MEDICAL UNIVERSITY „PROF. DR. PARASKEV STOYANOV“- VARNA
DEPARTMENT OF PROPAEDEUTICS OF INTERNAL DISEASES

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BLOOD LEVELS OF CIRCULATING LONG NON-CODING RIBONUCLEIC ACIDS (LncRNAs) IN CARDIOVASCULAR DISEASES

ABSTRACT

of the Dissertation for the Award of the Educational and Scientific Degree "PhD"

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The dissertation consists of 120 pages and includes 28 figures and 21 tables. The references include 299 titles.

The dissertation was discussed and recommended for defense by the Departmental Council of the Department of Propedeutics of Internal Diseases at the Medical University "Prof. Dr. Paraskev Stoyanov" – Varna. It has been scheduled for a public defense before an Academic Review Panel composed of the following members:

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СЪДЪРЖАНИЕ

ABBREVIATIONS USED

CVD	Cardiovascular diseases
WHO	World Health Organization
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
ACS	Acute coronary syndrome
ECG	Electrocardiogram
SCAG	Selective coronary angiography
AMI	Acute myocardial infarction
MI	Myocardial infarction
HF	Heart failure
cTn	Troponin
hsTn	High-sensitivity troponin
CK	Creatine kinase
CK-MB	Creatine kinase MB fraction
EF	Ejection fraction
LV	Left ventricle
IHD	Ischemic heart disease
SAP	Stable angina pectoris
EF	Ejection fraction
DM	Diabetes mellitus
CKD	Chronic kidney disease
lncRNAs	Long non-coding ribonucleic acids
microRNAs/miRNAs	Micro ribonucleic acids
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
mRNA	Messenger ribonucleic acid

STEMI	ST elevation myocardial infarction
NSTEMI	non ST elevation myocardial infarction
NSTE-ACS	non ST elevation acute coronary syndrom
lncRNA ANRIL	antisense long non-coding RNA in gene locus cyclin-dependent kinase inhibitors – INK4
lncRNA HOTAIR	HOX gene cluster antisense intergenic long non-coding RNA
lncRNA NRF	long non coding RNA necrosis related factor
lncRNA MIAT	myocardial infarction-associated transcript
IL 6	interleukin 6
BNP	brain natriuretic peptide
TNF α	tumor necrosis factor alpha
ISR	in-stent restenosis
LAD	left anterior descending artery
lncRNA Wisper	Wisp2 – signaling pathway protein super enhancer–associated RNA
lncRNA HIF1A	AS2/endogenous hypoxia-inducible factor 1α antisense RNA 2
lncRNA aHIF	hypoxia
I/R	ischemia reperfusion
RIPK1	receptor-interacting serine/threonine-protein kinase 1
RIPK3	receptor-interacting serine/threonine-protein kinase 3

INTRODUCTION

During the investigation of the human transcriptome sequence, a novel class of ribonucleic acids, termed long non-coding ribonucleic acids (lncRNAs), has been discovered.

This class of ribonucleic acids differs from other non-coding ribonucleic acids, such as microribonucleic acids (microRNAs/miRNAs), in their size, sequence, and structural characteristics (Hennessy, 2022).

Long non-coding ribonucleic acids represent a heterogeneous class of ribonucleic acids, generally defined as transcripts exceeding 200 nucleotides in length, which do not contain protein-coding sequences and are not classified as ribosomal, transfer, or small nuclear ribonucleic acids (Mattick et al., 2023). This group of non-coding ribonucleic acids modulates the transcription of protein-coding genes. They possess the ability to bind to adjacent genes, rendering them potential target molecules (Kohlmaier et al., 2023).

Numerous studies have demonstrated the role of lncRNAs and their involvement in the pathogenesis and development of cardiovascular diseases (Hennessy, 2022). Recently, research concerning the significance of lncRNAs in cardiovascular pathology has expanded beyond cellular in vitro analyses to include comprehensive in vivo characterization (Kohlmaier et al., 2023).

Certain lncRNAs have been identified as participants in embryonic heart development, while others are expressed in cardiomyocytes and endothelial musculature, modulating cellular growth and differentiation.

In studies utilizing animal models with cardiovascular pathology, it has been established that lncRNAs participate in the physiology and maintenance of cardiovascular system homeostasis, as well as in the pathogenesis of various cardiovascular diseases, such as atherosclerosis and myocardial infarction (Kohlmaier et al., 2023).

The role of lncRNAs in the genesis and development of heart failure has also been documented, where through diverse mechanisms, they influence multiple pathophysiological processes (Li et al., 2024).

With advancements in sequencing technology, genomic engineering, and RNA quantitative polymerase chain reaction techniques, novel lncRNAs are continuously being discovered across various medical domains, as well as in-depth analysis is being performed on those already identified.

The number of lncRNAs and their significance for the cardiovascular system is substantial. lncRNAs specifically expressed in cardiac tissue and expressed to different degrees in CVD have been described.

These RNA molecules play a crucial role in multiple processes within cardiac tissue, including cellular growth and differentiation, proliferation, apoptosis, cellular autophagy, and fibrosis.

Thousands of long-chain RNA molecules with aberrant expression have been discovered. Some predominantly occur in cardiac tissue and cardiomyocytes in animal models, while others are expressed in bodily fluids such as human plasma, serum, and urine. Their specific expression profile varies depending on the disease's developmental dynamics.

