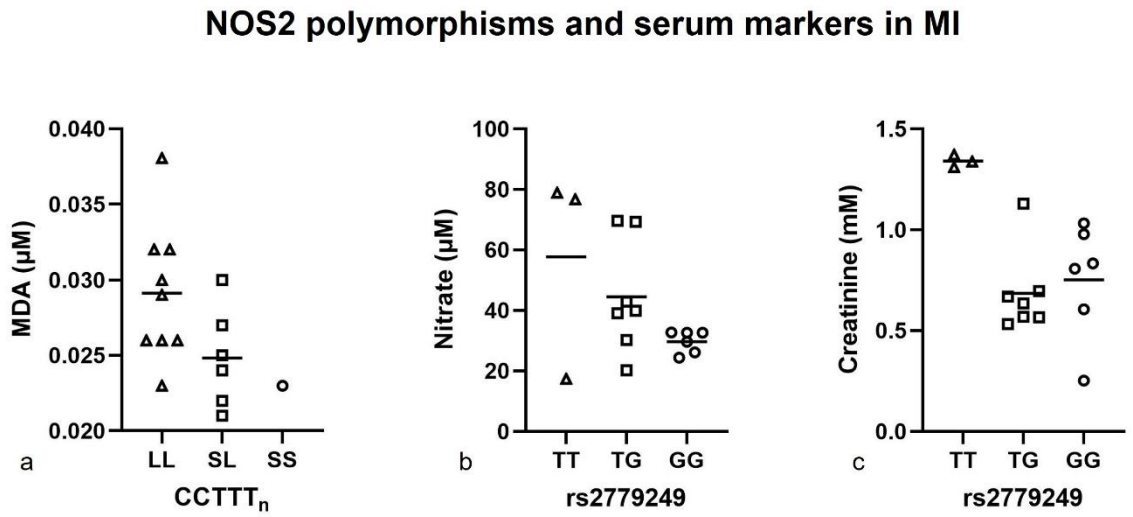


Figure 14: Serum MDA, nitrate and creatinine levels categorized according to $(CCTTT)_n$ repeat and the rs2779249, G>T *NOS2* polymorphisms in MI. Homozygous long $(CCTTT)_n$ repeats increased the serum MDA levels in comparison to heterozygous long repeats and homozygous short repeats, while the homozygous T allele at rs2779249, G>T increased the serum nitrate in comparison to homozygous GG carriers (p = 0,08) and significantly increased the creatinine levels in comparison to heterozygous T allele and homozygous GG carriers (p < 0,01). Horizontal bars depict the mean serum values in each group.

4.5 Discussion

4.5.1 *NOS2* risk polymorphisms in human MI hearts

The results in the present work reveal an accumulation of *NOS2* risk polymorphisms in the MI group in comparison to the control group. Even though no significant differences were found, the observations are in accordance with the findings of Sowjanya et al. [91]. They reported that *NOS2*

genotype risk groups in pre-cancerous and cancerous lesions showed higher levels of plasma NO_x and iNOS mRNA. They even detected a correlation of risk genotypes with plasma NO_x levels and tissue iNOS expression. Still, *NOS2* risk polymorphisms were not associated with cervical pre-cancer or cancer.

The findings of the present work did not reveal any influence of genetic polymorphisms on the iNOS mRNA expression. Within the control group, long $(CCTTT)_n$ repeats, as well as T alleles at rs2779249, G>T and at rs2297518, C>T and two or more *NOS2* polymorphisms combined, significantly increased the iNOS protein expression. This tendency towards increased iNOS protein levels was confirmed in the MI group, even though the difference in iNOS protein expression did not reach statistical significance between the different genotypes. Again these results are in accordance with Sowjanya et al. [91], who reported increased tissue iNOS expression in correlation with risk genotypes.

Hsu et al. [84] found an association of homozygous long $(CCTTT)_n$ repeats with atrial fibrillation in a tawainese study cohort and reported increased oxidative stress in patients with long homozygous repeat polymorphisms, which suggests an influence on iNOS expression and subsequent structural remodeling in atrial fibrillation.

This may suggest a predisposition to increased iNOS protein expression and therefor oxidative stress exposure, in carriers of risk *NOS2* polymorphism, upon cardiac event such as MI. Which may in turn lead to poorer outcome after MI.

4.5.2 *NOS2* risk polymorphisms increase serum markers in MI

Serum markers such as MDA and creatinine are extensively studied in the context of MI [94–98]. In the present study increased MDA levels in the serum of the MI group were detected, in comparison to healthy controls. This may suggest increased systemic oxidative stress after MI. However, the increase did not reach statistical significance. The heterogeneity of the MI group, consisting of acute and older infarctions, may be a reason for that. Still the seen enhanced MDA levels could have led to impaired prognosis after MI. According to Yin et al. [96], increased MDA levels after MI may even lead to complicated MI by varying degrees of arrhythmia.

We further detected increased creatinine levels in the MI group, compared to healthy controls. Matts et al. [101] reported that an increase in serum creatinine levels in normotensive, nonobese, normoglycemic survivors of MI without renal disease is an independent predictor of overall and atherosclerotic coronary heart disease mortality. The results in this study reveal that 0,1 mg/dL increment of baseline serum creatinine increase the risk for overall mortality by 36% and of atherosclerotic coronary heart disease mortality by 47%. Sorensen et al. [102] found that mortality in MI patients is increased when renal function is substantially reduced as indicated by creatinine clearance. However, they were not able to explain this phenomenon. Walsh et al. [98] associated elevated serum creatinine in patients with MI at presentation with risk of death over 1 year follow-up.

They hypothesized that in many patients elevated creatinine is the result of chronic renal dysfunction, which increases oxidation of low-density lipoproteins and may result in accelerated atherosclerosis and poor outcomes after MI.

In the current study, data of renal function were not collected, neither in the MI nor in the control group. Hence an influence of renal dysfunction on the elevated creatinine levels in the MI group cannot be ruled out. Still, Sibilitz et al. [97] detected that moderately elevated creatinine levels were associated with increased risk of MI, ischemic heart disease and early death in the general population. They hypothesized that creatinine may contribute to increased atherosclerosis by several mechanisms, such as vascular inflammation, altered macrophage behavior or altered protein turnover.

Levels of NO_x in blood and urine can act as markers of NOS activity [92,103]. Sowjanya et al. [91] found a correlation between iNOS mRNA levels and increased plasma NO_x levels in women with pre-cancerous lesions and invasive cancer. Akiyama et al. [93] detected that NO_x plasma levels were elevated in patients after acute MI and concluded that iNOS in heart muscle is one of the factors involved in this increase.

In the present study an increase of serum NO_x levels was detected in the MI group in comparison to the control group. However, no correlation between tissue iNOS levels and serum or urine NO_x levels was found. Therefore, it cannot be concluded that the enhanced serum NO_x levels are a marker of increased iNOS activity.

Despite, homozygous carriers of the rs2779249, G>T T allele showed strongly increased nitrate serum level, in comparison the heterozygous carriers and wildtype. Even though the MI group consisted of only three homozygous T allele carriers, these results suggest an influence of altered iNOS activity, on serum nitrate levels. Further, homozygous carriers of this T allele also showed significantly increased serum creatinine levels, in comparison to heterozygous carriers and wildtype. A direct link between iNOS expression and creatinine levels could not be found. However, a connection between vascular inflammation, altered macrophage behavior, iNOS and increased creatinine levels seems possible. Additionally, we found that homozygous carriers of long $(CCTTT)_n$ repeats showed higher MDA serum levels than heterozygous carriers or carriers of homozygous short repeats. Altered or increased iNOS activity through long repeats of $(CCTTT)_n$ and subsequently increased oxidative stress, as shown by enhanced serum MDA levels seems plausible.