A considerable number of studies demonstrate the involvement of long-chain RNAs in the pathogenesis and pathophysiology of socially significant diseases like myocardial infarction and heart failure.

For example, some RNA molecules have been described with increased expression in the early phase of myocardial infarction and heart failure, while others are associated with decreased expression. Long-chain RNAs have also been found to correlate with various laboratory and clinical parameters. This fact raises a wide field of discussion and makes them ideal candidates as excellent noninvasive biomarkers for the accurate diagnosis , prognosis and disease stratification in the field of cardiovascular pathology.

The development of novel approaches and in-depth analysis of lncRNAs' dynamic regulation enables the introduction of innovative therapeutic strategies.

In this context, large-scale and highly specific investigations are necessary to validate the significance of lncRNAs as diagnostic biomarkers and therapeutic targets in CVD.

These considerations motivate the selection of the dissertation's research theme.

I. Aim, Objectives, and Hypothesis

1. *Aim*

The aim of the study is to investigate and analyse the plasma expression of long non-coding ribonucleic acids – lncRNA Wisper and lncRNA NRF – in patients with heart failure (HF) and patients with ST-elevation myocardial infarction (STEMI).

2. *Objectives*

To achieve the aim, the following objectives have been established:

1. To investigate and compare the plasma expression levels of lncRNA Wisper in patients with heart failure compared to the control group.

2. To investigate and compare the plasma expression levels of lncRNA Wisper in patients with acute ST-elevation myocardial infarction within 12 hours of infarction onset compared to the control group.

3. To investigate and compare the plasma expression levels of lncRNA NRF in patients with heart failure compared to the control group.

4. To investigate and compare the plasma expression levels of lncRNA NRF in patients with acute ST-elevation myocardial infarction within 12 hours of infarction onset compared to the control group.

5. To explore and analyze the influence of additional factors such as age, gender, and comorbidity on the plasma expression of lncRNA Wisper and lncRNA NRF in patients with heart failure and acute myocardial infarction.

3. *Hypothesis*

LncRNA Wisper and LncRNA NRF demonstrate elevated expression levels in the blood plasma of patients with heart failure and patients with STEMI.

The plasma expression of LncRNA Wisper and LncRNA NRF is not influenced by factors such as gender, age, and comorbidities (diabetes mellitus, dyslipidemia, chronic renal insufficiency).

1. *Time and place of study*

This study has a retrospective nature. Biological material (blood samples) was collected from patients hospitalized at University Multiprofile Hospital for Active Treatment "Sveta Marina", specifically in the Internal Medicine Clinic and First Cardiology Clinic with Intensive Cardiology Department during the period 2014 – 2016. Biological material was stored in the hospital's material base in accordance with RNA molecule storage requirements at –80 °C. LncRNA Wisper and lncRNA NRF were investigated in the laboratory of the Department of "Medical Microbiology, Associate Prof. Dr. Ivan Gerbov Mitov, DSc", in collaboration with the Department of Physiology and Pathophysiology, Medical Faculty – Medical University, Sofia.

The study was conducted after approval from the Scientific Research Ethics Commission of Medical University "Prof. Dr. Paraskev Stoyanov" – Varna, in compliance with the requirements of the Helsinki Declaration. Participants were included after prior signing of informed consent.

2. *Subject of the Study*

To study the levels of circulating long-chain non-coding RNAs - LncRNA Wisper and LncRNA NRF in the blood plasma of patients with heart failure and patients with acute ST-elevation myocardial infarction.

3. *Object of the Study*

Patients were divided into three groups as follows: First group – patients with ST-elevation myocardial infarction – n = 37; Second group – patients diagnosed with heart failure NYHA class III – IV – n = 28; Third group – individuals without cardiovascular diseases, matching the inclusion and exclusion criteria, used as the control group – n = 15.

4. *Scope and Selection of the Patients Included in the Study*

For the purposes of this study, plasma expression of long circulating RNA molecules – lncRNA Wisper and lncRNA NRF – was examined using real-time reverse transcription polymerase-chain reaction-RT-qPCR method in a total of 80 individuals.

Patients were selected according to precisely defined inclusion and exclusion criteria.

First Group (n = 37) consisted of hospitalized patients with ST-elevation myocardial infarction (STEMI). Venous blood for investigation was collected within the first 12 hours from the onset of AMI symptoms.

Second Group consisted of patients (n = 28) hospitalized for exacerbated heart failure (NYHA functional class III-IV). For the majority of patients, the cause of heart failure was ischemic heart disease – with 19 patients (67.9%) having experienced a previous myocardial infarction, 5 patients (17.9%) with idiopathic dilated cardiomyopathy. Among the remaining 4 patients (14.3%), the causes were valvular pathology or rhythm disorders. The average ejection fraction of the HF group was 35.46% ± 7.48. Of these, 4 (14.2%) were in NYHA functional class IV.

Third Group consisted of individuals without cardiovascular diseases, matching the inclusion and exclusion criteria, used as the control group (n = 15).

Inclusion Criteria for STEMI Patient Group:

Individuals hospitalized at University Multiprofile Hospital for Active Treatment "Sveta Marina" EAD, who signed the general Declaration of Informed Consent and Declaration of Study Participation.

Patients over 18 years of age.

Patients with chest pain showing significant (minimum 2 mm) ST segment elevation in at least two adjacent electrocardiographic leads and/or significant increase in cardiac troponin (above the 99th percentile of the upper reference limit (URL)); <12 hours from symptom onset to primary PCI..

Inclusion Criteria for HF Patient Group:

Individuals hospitalized at University Multiprofile Hospital for Active Treatment "Sveta Marina" EAD, who signed the general Declaration of Informed Consent and Declaration of Study Participation.

Patients over 18 years of age.

Clinical and echocardiographic (EF<40%) criteria for HF. The diagnosis of heart failure was established according to the recommendations of the European Society of Cardiology (Ponikowski et al., 2014), which defines heart failure as a clinical syndrome with a typical clinical presentation (dyspnea, ankle swelling, fatigue), accompanied by existing physical findings (jugular venous congestion, pulmonary congestion, and peripheral oedema), caused by structural cardiac changes resulting in reduction of stroke volume and/or increased preload, preliminary load at rest or during physical exertion.

Inclusion Criteria for the Third Group:

Patients over 18 years of age.

Patients who signed the Declaration of Study Participation

Individuals without evidence of cardiovascular diseases

Exclusion Criteria for Study Participation:

Individuals under 18 years of age

Clinical and laboratory evidence of active infection. Inflammatory diseases.

Oncological diseases.

5 *Laboratory Methods*

For the study purposes, peripheral venous blood stored at –80°C in the University Multiprofile Hospital for Active Treatment "Sveta Marina" storage unit was used from patients distributed across three groups.

Each blood sample – 2 mL – was distributed in vacutainers, which were centrifuged within 15 minutes at 2500 g for 20 minutes, after which the biological material was stored according to RNA molecule storage requirements – at –80°C until the day of analysis.

For this investigation, samples were transported in a special cooling bag equipped with dry ice to the laboratory of "Medical Microbiology, Corresponding Member Prof. Dr. Ivan Gerbov Mitov, DSc", where, in collaboration with the Department of "Physiology and Pathophysiology", Medical Faculty, Medical University, Sofia, the plasma expression analysis of long-chain RNAs – lncRNA Wisper and lncRNA NRF – was performed using RT-qPCR. The method includes the following stages:

RNA Isolation from Plasma

Materials

- Quick-DNA/RNA Miniprep Kit (Zymo Research)
- Plasma samples
- Microcentrifuge tubes (2 mL)
- Pipettes and sterile tips
- Centrifuge
- Ethanol (95-100%)
- RNase-free water

Required Equipment

- Micro centrifuge
- Vortex
- Thermal block

Method

1. Preliminary Preparation
    - Add ethanol to DNA/RNA Wash Buffer according to manufacturer's instructions
    - All centrifugation steps performed at room temperature
2. Sample Lysis
    - Add 800 µL QIAzol Lysis Reagent to 200 µL plasma
    - Vortex for 15 seconds
    - Incubate at room temperature for 15-20 minutes
3. RNA Binding
    - Transfer lysate to Zymo-Spin™ IC Column
    - Centrifuge at 12,000 × g for 1 minute
    - Discard filtrate
4. Washing
    - Add 400 µL DNA/RNA Prep Buffer
    - Centrifuge at 12,000 × g for 1 minute
    - Discard filtrate
    - Add 400 µL DNA/RNA Wash Buffer
    - Repeat washing once again
5. RNA Elution
    - Transfer the column to a new sterile tube
    - Add 32 µL DNase/RNase-Free Water directly onto the matrix
    - Incubate for 1 minute at room temperature
    - Centrifuge at 12,000 × g for 1 minute

Reverse Transcription

Materials

- miScript II RT Kit (Qiagen)
- Isolated RNA
- PCR tubes (0.2 mL)
- Pipettes and sterile tips
- RNase-free water

Equipment

- Thermal block
- Mini-centrifuge
- Vortex

Method

1. Preparation of Reaction Mix (for one reaction):

- 4 µL 5x miScript HiSpec Buffer
- 2 µL 10x miScript Nucleics Mix
- 2 µL miScript Reverse Transcriptase Mix
- Isolated RNA (up to 12 µL; 100ng/µl)
- RNase-free water to final volume 20 µL

2. Reverse Transcription
    - Carefully mixing by pipetting
    - Brief centrifugation to collect liquid
    - Incubate in thermal block under following conditions:
        - 37°C for 60 minutes (cDNA synthesis)
        - 95°C for 5 minutes (reverse transcriptase inactivation)
        - Cooling to 4°C
3. Storage
    - Obtained cDNA stored at -20°C for short-term
    - Long-term storage recommended at -80°C

RT-qPCR Performance

Preparation of RT-qPCR Reaction Mix (for one reaction, total volume 10 µL):

- 5 µL 2× QuantiTect SYBR Green PCR Master Mix
- 0.5 µL forward primer (final concentration 10 pM)
- 0.5 µL reverse primer (final concentration 10 pM)
- 2 µL cDNA template
- 2 µL RNase-free water

Reaction Preparation:

- Distribute reaction mix into wells of a 384-well plate
- Include three technical replicates for each sample
- Include negative control (water instead of cDNA)
- Seal plate with optical foil
- Brief centrifugation to collect liquid at bottom

Thermal Profile for RT-qPCR:

1. Initial Denaturation and Activation:
    - 95°C for 15 minutes
2. 45 Amplification Cycles:
    - Denaturation: 94°C for 15 seconds
    - Annealing: 55°C for 30 seconds

- Elongation: 72°C for 30 seconds

RNA Isolation from Plasma

RNA was isolated from plasma samples using the Quick-DNA/RNA Miniprep Kit (Zymo Research) according to the manufacturer's protocol with specific modifications. To 200 µL plasma, 800 µL QIAzol Lysis Reagent was added, followed by homogenization via vortexing for 15 seconds and incubation at room temperature for 15-20 minutes. The resulting lysate was transferred to a Zymo-Spin™ IC Column and centrifuged at 12,000 × g for 1 minute. After filtrate removal, RNA bound to the column was washed twice with 700 µL DNA/RNA Wash Buffer by centrifugation at 12,000 × g for 1 minute. For RNA elution, the column was transferred to a new sterile tube, and 32 µL DNase/RNase-Free Water was added. After one-minute incubation at room temperature, RNA was eluted by centrifugation at 12,000 × g for 1 minute.

Reverse Transcription

Isolated RNA was reverse transcribed using the miScript II RT Kit (Qiagen). The 20 µL reaction mix contained 4 µL 5x miScript HiSpec Buffer, 2 µL 10x miScript Nucleics Mix, 2 µL miScript Reverse Transcriptase Mix, and isolated RNA (up to 12 µL; 100ng/µl). The volume was adjusted to 20 µL with RNase-free water as needed. After careful mixing and brief centrifugation, the reaction mix was incubated at 37°C for 60 minutes for cDNA synthesis, followed by a 5-minute incubation at 95°C to inactivate the reverse transcriptase. The obtained cDNA was stored at -20°C for short-term or -80°C for long-term preservation.

All steps were performed under strict RNA handling conditions, using RNase-free consumables and reagents. Multiple freeze-thaw cycles of samples were avoided.

RT-qPCR Performance

RT-qPCR analysis was performed using the QuantiTect SYBR Green PCR Kit (Qiagen). The 10 µL reaction mix contained 5 µL 2× QuantiTect SYBR Green PCR Master Mix, 0.5 µL each of forward and reverse primers (final concentration 10 pM), 2 µL cDNA matrix, and 2 µL RNase-free water. The reaction was conducted in 384-well plates, with each sample analysed in three technical replicates. Negative controls containing water instead of cDNA matrix were included to detect potential contamination.

PCR amplification was performed on a 7900 HT Fast Real-Time PCR system (Applied Biosystems) under the following conditions: initial denaturation and polymerase activation at 95°C for 15 minutes, followed by 45 cycles including denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and elongation at 72°C for 30 seconds.

Sequence of Utilized Primers:

Primer Name	Sequence (5'→3')
lncNRF1_F	CACTCCATCCTCCCACCTT
lncNRF1_R	GTGTATTCTCAGCCTGGGTGA
lncRNA_Wisper_F	TTCCTTTGGAGCATCTGGAC
lncRNA_Wisper_R	ACTGGCTTTCGGACTGGTG
GAPDH-201_F	TGGTCTCCTCTGACTTCAACAG
GAPDH-201_R	GCTTGACAAAGTGGTCGTTG

As a reference gene (endogenous control) for gene expression analysis, GAPDH gene was utilized.

The RQ value indicates by how many times the expression is changed compared to the calibrator. The calibrator has an RQ value of 1. All samples are compared to the calibrator. An RQ value of 10 means that the investigated lncRNA is 10-fold more strongly expressed in the given sample compared to the calibrator sample. An RQ value of 0.1 indicates that the investigated lncRNA has 10-fold lower expression.

It is considered that the RQ value is significant when it is > 2 or less than 0.50. An RQ value above 2 indicates a positive change in expression (increase) of the respective studied lncRNA. An RQ value < 0.50 indicates a negative change in expression (decrease in expression).

The plasma expression of the indicated lncRNAs was compared with the concentrations of troponin I (ng/ml) (Immulite 2000, Siemens, Erlangen, Germany), as well as HMGB1 (ng/ml) (ELISA Shino-Test Corporation, Kanagawa, Japan), and RIPK3 (ng/ml) (ELISA CUSABIO, Wuhan, China).

6. Echocardiographic Methods

Left ventricular ejection fraction was assessed using the Simpson method, in accordance with the current recommendations for evaluating cardiac chambers, proposed and described by the European Association of Cardiovascular Imaging of the European Society of Cardiology.

7. Statistical Methods

The statistical analysis of the study was performed using SPSS Statistics V.23. Categorical characteristics were summarized in frequency and percentages.

The following statistical methods were used:

1. Descriptive analysis to determine statistical variables: mean value, standard error of the mean, minimal and maximal values, median.

2. Kolmogorov–Smirnov Test/Shapiro-Wilk Test: a test to verify the null hypothesis and determine the distribution type of values/sample normality distribution.

3. Graphical method – linear and planar graphical representations, circular and sector diagrams, stereograms, and symbolic diagrams were utilized.