4.6 Conclusion

The present study revealed an accumulation of *NOS2* risk polymorphisms in the MI group in contrast to healthy controls. We further detected an increase in the number of iNOS protein producing cells in *NOS2* risk polymorphism groups and increased MDA, creatinine and nitrate serum levels seem to be connected to *NOS2* risk polymorphisms. However, further studies are needed to examine the

relationship between cardiac iNOS expression, altered iNOS activity by gene polymorphisms and serum markers of iNOS activity in MI.

5. REFERENCES

1. Yu X, Ge L, Niu L, et al. (2018) The Dual Role of Inducible Nitric Oxide Synthase in Myocardial Ischemia/Reperfusion Injury: Friend or Foe? *Oxidative Medicine and Cellular Longevity* 2018: 1–7.
2. Kim Y, Nurakhayev S, Nurkesh A, et al. (2021) Macrophage Polarization in Cardiac Tissue Repair Following Myocardial Infarction. *IJMS* 22: 2715.
3. Zimmer A, Bagchi AK, Vinayak K, et al. (2019) Innate immune response in the pathogenesis of heart failure in survivors of myocardial infarction. *American Journal of Physiology-Heart and Circulatory Physiology* 316: H435–H445.
4. Pouleur A-C, Barkoudah E, Uno H, et al. (2010) Pathogenesis of Sudden Unexpected Death in a Clinical Trial of Patients With Myocardial Infarction and Left Ventricular Dysfunction, Heart Failure, or Both. *Circulation* 122: 597–602.
5. Mehta LS, Beckie TM, DeVon HA, et al. (2016) Acute Myocardial Infarction in Women: A Scientific Statement From the American Heart Association. *Circulation* 133: 916–947.
6. Aydin S, Ugur K, Aydin S, et al. (2019) Biomarkers in acute myocardial infarction: current perspectives. *VHRM* Volume 15: 1–10.
7. Berezin AE, Berezin AA (2020) Adverse Cardiac Remodelling after Acute Myocardial Infarction: Old and New Biomarkers. *Disease Markers* 2020: 1–21.
8. Vannini F, Kashfi K, Nath N (2015) The dual role of iNOS in cancer. *Redox Biology* 6: 334–343.
9. Feng Q, Lu X, Jones DL, et al. (2001) Increased Inducible Nitric Oxide Synthase Expression Contributes to Myocardial Dysfunction and Higher Mortality After Myocardial Infarction in Mice. *Circulation* 104: 700–704.
10. Heiss C, Rodriguez-Mateos A, Kelm M (2015) Central Role of eNOS in the Maintenance of Endothelial Homeostasis. *Antioxidants & Redox Signaling* 22: 1230–1242.
11. Li H, Horke S, Förstermann U (2014) Vascular oxidative stress, nitric oxide and atherosclerosis. *Atherosclerosis* 237: 208–219.
12. Muller G, Morawietz H (2009) Nitric Oxide, NAD(P)H Oxidase, and Atherosclerosis. *Antioxidants & Redox Signaling* 11: 1711–1731.
13. Strasen J, Ritter O (2011) Role of nNOS in Cardiac Ischemia-Reperfusion Injury. *Trends in Cardiovascular Medicine* 21: 58–63.
14. Lind M, Hayes A, Caprnda M, et al. (2017) Inducible nitric oxide synthase: Good or bad? *Biomedicine & Pharmacotherapy* 93: 370–375.
15. Liu Y-H, Carretero OA, Cingolani OH, et al. (2005) Role of inducible nitric oxide synthase in cardiac function and remodeling in mice with heart failure due to myocardial infarction. *American Journal of Physiology-Heart and Circulatory Physiology* 289: H2616–H2623.
16. Chartrain NA, Geller DA, Koty PP, et al. (1994) Molecular cloning, structure, and chromosomal localization of the human inducible nitric oxide synthase gene. *Journal of Biological Chemistry* 269: 6765–6772.
17. Morris SM, Billiar TR (1994) New insights into the regulation of inducible nitric oxide synthesis. *American Journal of Physiology-Endocrinology and Metabolism* 266: E829–E839.
18. Lin K, Baritaki S, Vivarelli S, et al. (2022) The Breast Cancer Protooncogenes HER2, BRCA1 and BRCA2 and Their Regulation by the iNOS/NOS2 Axis. *Antioxidants* 11: 1195.
19. Weigert A, Brüne B (2008) Nitric oxide, apoptosis and macrophage polarization during tumor progression. *Nitric Oxide* 19: 95–102.
20. Liu Y, Xu R, Gu H, et al. (2021) Metabolic reprogramming in macrophage responses. *Biomark Res* 9: 1.
21. Qidwai T, Jamal F (2010) Inducible Nitric Oxide Synthase (iNOS) Gene Polymorphism and Disease Prevalence: Inducible Nitric Oxide Synthase. *Scandinavian Journal of Immunology* 72: 375–387.
22. Hobbs MR, Udhayakumar V, Levesque MC, et al. (2002) A new NOS2 promoter polymorphism associated with increased nitric oxide production and protection from severe malaria in Tanzanian and Kenyan children. *The Lancet* 360: 1468–1475.