4. Statistical investigation of dependencies through correlation analysis: by determining the Spearman coefficient (non-parametric analysis), which tests the existing correlation - direct or inverse proportional relationship between two variables.

5. Pairwise comparison – a statistical method used to compare the relationship between mean values in a population when group comparisons are performed.

6. Mann-Whitney U test for comparing mean values.

7. The specificity and sensitivity of long-chain RNAs for differentiating patients with heart failure, STEMI, and healthy control individuals were evaluated using receiver operating characteristic (ROC) curve analysis. The diagnostic accuracy of biomarkers was determined by obtaining the largest possible area under the ROC curve (AUC).

Presented values include mean ± standard deviation (SD). In data processing, differences with $p \leq 0.05$ were considered statistically significant.

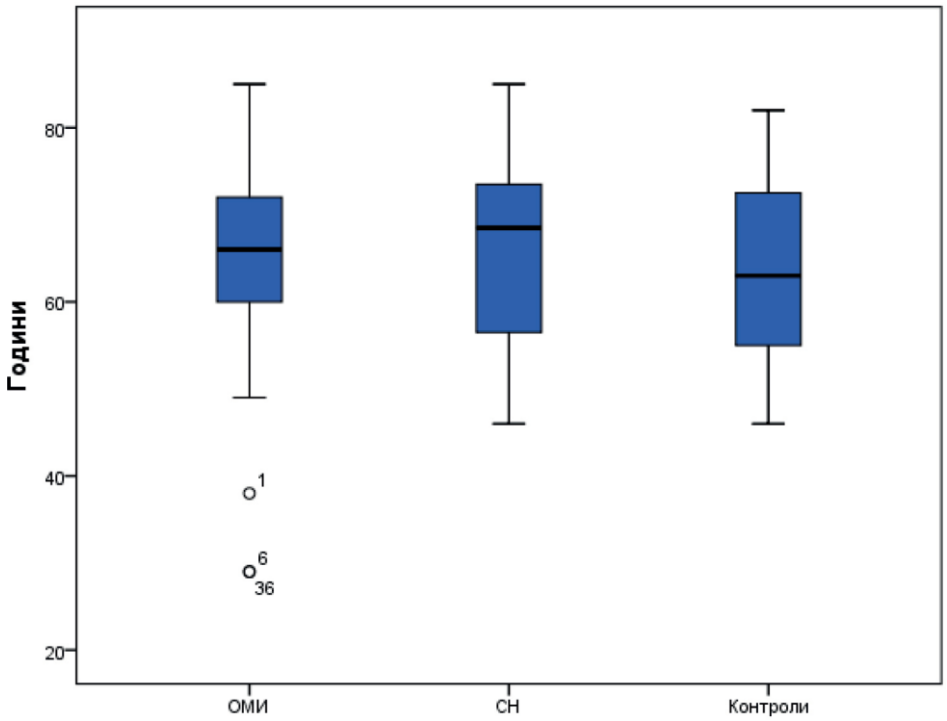
III. RESULTS

1 Demographic Characteristics of the Studied Patient Groups

- **Distribution of Patients by Age in the Studied Groups**

The analysis of the age of the patients included in the scientific study shows that the mean age of patients with HF is 66.2 ± 11 years, with a minimum age of 46 years and a maximum of 85 years. The mean age of patients with STEMI is 64 ± 13.2 years, with a minimum age of 29 years and a maximum of 85 years. The mean age of the control group is 64.3 ± 11.3 years, with a minimum of

46 years and a maximum of 78 years. No significant difference was observed in age between patients with ST-Elevation AMI and the control group (p = 0.769), nor in the age of patients with HF and the control group (p = 0.515) (Fig. 1).



Legend:
Години:Years
ОМИ-AMI
СН- HF
Контроли- Controls

Figure 1. Distribution of Patients in the Study Groups by Age

▶ **Distribution of Heart Failure Patients by Gender**

In the HF patient group, males predominate, accounting for 67.9% compared to females, who represent 32.1% (Fig. 2).

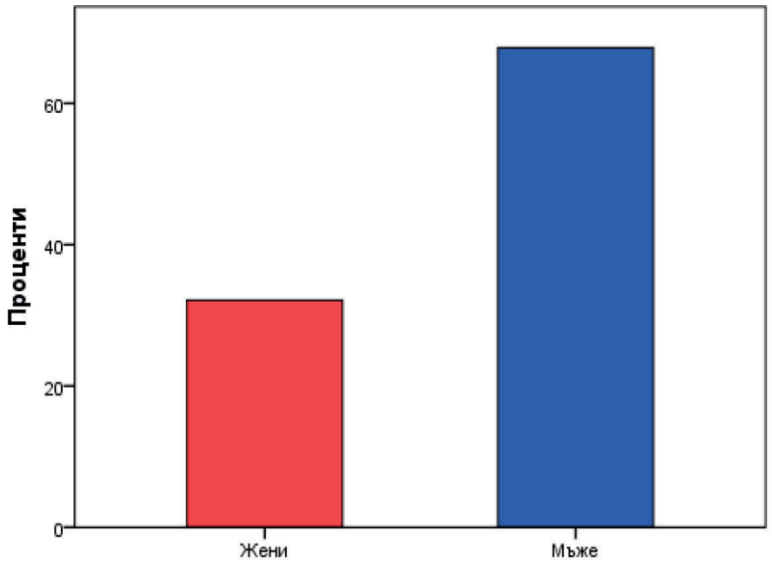


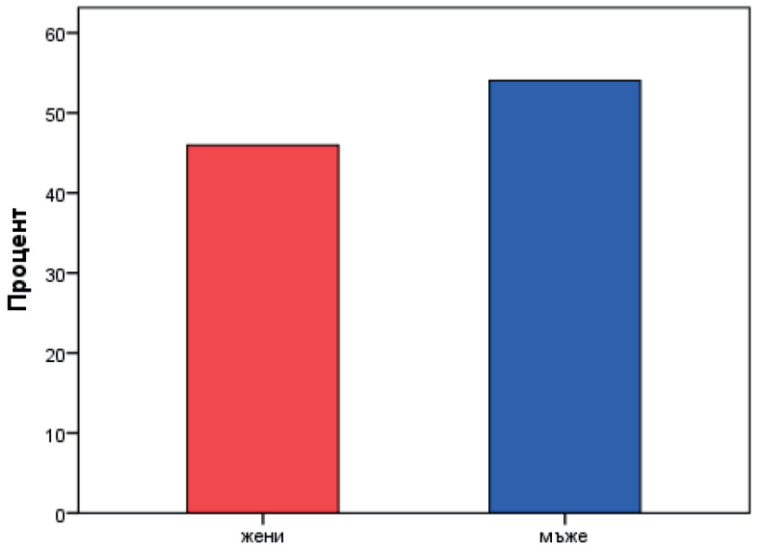
Figure 2. Distribution of HF Patients by Gender

Legend:
Проценти- Percents
Жени-Female
Мъже- Male

▶ **Distribution of Acute Myocardial Infarction Patients by Gender**

In the AMI patient group, 45.9% are female and 54.1% are male (Fig. 3).

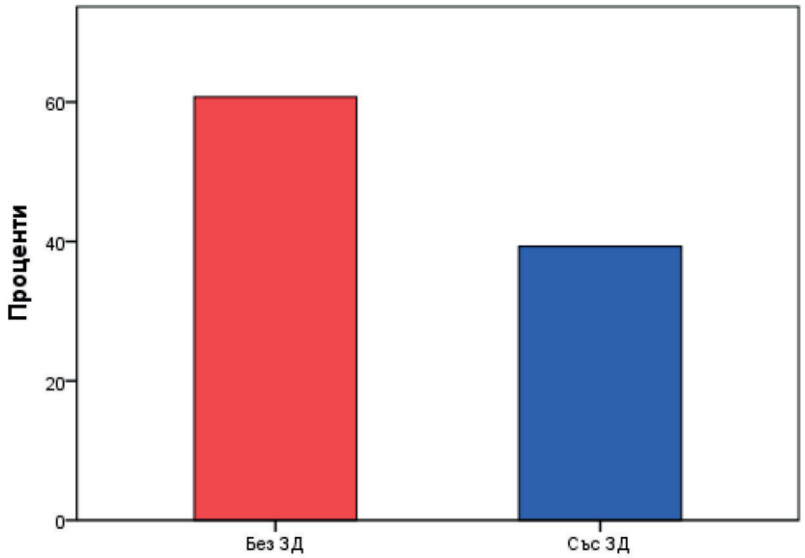
Figure 3. Distribution of AMI Patients by Gender



Legend:
Проценти- Percents
Жени-Female
Мъже- Male

2. Comorbidities in the Heart Failure Patient Group

1. All studied patients with HF also have accompanying Arterial Hypertension (AH).
2. Approximately one-third of patients with HF are also diagnosed with DM (39.3%) (Fig. 4).



Legend:
Проценти- Percents
Без ЗД- without DM
Със ЗД- with DM

Figure4. Distribution of HF Patients according to the frequency of Diabetes Mellitus

3. Part of the HF patients also have accompanying Chronic Kidney Disease (17.9%), while for the remaining 82.1%, such condition was not established (Fig. 5).

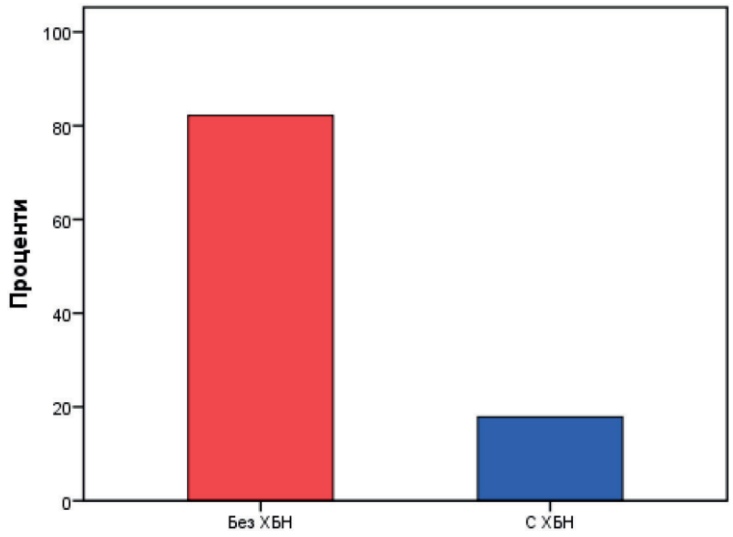


Figure 5. Distribution of HF Patients According to the Frequency of CKD

4. The analysis of results shows that one-quarter of the studied HF patients have established dyslipidemia (25%) (Fig. 6).