23. Kashfi K, Kannikal J, Nath N (2021) Macrophage Reprogramming and Cancer Therapeutics: Role of iNOS-Derived NO. *Cells* 10: 3194.
24. de la Cruz-Ojeda P, Flores-Campos R, Dios-Barbeito S, et al. (2021) Role of Nitric Oxide in Gene Expression Regulation during Cancer: Epigenetic Modifications and Non-Coding RNAs. *IJMS* 22: 6264.
25. Gredic M, Wu C-Y, Hadzic S, et al. (2022) Myeloid-cell-specific deletion of inducible nitric oxide synthase protects against smoke-induced pulmonary hypertension in mice. *Eur Respir J* 59: 2101153.
26. Suzuki H, Wildhirt SM, Dudek RR, et al. (1996) Induction of apoptosis in myocardial infarction and its possible relationship to nitric oxide synthase in macrophages. *Tissue and Cell* 28: 89–97.
27. Petros A, Lamb G, Leone A, et al. (1994) Effects of a nitric oxide synthase inhibitor in humans with septic shock. *Cardiovascular Research* 28: 34–39.
28. Chauhan SD, Seggara G, Vo PA, et al. (2003) Protection against lipopolysaccharide-induced endothelial dysfunction in resistance and conduit vasculature of iNOS knockout mice. *FASEB j* 17: 773–775.
29. Mungrue IN, Gros R, You X, et al. (2002) Cardiomyocyte overexpression of iNOS in mice results in peroxynitrite generation, heart block, and sudden death. *J Clin Invest* 109: 735–743.
30. Soskic SS (2011) Regulation of Inducible Nitric Oxide Synthase (iNOS) and its Potential Role in Insulin Resistance, Diabetes and Heart Failure. *TOCMJ* 5: 153–163.
31. Searles CD (2002) The Nitric Oxide Pathway and Oxidative Stress in Heart Failure. *Congestive Heart Failure* 8: 142–155.
32. Gori T, Münzel T (2011) Oxidative stress and endothelial dysfunction: Therapeutic implications. *Annals of Medicine* 43: 259–272.
33. Jones DP (2008) Radical-free biology of oxidative stress. *American Journal of Physiology-Cell Physiology* 295: C849–C868.
34. Münzel T, Gori T, Bruno RM, et al. (2010) Is oxidative stress a therapeutic target in cardiovascular disease? *European Heart Journal* 31: 2741–2748.
35. Gosslau A, Rensing L (2002) Oxidativer Stress, altersabhängige Zellschädigungen und antioxidative Mechanismen. *Z Gerontol Geriat* 35: 139–150.
36. Takimoto Y, Aoyama T, Keyamura R, et al. (2000) Differential expression of three types of nitric oxide synthase in both infarcted and non-infarcted left ventricles after myocardial infarction in the rat. *International Journal of Cardiology* 76: 135–145.
37. Shears LL, Kawaharada N, Tzeng E, et al. (1997) Inducible nitric oxide synthase suppresses the development of allograft arteriosclerosis. *J Clin Invest* 100: 2035–2042.
38. Brunner M, Gruber M, Schmid D, et al. (2015) Proliferation of macrophages due to the inhibition of inducible nitric oxide synthesis by oxidized low-density lipoproteins. *EXCLI Journal; 14:Doc439; ISSN 1611-2156*.
39. Nussler AK, Billiar TR (1993) Inflammation, immunoregulation, and inducible nitric oxide synthase. *Journal of Leukocyte Biology* 54: 171–178.
40. Wilmes V, Kur IM, Weigert A, et al. (2023) iNOS expressing macrophages co-localize with nitrotyrosine staining after myocardial infarction in humans. *Front Cardiovasc Med* 10: 1104019.
41. Zhang P, Xu X, Hu X, et al. (2007) Inducible Nitric Oxide Synthase Deficiency Protects the Heart From Systolic Overload–Induced Ventricular Hypertrophy and Congestive Heart Failure. *Circulation Research* 100: 1089–1098.
42. Vinten-Johansen J (2000) Physiological Effects of Peroxynitrite: Potential Products of the Environment. *Circulation Research* 87: 170–172.
43. Fysikopoulos A, Seimetz M, Hadzic S, et al. (2021) Amelioration of elastase-induced lung emphysema and reversal of pulmonary hypertension by pharmacological iNOS inhibition in mice. *Br J Pharmacol* 178: 152–171.

44. Whiteman M, Halliwell B (1997) Prevention of Peroxynitrite-Dependent Tyrosine Nitration and Inactivation of α_1-Antiproteinase by Antibiotics. *Free Radical Research* 26: 49–56.
45. Okamoto T, Akaike T, Sawa T, et al. (2001) Activation of Matrix Metalloproteinases by Peroxynitrite-induced Protein S-Glutathiolation via Disulfide S-Oxide Formation. *Journal of Biological Chemistry* 276: 29596–29602.
46. Creemers EEJM, Cleutjens JPM, Smits JFM, et al. (2001) Matrix Metalloproteinase Inhibition After Myocardial Infarction: A New Approach to Prevent Heart Failure? *Circulation Research* 89: 201–210.
47. Turillazzi E, Cerretani D, Cantatore S, et al. (2017) Myocardial oxidative damage is induced by cardiac Fas-dependent and mitochondria-dependent apoptotic pathways in human cocaine-related overdose. *Sci Rep* 7: 44262.
48. Frustaci A, Russo MA, Morgante E, et al. (2015) Oxidative myocardial damage in human cocaine-related cardiomyopathy: Oxidative stress in cocaine cardiomyopathy. *Eur J Heart Fail* 17: 283–290.
49. Wildhirt SM, Dudek RR, Suzuki H, et al. (1995) Involvement of inducible nitric oxide synthase in the inflammatory process of myocardial infarction. *International Journal of Cardiology* 50: 253–261.
50. Troidl C, Möllmann H, Nef H, et al. (2009) Classically and alternatively activated macrophages contribute to tissue remodelling after myocardial infarction. *Journal of Cellular and Molecular Medicine* 13: 3485–3496.
51. Frantz S, Nahrendorf M (2014) Cardiac macrophages and their role in ischaemic heart disease. *Cardiovascular Research* 102: 240–248.
52. Duncan SE, Gao S, Sarhene M, et al. (2020) Macrophage Activities in Myocardial Infarction and Heart Failure. *Cardiology Research and Practice* 2020: 1–16.
53. Ma Y, Mouton AJ, Lindsey ML (2018) Cardiac macrophage biology in the steady-state heart, the aging heart, and following myocardial infarction. *Translational Research* 191: 15–28.
54. Heidt T, Courties G, Dutta P, et al. (2014) Differential Contribution of Monocytes to Heart Macrophages in Steady-State and After Myocardial Infarction. *Circ Res* 115: 284–295.
55. Novak ML, Koh TJ (2013) Macrophage phenotypes during tissue repair. *Journal of Leukocyte Biology* 93: 875–881.
56. Shimojo T, Nishikawa T, Ishiyama S, et al. (1998) Participation of Nitric Oxide and Peroxynitrite in the Development of Myocardial Tissue Damage in Acute Myocardial Infarction. *Cardiovascular Pathology* 7: 25–30.
57. Wilmes V, Lux C, Niess C, et al. (2020) Changes in gene expression patterns in postmortem human myocardial infarction. *Int J Legal Med* 134: 1753–1763.
58. Koppelkamm A, Vennemann B, Fracasso T, et al. (2010) Validation of adequate endogenous reference genes for the normalisation of qPCR gene expression data in human post mortem tissue. *Int J Legal Med* 124: 371–380.
59. Pilbrow AP, Ellmers LJ, Black MA, et al. (2008) Genomic selection of reference genes for real-time PCR in human myocardium. *BMC Med Genomics* 1: 64.
60. Ramakers C, Ruijter JM, Deprez RHL, et al. (2003) Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters* 339: 62–66.
61. Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative CT method. *Nat Protoc* 3: 1101–1108.
62. Ramanujam D, Schön AP, Beck C, et al. (2021) MicroRNA-21–Dependent Macrophage-to-Fibroblast Signaling Determines the Cardiac Response to Pressure Overload. *Circulation* 143: 1513–1525.
63. Silverman MG, Yeri A, Moorthy MV, et al. (2020) Circulating miRNAs and Risk of Sudden Death in Patients With Coronary Heart Disease. *JACC: Clinical Electrophysiology* 6: 70–79.
64. Colpaert RMW, Calore M (2019) MicroRNAs in Cardiac Diseases. *Cells* 8: 737.
65. Sun T, Dong Y-H, Du W, et al. (2017) The Role of MicroRNAs in Myocardial Infarction: From Molecular Mechanism to Clinical Application. *IJMS* 18: 745.

66. Kura B, Szeiffova Bacova B, Kalocayova B, et al. (2020) Oxidative Stress-Responsive MicroRNAs in Heart Injury. *IJMS* 21: 358.
67. Dlouhá D, Hubáček JA (2017) Regulatory RNAs and Cardiovascular Disease – With a Special Focus on Circulating MicroRNAs. *Physiol Res* S21–S38.
68. Yuan J, Chen H, Ge D, et al. (2017) Mir-21 Promotes Cardiac Fibrosis After Myocardial Infarction Via Targeting Smad7. *Cell Physiol Biochem* 42: 2207–2219.
69. Hinkel R, Ramanujam D, Kaczmarek V, et al. (2020) AntimiR-21 Prevents Myocardial Dysfunction in a Pig Model of Ischemia/Reperfusion Injury. *Journal of the American College of Cardiology* 75: 1788–1800.
70. Keller T, Boeckel J-N, Groß S, et al. (2017) Improved risk stratification in prevention by use of a panel of selected circulating microRNAs. *Sci Rep* 7: 4511.
71. Pinchi E, Frati P, Aromatario M, et al. (2019) miR-1, miR-499 and miR-208 are sensitive markers to diagnose sudden death due to early acute myocardial infarction. *J Cell Mol Med* 23: 6005–6016.
72. Guo Z, Shao L, Zheng L, et al. (2012) miRNA-939 regulates human inducible nitric oxide synthase posttranscriptional gene expression in human hepatocytes. *Proc Natl Acad Sci USA* 109: 5826–5831.
73. Warren SE, Royal HD, Markis JE, et al. (1988) Time course of left ventricular dilation after myocardial infarction: Influence of infarct-related artery and success of coronary thrombolysis. *Journal of the American College of Cardiology* 11: 12–19.
74. Foster CR, Daniel LL, Daniels CR, et al. (2013) Deficiency of Ataxia Telangiectasia Mutated Kinase Modulates Cardiac Remodeling Following Myocardial Infarction: Involvement in Fibrosis and Apoptosis. *PLoS ONE* 8: e83513.
75. Chen Y, Yin Y, Jiang H (2021) miR-30e-5p Alleviates Inflammation and Cardiac Dysfunction After Myocardial Infarction Through Targeting PTEN. *Inflammation* 44: 769–779.
76. Masè M, Grasso M, Avogaro L, et al. (2017) Selection of reference genes is critical for miRNA expression analysis in human cardiac tissue. A focus on atrial fibrillation. *Sci Rep* 7: 41127.
77. Bejerano T, Etzion S, Elyagon S, et al. (2018) Nanoparticle Delivery of miRNA-21 Mimic to Cardiac Macrophages Improves Myocardial Remodeling after Myocardial Infarction. *Nano Lett* 18: 5885–5891.
78. Li H, Liao Y, Gao L, et al. (2018) Coronary Serum Exosomes Derived from Patients with Myocardial Ischemia Regulate Angiogenesis through the miR-939-mediated Nitric Oxide Signaling Pathway. *Theranostics* 8: 2079–2093.
79. Chen C, Zong M, Lu Y, et al. (2020) Differentially expressed lnc-NOS2P3-miR-939-5p axis in chronic heart failure inhibits myocardial and endothelial cells apoptosis via iNOS/TNFα pathway. *J Cell Mol Med* 24: 11381–11396.
80. Pu L, Kong X, Li H, et al. (2021) Exosomes released from mesenchymal stem cells overexpressing microRNA-30e ameliorate heart failure in rats with myocardial infarction. *Am J Transl Res* 13: 4007–4025.
81. Yiping Wang, Yingqiong Huang, Min Zhang, et al. (2018) Bioinformatic Analysis of the Possible Regulative Network of miR-30a/e in Cardiomyocytes 2 Days Post Myocardial Infarction. *Acta Cardiologica Sinica* 34.
82. Cheng N, Li L, Wu Y, et al. (2021) microRNA-30e up-regulation alleviates myocardial ischemia-reperfusion injury and promotes ventricular remodeling via SOX9 repression. *Molecular Immunology* 130: 96–103.
83. Kröncke K-D, Fehsel K, Kolb-Bachofen V (2001) Inducible nitric oxide synthase in human diseases. *Clinical and Experimental Immunology* 113: 147–156.
84. Hsu L-A, Yeh Y-H, Chen W-J, et al. (2017) Association evidence of CCTTT repeat polymorphism in the iNOS promoter and the risk of atrial fibrillation in Taiwanese. *Sci Rep* 7: 42388.
85. Xu W, Humphries S, Tomita M, et al. (2000) Survey of the Allelic Frequency of a NOS2A Promoter Microsatellite in Human Populations: Assessment of the NOS2A Gene and Predisposition to Infectious Disease. *Nitric Oxide* 4: 379–383.

86. Morris BJ, Markus AM, Glenn CL, et al. (2002) Association of a functional inducible nitric oxide synthase promoter variant with complications in type 2 diabetes. *J Mol Med* 80: 96–104.
87. Yocum GT, Gaudet JG, Lee SS, et al. (2009) Inducible Nitric Oxide Synthase Promoter Polymorphism Affords Protection Against Cognitive Dysfunction After Carotid Endarterectomy. *Stroke* 40: 1597–1603.
88. Nikkari ST, Määttä KM, Kunnas TA (2015) Functional Inducible Nitric Oxide Synthase Gene Variants Associate With Hypertension: A Case–Control Study in a Finnish Population—The TAMRISK Study. *Medicine* 94: e1958.
89. Fu L, Zhao Y, Lu J, et al. (2009) Functional single nucleotide polymorphism-1026C/A of inducible nitric oxide synthase gene with increased YY1-binding affinity is associated with hypertension in a Chinese Han population. *Journal of Hypertension* 27: 991–1000.
90. Dhillon SS, Mastropaolo LA, Murchie R, et al. (2014) Higher Activity of the Inducible Nitric Oxide Synthase Contributes to Very Early Onset Inflammatory Bowel Disease. *Clinical and Translational Gastroenterology* 5: e46.
91. Sowjanya AP, Rao M, Vedantham H, et al. (2016) Correlation of plasma nitrite/nitrate levels and inducible nitric oxide gene expression among women with cervical abnormalities and cancer. *Nitric Oxide* 52: 21–28.
92. Bryan NS (2006) Nitrite in nitric oxide biology: Cause or consequence? *Free Radical Biology and Medicine* 41: 691–701.
93. Akiyama K, Kimura A, Suzuki H, et al. (1998) Production of oxidative products of nitric oxide in infarcted human heart. *Journal of the American College of Cardiology* 32: 373–379.
94. Sigala F, Kotsinas A, Savari P, et al. (2010) Oxidized LDL in human carotid plaques is related to symptomatic carotid disease and lesion instability. *Journal of Vascular Surgery* 52: 704–713.
95. Madole MB, Bachewar NP, Aiyar CM (2015) Study of oxidants and antioxidants in patients of acute myocardial infarction. *Adv Biomed Res* 4: 241.
96. Yin Y, Han W, Cao Y (2019) Association between activities of SOD, MDA and Na+-K+-ATPase in peripheral blood of patients with acute myocardial infarction and the complication of varying degrees of arrhythmia. *Hellenic Journal of Cardiology* 60: 366–371.
97. Sibilitz KL, Benn M, Nordestgaard BG (2014) Creatinine, eGFR and association with myocardial infarction, ischemic heart disease and early death in the general population. *Atherosclerosis* 237: 67–75.
98. Walsh CR, O'Donnell CJ, Camargo CA, et al. (2002) Elevated serum creatinine is associated with 1-year mortality after acute myocardial infarction. *American Heart Journal* 144: 1003–1011.
99. Hanff E, Eisenga MF, Beckmann B, et al. (2017) Simultaneous pentafluorobenzyl derivatization and GC-ECNICI-MS measurement of nitrite and malondialdehyde in human urine: Close positive correlation between these disparate oxidative stress biomarkers. *Journal of Chromatography B* 1043: 167–175.
100. Hanff E, Lützow M, Kayacelebi AA, et al. (2017) Simultaneous GC-ECNICI-MS measurement of nitrite, nitrate and creatinine in human urine and plasma in clinical settings. *Journal of Chromatography B* 1047: 207–214.
101. Matts JP, Karnegis JN, Campos CT, et al. (1993) Serum creatinine as an independent predictor of coronary heart disease mortality in normotensive survivors of myocardial infarction. POSCH Group. *J Fam Pract* 36: 497–503.
102. Sørensen C (2002) The prognostic importance of creatinine clearance after acute myocardial infarction. *European Heart Journal* 23: 948–952.
103. Kleinbongard P, Dejam A, Lauer T, et al. (2003) Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free Radic Biol Med* 35: 790–796.