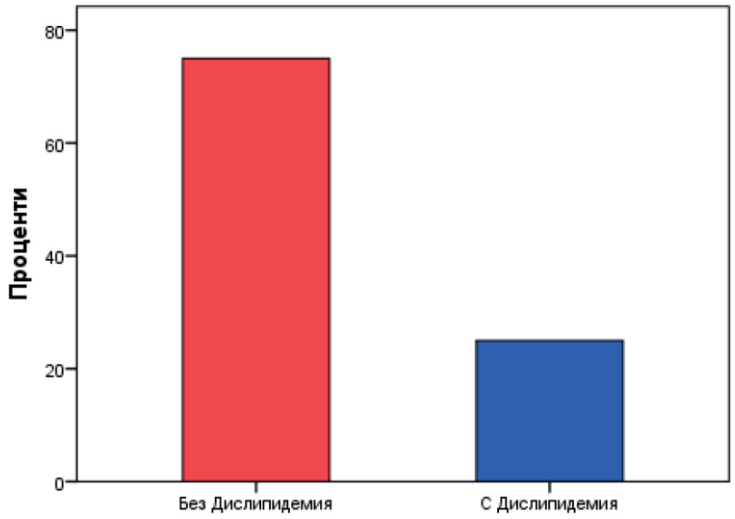
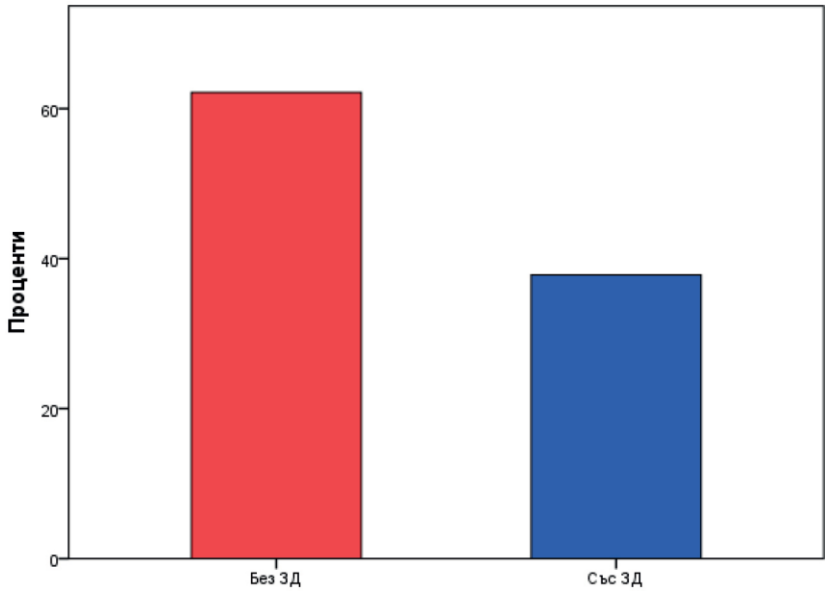


Figure 6. Distribution of HF Patients according to the frequency of Dyslipidemia

Legend:
Проценти- Percents
Без дислипидемия - without dyslipidemia
Със дислипидемия - with dyslipidemia

3. Comorbidities in the Acute Myocardial Infarction Patient Group

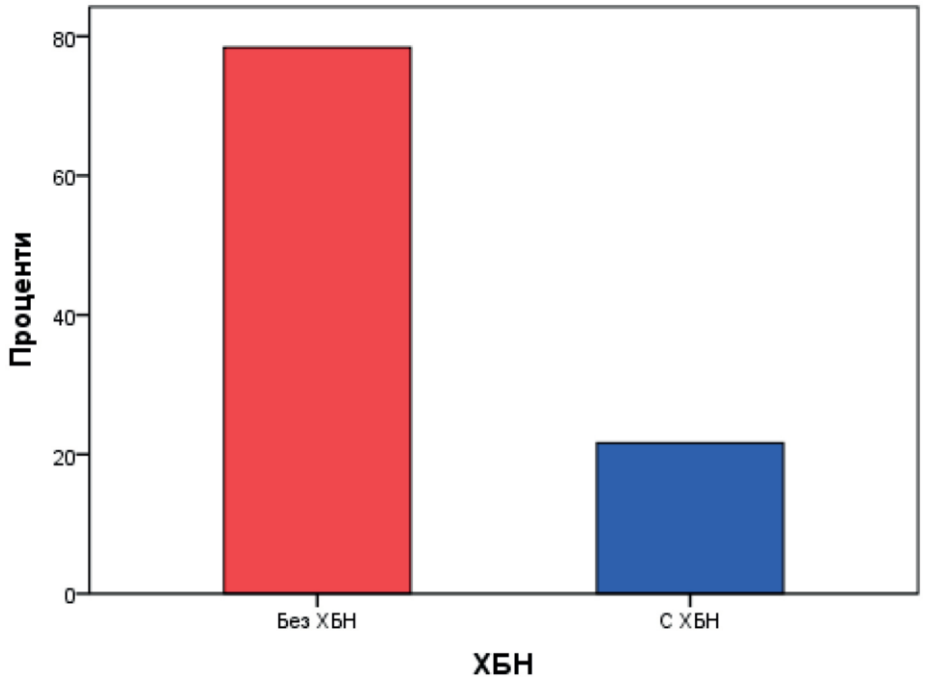
1. All AMI patients have Arterial Hypertension (100%).
2. Among AMI patients, 37.8% are diagnosed with DM, while for the remaining 62.2%, such condition was not established (Fig. 7).