SUMMARY

Cardiovascular diseases (CVDs), which include disorders of the heart and its blood vessels, such as myocardial infarction (MI), are the leading cause of death globally. During MI necrosis of myocardial cells leads to inflammation and fibrosis. Cardiac scarring and subsequently adverse remodeling, negatively impact myocardial healing and may lead to further disease states after MI, such as hypertension, heart failure, and death. While elevated levels of cardiac-specific troponins (cTnT and cTnI), as well as cardiac specific creatine kinase (CK-MB) are central biomarkers for the diagnosis of MI, interleukins, natriuretic peptides, matrix metallopeptidases (MMPs) and noncoding RNAs are currently discussed as biomarkers for adverse cardiac remodeling after MI. Nevertheless, a thorough understanding of the molecular processes during MI may lead to further biomarkers or improved therapy options.

Inducible nitric oxide synthase (iNOS) is one of the three NOS isoforms that produce nitric oxide (NO). It is induced by stimuli such as inflammation and hypoxia and has been described in patients with end-stage heart failure, cardiomyopathy and ischemic heart disease. In murine and rabbit models of MI increased iNOS expression is suggested to contribute to left ventricular dysfunction, heart failure progression, myocardial injury and extent of infarct size, even late after ischemia and reperfusion. These detrimental effects are due to the reaction of iNOS derived NO with superoxide anion (O_2^-), which leads to reactive intermediates that can nitrate, nitrosate or oxidize their biological environment. This oxidative nitrosative stress promotes DNA damage, suppresion of DNA-repair enzymes and post-translational modifications of proteins. Specifically, peroxynitrite ($ONOO^-$) is formed by the reaction of NO with O_2^- and is a strong oxidant causing nitrosylation of proteins and the formation of nitrotyrosine. Further peroxynitrite inactivates anti-proteases and activates MMPs, that are known for degrading and remodeling extracellular matrix under physiological and pathological conditions, leading to left ventricular remodeling after MI. Hence, understanding the role of iNOS in MI may lead to improved therapy options and to the development of biomarkers for the progression of heart failure after MI.

In the present thesis the role of iNOS in human MI is thoroughly examined for the first time. It includes genetic and cellular approaches to determine the iNOS expression and factors influencing iNOS expression in human MI tissue. Further, methods of measuring iNOS derived oxidative stress are established. Especially, the present work emphasizes the differences in iNOS expression between animal models of MI and human MI.

The first study focused on examining iNOS mRNA and protein levels in postmortem human MI hearts. A significant increase of iNOS mRNA in infarcted and non-infarcted tissue was detected, in comparison to healthy controls. Further, significantly increased iNOS protein levels were found in infarcted and non-infarcted regions. Interestingly, iNOS was predominantly found in “M2” resident macrophages and to a smaller amount in “M1” inflammatory macrophages. Accordingly, the

significant increase in iNOS protein in infarcted regions was seen in the “M2” resident macrophages. In cardiomyocytes no iNOS protein expression was detected. A distance analysis between iNOS+ and nitrotyrosine (NT)+ cells revealed that NT+ cells peak within 10-15 µm of iNOS, suggesting a dependence and a range of activity by iNOS produced NO.

The second study examined microRNAs as posttranscriptional regulators of the iNOS expression. The expression of miR-939, miR-21 and miR-30e was measured and correlated with iNOS mRNA and protein levels in postmortem human MI hearts. miR-939 is known to decrease cytokine induced iNOS protein expression and NO synthesis in human hepatocytes. miR-21 has its strongest expression in cardiac macrophages in mice, where it is the strongest expressed miRNA among all cardiac miRNAs. The function of miR-21 in fibroblasts comprises of regulating fibroblast proliferation and fibrosis. miR-30e is described as downregulated in hypoxic cardiomyocytes and MI tissue of rats to protect the myocardium via enhancing cardiomyocyte viability and inhibiting apoptosis. The results revealed significant upregulation of miR-939, miR-21 and miR-30e in infarcted and non-infarcted regions of postmortem human MI hearts in comparison to healthy controls. Correlation analysis showed a significant correlation between miR-939 expression and the iNOS mRNA expression in the control group and the infarcted regions, which was more pronounced in the controls. A massive iNOS activation might exceed the capability of miR-939 to keep its expression in balance. On the other hand, miR-30e and miR-21 did not seem to influence cardiac iNOS levels in MI or macrophage polarization.

In the third study, the iNOS gene *NOS2* was genotyped for the $(CCTTT)_n$ repeat polymorphism in the promoter region, the rs2779249, G>T single nucleotid polymorphism (SNP) -1026 basepairs (bp) before transcription start and the rs2297518, C>T SNP at exon 16. These polymorphisms are known to influence the iNOS expression and subsequently oxidative stress in atrial fibrilliation and hypertension amongst others. In the MI group we found an accumulation of *NOS2* risk polymorphisms, such as long $(CCTTT)_n$ repeat polymorphisms or the T alleles at rs2779249, G>T and rs2297518, C>T in contrast to healthy controls. Additionally, we detected an increase in iNOS protein levels, in the control and MI group, when *NOS2* risk polymorphisms were present.

Serum levels of malondialdehyde (MDA), a marker of oxidative stress, of creatinine, an independent risk factor for and after MI and of nitrate/nitrite (NO_x), which are known the reflect the activity of iNOS, were increased in the MI group in comparison to the controls. Further, in the MI group homozygous carriers of the long $(CCTTT)_n$ repeat had increased serum MDA levels in comparison to heterozygote carriers or carriers of short $(CCTTT)_n$ repeats. Homozygous carriers of the T allele at rs2779249, G>T in the MI group showed increased serum levels of nitrate and creatinine. This suggests a connection between serum markers and *NOS2* risk polymorphism in MI, however, no direct influence of risk polymorphisms on cardiac iNOS levels was observed.

In conclusion the present thesis revealed increased iNOS mRNA expression in postmortem human infarction hearts and increased iNOS protein production. Interestingly, iNOS is predominantly

expressed in “M2”-resident macrophages and upon MI, the iNOS production is even more increased in these macrophages. Since NT+ cells peak within 10-15µm of iNOS+ cells, this could represent an activity range of iNOS and subsequent oxidative stress production within that perimeter.

miR-939 seems to regulate the iNOS expression posttranscriptional, however, upon MI this regulation is not efficiently to keep the iNOS mRNA level at bay.

Risk polymorphisms in the *NOS2* gene accumulate in the MI group and seem to enhance iNOS protein expression. This may lead to increased oxidative stress and adverse cardiac remodeling after MI. The seen increased serum levels of MDA, creatinine and NO_x in the MI group could not be correlated with cardiac iNOS mRNA and protein levels. However, long $(CCTTT)_n$ repeats and the homozygous T-allele at rs2779249, G>T seem to influence serum MDA, creatinine and nitrate level in the MI group. The role of iNOS in MI seems complex, however we could reveal increased oxidative stress upon MI, by increased iNOS production. Hence, increased iNOS expression and activity due to certain risk polymorphisms may impair the healing process after MI, lead to adverse remodeling and further heart diseases such as heart failure.

ZUSAMMENFASSUNG

Kardiovaskuläre Erkrankungen, zu denen der Myokardinfarkt gehört, sind weltweit die führende Todesursache. Beim Myokardinfarkt kommt es zur Nekrose von Kardiomyozyten, was zu Entzündungen und Fibrose führt. Die Folgen sind ein Umbau des Gewebes und Narbengewebe, welche den Heilungsprozess beeinträchtigen und zu weiteren Erkrankungen wie Herzrhythmusstörungen und Herzinsuffizienz führen können. Erhöhte Blutkonzentrationen von myokardialem Troponin (cTnT und cTnI) und der Creatinkinase (CK-MB) sind zentrale Biomarker bei der Diagnostizierung eines Herzinfarktes. Zur Erkennung des kardialen Remodeling und der möglichen Folgeerkrankungen werden Interleukine, natiuretische Peptide, Matrixmetallopeptidasen und nichtkodierende RNAs erforscht. Ein verbessertes Verständnis der molekularen Prozesse bei Myokardinfarkt kann zur Entdeckung weiterer Biomarker oder zu weiteren Therapieoptionen nach Myokardinfarkt beitragen.

Die induzierbare Stickoxidsynthase (iNOS) ist eine der drei NOS Isoformen, die Stickstoffmonoxid (NO) produzieren. iNOS wird durch Entzündungen oder Hypoxie induziert und die iNOS Expression wurde in Patienten mit diversen kardiovaskulären Erkrankungen nachgewiesen. Zur iNOS Expression im Myokardinfarkt gibt es bislang nur Tierstudien, die einen Beitrag der iNOS zur linksventrikulären Dysfunktion, fortschreitender Herzinsuffizienz, myokardialen Verletzungen und größeren Infarktnarben beschreiben. Diese schädlichen Effekte der iNOS werden der Reaktion von NO mit dem Superoxidanion (O_2^-) zugeschrieben, was die Entstehung von Peroxynitrit ($ONOO^-$) zur Folge hat. Peroxynitrit hat wie alle Sauerstoffradiale eine Nitrierung oder Oxidierung der biologischen Umgebung zur Folge. Oxidativer bzw. nitrosativer Stress dieser Art schädigt die DNA, DNA-Reparaturenzyme und führt zu posttranslationalen Proteinmodifizierungen. Insbesondere Peroxynitrit führt zur Nitrosylierung von Tyrosinresten der Proteine, wodurch Nitrotyrosin entsteht. Des Weiteren aktiviert Peroxynitrit die Matrixmetallopeptidasen, was einen Ab- und Umbau der extrazellulären Matrix zur Folge hat, wodurch linksventrikuläres Remodeling entstehen kann. Dementsprechend stellt ein verbessertes Verständnis der Rolle der iNOS bei Myokardinfarkt einen wichtigen Baustein dar, für verbesserte Therapieoptionen, aber auch für die Entwicklung von Biomarkern für fortschreitende Herzinsuffizenz nach Myokardinfarkt.

In der vorliegenden Doktorarbeit wurde zum ersten Mal die Rolle der iNOS im humanen Myokardinfarkt untersucht. Es wurden sowohl genetische, als auch zelluläre Methoden angewendet, um die iNOS Expression, sowie regulierende Faktoren der iNOS Expression zu bestimmen. Außerdem wurden Methoden etabliert, um den iNOS abhängigen oxidativen Stress zu messen. Die Ergebnisse der Arbeit weisen insbesondere auf die Unterschiede in der iNOS Expression zwischen Tiermodellen und humanem Myokardinfarkt hin.

In einer ersten Studie lag der Fokus auf der Bestimmung und Quantifizierung der iNOS mRNA und des iNOS Proteins in postmortalen humanen Infarktherzen. Ein signifikanter Anstieg der iNOS mRNA

in infarzierten und nicht infarzierten Regionen, im Vergleich zu gesunden Kontrollherzen wurde festgestellt. Zusätzlich wurde auch ein signifikanter Anstieg des iNOS Proteins in infarzierten und nicht-infarzierten Regionen beobachtet. Hauptsächlich wurden gewebeständige “M2” Makrophagen als die iNOS exprimierenden Zellen im humanen Myokardinfarkt festgestellt, zu einem kleineren Anteil fand sich die iNOS Expression auch in inflammatorischen “M1” Makrophagen. Dementsprechend war der signifikante Anstieg der iNOS Protein Expression in den infarzierten Regionen in den gewebeständigen Makrophagen zu finden. Die Kardiomyozyten wiesen dagegen keine iNOS Expression auf. Abschließend wurde eine Distanzanalyse zwischen iNOS+ und Nitrotyrosin+ Zellen durchgeführt, die einen Höchstwert an Nitrotyrosin+ Zellen im Umkreis von 10-15µm von iNOS exprimierenden Zellen aufwies. Damit ist von einem Aktivitätsradius der iNOS in diesem Umkreis auszugehen und der durch Nitrotyrosin nachgewiesene oxidative Stress möglicherweise auf iNOS zurückzuführen.

In einer zweiten Studie wurden micro RNAs (miRNAs) als mögliche posttranskriptionale Regulatoren der iNOS Expression untersucht. In human Hepatozyten ist beschrieben, dass miR-939 die Zytokin-induzierte iNOS Protein Expression, sowie die NO-Synthese verringert. miR-21 ist die am stärksten exprimierte kardiale miRNA und vorranging in kardialen Makrophagen zu finden. Auch in Fibroblasten ist miR-21 exprimiert und reguliert die Proliferation der Fibroblasten. miR-30 ist herunterreguliert in hypoxischen Kardiomyozyten und in Infarktgewebe von Rattenherzen, um das Myokardium u.a. durch ein Inhibieren der Apoptose zu schützen. Die Expressionen von miR-939, miR-21 und miR-30e wurden gemessen und mit iNOS mRNA und Protein Expression korreliert. Die Ergebnisse zeigen eine signifikante Hochregulierung der miR-939, miR-21 und miR-30e in infarzierten und nicht-infarzierten Regionen der postmortalen humanen Infarktherzen im Vergleich zu gesunden Kontrollen. Korrelationsanalysen zeigten eine signfikante Korrelation zwischen miR-939 Expression und der iNOS Genexpression in der Kontrollgruppe und den infarzierten Regionen. In der Kontrollgruppe war die Korrelation stärker ausgeprägt. Eine massive iNOS Aktivierung in den infarzierten Regionen könnte die Kapazität von miR-939 übersteigen, die iNOS Expression in Balance zu halten. miR-21 und miR-30e zeigten keinen Einfluss auf die iNOS Expression oder die Makrophagenpolarisierung.

In einer dritten Studie wurde das iNOS Gen *NOS2* genotypisiert auf den $(CCTTT)_n$ Längenpolymorphismus in der Promotorregion, den rs2779249, G>T Single Nukleotid Polymorphismus (SNP) -1026 Basenpaare (bp) vor Transkriptionsstart und den rs2297518, C>T SNP in Exon 16. Diese Polymorphismen beeinflussen die iNOS Expression und oxidativen Stress u.a. bei Vorhofflimmern und Bluthochdruck. In der Infarktgruppe wurde eine Häufung der *NOS2* Risikopolymorphismen, wie längere $(CCTTT)_n$ Längenpolymorphismen und T-Allele an den Positionen rs2779249, G>T und rs2297518, C>T im Vergleich zur Kontrollgruppe gefunden. Zusätzlich wurde eine Anstieg an iNOS Protein gefunden, sowohl in der Kontroll- als auch in der Infarktgruppe, wenn *NOS2* Risikopolymorphismen vorhanden waren. Serumkonzentrationen von

Malondialdehyd (MDA), einem Marker für oxidativen Stress, von Kreatinin, einem unabhängigen Risikofaktor für und nach Myokardinfarkt, und von Nitrat/Nitrit (NO_x), die die Aktivität der iNOS reflektieren, waren in der Infarktgruppe erhöht, im Vergleich zur Kontrollgruppe. Des Weiteren hatten homozygote Träger von längeren $(CCTTT)_n$ Längenpolymorphismen in der Infarktgruppe höhere Serum MDA Konzentrationen als heterozygote Träger oder homozygote Träger von kürzeren $(CCTTT)_n$ Längenpolymorphismen. Homozygote Träger des T-Allels an Position rs2779249, G>T zeigten in der Infarktgruppe erhöhte Serumkonzentrationen von Nitrat und Kreatinin. Das lässt auf einen Zusammenhang zwischen Serummarkern und *NOS2* Risikopolymorphismen bei Myokardinfarkt schließen, dennoch wurde kein direkter Einfluss von *NOS2* Risikopolymorphismen auf kardiale iNOS Level beobachtet.

Zusammenfassend zeigt die vorliegende Doktorarbeit einen signifikanten Anstieg der iNOS Expression in postmortalen humanen Infarktherzen, die vorranging von den gewebeständigen "M2" Makrophagen ausgeht. Außerdem konnte ein Aktivitätsradius der iNOS im Umkreis von 10-15µm detektiert werden, in dem es zu einem Höchstwert an Nitrotyrosin+ Zellen kommt. miR-939 scheint die iNOS Expression posttranskriptional zu regulieren, aber bei Myokardinfarkt übersteigt die iNOS Aktivität den regulatorischen Effekt von miR-939. Des Weiteren gibt es eine Häufung von *NOS2* Risikopolymorphismen in der Infarktgruppe, die die iNOS Protein Expression erhöhen. Das kann zu gesteigertem oxidativem Stress und einem kardialen Remodeling nach Myokardinfarkt führen. Die in der Infarktgruppe festgestellten erhöhten Serumkonzentrationen von MDA, Kreatinin und NO_x konnten weder mit kardialer iNOS mRNA oder Protein Expression korreliert werden, allerdings scheinen längere $(CCTTT)_n$ Längenpolymorphismen und homozygote T Allele an Position rs2779249, G>T die Serummarker zu beeinflussen.

Die Rolle der iNOS bei humanem Myokardinfarkt ist komplex, allerdings konnten wir gesteigerten oxidativen Stress im Gewebe nachweisen, der direkt auf die erhöhte iNOS Produktion zurückzuführen ist. Demnach könnte eine erhöhte iNOS Aktivität aufgrund gewisser Risikopolymorphismen den Heilungsprozess nach Myokardinfarkt beeinflussen, zu kardialem Remodeling führen und somit Folgeerkrankungen begünstigen wie eine chronische Herzinsuffizienz.

LIST OF FIGURES

LIST OF ABBREVIATIONS

bp	basepair
BSA	Bovine Serum Albumin
cDNA	complementary DNA
CK-MB	Cardiac Specific Creatine Kinase
COPD	Chronic Obstructive Pulmonary Disease
CVD	Cardiovascular Diseases
cTnI	Inhibitory Cardiac Specific Troponin
cTnT	Cardiac Specific Troponin T
DAPI	4′,6-diamidino-2-phenylindole
DNA	desoxyribonucleid acid
eNOS	endothelial Nitric Oxide Synthase
FFPE	Formalin-fixed paraffin embedded
GC-MS	Gas-Chromatography/Mass Spectrometry
HRP	Horseradish Peroxidase
iNOS	Inducible Nitric Oxide Synthase
IHC	immunhistological
IPC	Ischemic Preconditioning
MDA	Malondialdehyde
MI	Myocardial Infarction
miRNAs	micro ribonucleid acids
MMP	Matrix Metallopeptidases
mRNA	messenger ribonucleid acid
nNOS	Neuronal Nitric Oxide Synthase

NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NOS2	nitric oxide synthase 2 gene
NO_x	Nitrate and nitrite levels
NT	Nitrotyrosine
PASMCs	pulmonary artery smooth muscle cells
PBS	Phospahte Buffered Saline
PCR	Polymerase Chain Reaction
PMI	postmortem interval
PPIA	Peptidylprolyl isomerase A
PTEN	Phosphatase and Tensin homologue
qPCR	Quantitative real time PCR
RNA	ribonucleid acid
SCD	Sudden Cardiac Death
SNP	Single Nucleotid Polymorphism
TBP	TATA-box binding protein
TBS	Tris Buffered Saline
TPT1	tumor protein translationally controlled 1
WHO	World Health Organization
3'UTR	3' untranslated region

OWN WORK

Experiments, data analysis and writing of the present thesis were done by myself, with exception of the following:

- The immunhistological staining of iNOS was done by Christine Elbert, technician at the Institute of Legal Medicine in Frankfurt, Goethe University, Frankfurt am Main, Germany
- The immunhistological staining of CD68 was done by technicians at the Dr. Senckenberg Institute of Pathology, University Hospital Frankfurt, Goethe University, Frankfurt am Main, Germany
- Immunofluorescence stainings with the BOND-RX Multiplex Stainer system were carried out by technicians at the Institute of Biochemistry I, Faculty of Medicine, Goethe University, Frankfurt am Main, Germany
- GC-MS analysis and data evaluation were carried out in the working group of Prof. Dr. Dimitrios Tsikas at the Institute of Clinical Pharmacology, Hannover Medical School

EHRENWÖRTLICHE ERKLÄRUNG

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit entsprechend den Regeln guter wissenschaftlicher Praxis selbstständig und ohne unzulässige Hilfe Dritter angefertigt habe.

Sämtliche aus fremden Quellen direkt oder indirect übernommenen Gedanken, sowie sämtliche von Anderen direkt oder indirekt übernommenen Daten, Techniken und Materialien sind als solche kenntlich gemacht. Die Arbeit wurde bisher bei keiner anderen Hochschule zu Prüfungszwecken eingereicht.

Die eingereichte elektronische Version stimmt mit der schriftlichen Version überein.

Darmstadt, den _____

_____

Verena Wilmes

DANKSAGUNG

Bei Herrn **Prof. Dr. Gerhard Thiel** möchte ich mich für die Bereitschaft bedanken, die Betreuung und Begutachtung meiner Arbeit zu übernehmen.

Frau **Prof. Dr. Silke Kauferstein** hat die externe Betreuung und Begutachtung meiner Arbeit übernommen. Vielen Dank für die lehrreichen letzten Jahre, die wertvollen Erfahrungen und Kontakte und die Unterstützung bei kniffligen Fragen.

Herrn **Prof. Dr. Marcel A. Verhoff**, dem Direktor des Instituts für Rechtsmedizin, danke ich für die Bereitstellung sämtlicher Materialien und Geräte, ohne die diese Arbeit nicht möglich gewesen wäre.

Ein großer Dank gilt Frau **Dr. Elise Gradhand**, Oberärztin der Pathologie des Universitätsklinkums Frankfurt, für die exzellente histologische Unterstützung, ohne die diese Arbeit so nicht entstanden wäre.

Ebenso möchte ich mich bei **Prof. Dr. Andreas Weigert** und **Dr. Ivan M. Kur** vom Institut für Biochemie I des Universitätsklinikums Frankfurt für die sofortige Bereitschaft an der Arbeit mitzuwirken und die konstruktive, lehrreiche und spannende Zusammenarbeit bedanken.

Herrn **Prof. Dr. Dimitrios Tsikas und seiner gesamten Arbeitsgruppe** der Medizinischen Hochschule Hannover danke ich für die Durchführung der GC-MS Analysen und die Auswertung der Ergebnisse.

Bei **Luise Mildeberger**, meiner ehemaligen Masterstudentin, möchte ich mich vor allem für die Etablierung des microRNA Workflows bedanken, der meine eigenen Analysen vereinfacht hat. Außerdem möchte ich mich für die sehr schöne gemeinsame Zeit bedanken.

Generell möchte ich mich ganz lieb bei allen **Kolleg*innen der Rechtsmedizin Frankfurt** bedanken, besonders bei den gegenwärtigen und ehemaligen Mitarbeiterinnen der Forensischen Biologie. Bei **Dr. Stefanie Scheiper-Welling**, **Katharina Hartmann**, **Valentina Birne**, **Dr. Lena Lutz** und **Luise Thümmel** möchte ich mich für die gegenseitige Unterstützung und die schöne Arbeitsatmosphäre bedanken. Vielen Dank auch an **Christine Elbert**, **Romy Weber**, **Sena Yayciouglu**, **Svenja Schuett** und ehemalige Studentinnen wie **Monika Tabunscik** und **Jasmin Köffer**. Mit euch gab es sehr viele schöne Momente und viel emotionalen Rückhalt.

Zum Schluss möchte ich mich bei **meiner Familie** bedanken, dir mir all das ermöglicht hat.

WISSENSCHAFTLICHE PUBLIKATIONEN

Wilmes V, Kur I, Weigert A, Verhoff MA, Gradhand E*, Kauferstein S*. (2023) iNOS expressing macrophages co-localize with nitrotyrosine staining after myocardial infarction in humans. Frontiers in Cardiovascular Medicine. 10:1104019. *Authors contributed equally

Mildeberger L, Bueto J, **Wilmes V**, Scheiper-Welling S, Niess C, Gradhand E, Verhoff MA, Kauferstein S. Suitable biomarkers for post-mortem differentiation of cardiac death causes: Quantitative analysis of miR-1, miR-133a and miR-26a in heart tissue and whole blood. (2023) Forensic Science International: Genetics. 102867

Wilmes V, Lux C, Niess C, Gradhand E, Verhoff MA, Kauferstein S. Changes in gene expression patterns in postmortem human myocardial infarction. Internal Journal of Legal Medicine. (2020) 134:1753-1763.

Wilmes V, Scheiper S, Roehr W, Niess C, Kippenberger S, Steinhorst K, Verhoff MA, Kauferstein S. (2020) Increased inducible nitric oxide synthase (iNOS) expression in human myocardial infarction. International Journal of Legal Medicine. 134:575-581.

TEILNAHME UND BEITRÄGEN AN KONGRESSEN

100. Jahrestagung der Deutschen Gesellschaft für Rechtsmedizin
vom 10.-16. September 2021 in München, Deutschland. **Wilmes V**, Niess, C, Gradhand, E, Verhoff MA, Kauferstein S. iNOS Expressionstudie in postmortalen humanen Infarktherzen zum Nachweis von oxidativem Stress. **(Vortrag)**

30. Frühjarhstagung der Deutschen Gesellschaft für Rechtsmedizin (digital)
am 24. April 2021 in Frankfurt, Deutschland (digital). **Wilmes V**, Lux C, Niess C, Gradhand E, Verhoff MA, Kauferstein S. Genexpressionsstudie in postmortalen humanen Infarktherzen zum Nachweis von oxidativem Stress. **(Vortrag)**

98. Jahrestagung der Deutschen Gesellschaft für Rechtsmedizin
vom 17.-21. 2019 September in Hamburg, Deutschland. **Wilmes V**, Scheiper S, Roehr W, Niess C, Kippenberger S, Steinhorst K, Verhoff MA, Kauferstein S. Vergleichende Genexpressionsstudie zum Nachweis von oxidativem Stress bei Myokardinfarkt. **(Poster)**

39. Spurenworkshop der Deutschen Gesellschaft für Rechtsmedizin
vom 22. - 23. Februar 2019 in Jena, Deutschland

Verena Wilmes

Details

29.10.1992 in Bad Soden, Germany

Education

Since 01/2021	**PhD candidate (Dr. rer nat)** Department Biology at Technical University Darmstadt, Germany "The role of iNOS in human myocardial infarction"
01/2016-04/2018	**Master of Science Degree Physical Biology of Cells and Cell Interactions**, Goethe University Frankfurt, Germany (Grade: 1,6) "Gene Expression of Arginine Glycine Amidino Transferase (AGAT) and inducible Nitric Oxide Synthase (iNOS) in Myocardial Infarction."
10/2015-01/2016	**Master of Science Degree Molecular Biology** (1 Semester), Goethe University Frankfurt, Germany
10/2012-09/2015	**Bachelor of Science Degree Biology**, Goethe University Frankfurt, Germany (Grade: 2,0) "Untersuchungen zur intra- und interspezifischen Kommunikation der Larven forensisch relevanter Schmeißfliegen (Diptera:Calliphoridae)."
09/2003-06/2012	Graduating from Albert-Einstein-Schule, Schwalbach, Germany (Grade: 2,3)

Experience

Since 05/2019	**Research assistant** at the Institute of Legal Medicine in Frankfurt, Germany - DNA/RNA extraction of human tissue and whole blood - Analyses on oxidative stress, immunhistochemical stainings and mRNA/miRNA expression - Sanger sequencing - Supervision and planning of Bachelor- and Master Theses - Presentation of results in publications and PowerPoint presentations

10/2018-03/2019	**Technical Assistant** in forensic case work at the Institute of Legal Medicine in Frankfurt, Germany
06/2018-09/2018	**HiWi** at the Institute of Legal Medicine in Frankfurt, Germany - mRNA expression in human myocardial infarction
07/2017-04/2018	**Master Thesis** at the Institute of Legal Medicine in Frankfurt, Germany -RNA extraction of native human cardiac tissue
02/2017	**Internship** at the Paul-Ehrlich-Institute in Langen, Germany - Novel vaccine strategies
10/2016	**Internship** at the Max-Planck-Institute in Bad Nauheim, Germany - Development of the cardiovascular system - Working with CRISPR/Cas9
08/2016-09/2016	**Internship** at the Institute of Legal Medicine Frankfurt, Germany -mtDNA Analyses
04/2015-09/2015	**Bachelor Thesis** at the Institute of Legal Medicine in Frankfurt, Germany