Legend:
Проценти- Percents
Без ЗД- without DM
Със ЗД- with DM

Figure 7. Distribution of AMI Patients according to the frequency of Diabetes Mellitus

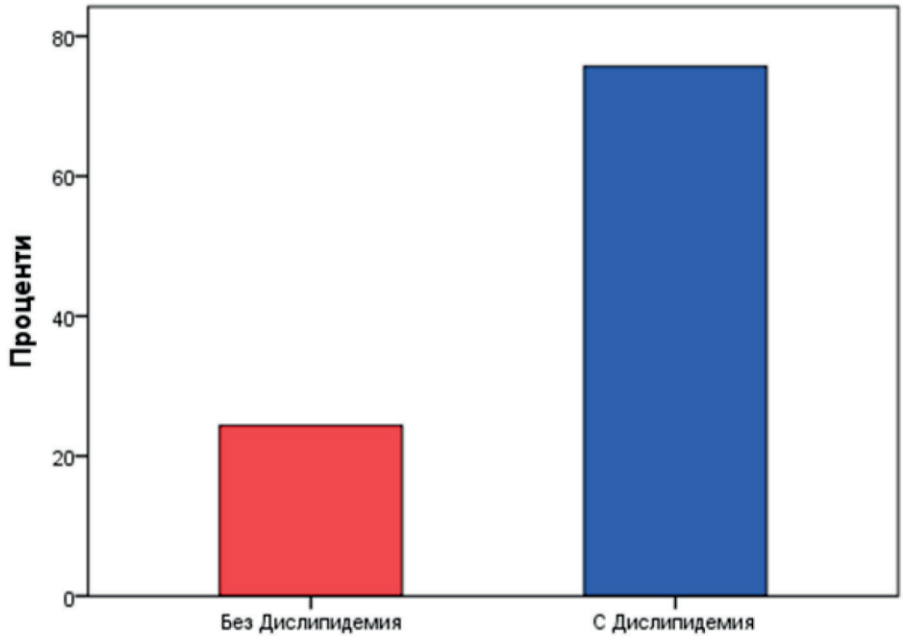
3. Chronic Kidney Disease (CKD) was found in 21.6% of AMI patients (Fig. 8).



Legend:
Проценти- Percents
Без ХБН- without CKD
Със ХБН- with CKD

Figure 8. Distribution of AMI Patients according to the frequency of CKD

Among AMI patients, dyslipidemia was established in 75.7%, while for the remaining 24.3%, such condition was not proven (Fig. 9).



Legend:
Проценти- Percents
Без дислипидемия - without dyslipidemia
Със дислипидемия - with dyslipidemia

Figure 9. Distribution of AMI Patients according to the frequency of Dyslipidemia

4. Plasma Expression of lncRNA Wisper of the Studied Patient Groups

Within the dissertation work, the expression level of lncRNA Wisper was investigated in the plasma of 28 patients with HF III - IV functional class according to NYHA, 37 patients with ST-Elevation Myocardial Infarction (STEMI), and 15 patients serving as the control group.

The Shapiro-Wilk test for abnormality revealed a non-homogeneous distribution of lncRNA Wisper expression among the studied groups (p = 0.000) (Table 1).

Table 1. Distribution of lncRNA Wisper Expression in the Studied Groups

	Study Group	Number	P
lncRNA Wisper	AMI	37	,000
	HF	28	,000
	Controls	15	,000

In a subsequent analysis, the mean value and standard deviation of lncRNA Wisper were evaluated for the three patient groups. The mean value in the Acute Myocardial Infarction (AMI) group is mean = 101.6 ± 529.92, in the Heart Failure (HF) patients is mean = 90.5 ± 431.1, and in the control group – mean = 25.2 ± 63.8 (Table 2).

Table 2. Mean Values of lncRNA Wisper in the Studied Groups

Groups	Mean	SD
AMI	101,66382187345	529,9245120789274
HF	90,524439826586	431,1765401446110
Controls	25,276000000000	63,84884256657464

In the STEMI patient group, the expression levels of lncRNA Wisper were significantly higher compared to the control group (p = 0.03). A significant difference in the long-chain RNA expression was also noted when comparing the Heart Failure patient group with the control group (p = 0.021). No significant difference was found in lncRNA Wisper expression between patients with heart failure and those with acute myocardial infarction (p = 1.0) (Table 3, Fig. 10).

Table 3. Comparative Analysis of lncRNA Wisper Expression in the Studied Groups

	Test Statistic	Std. Error	Std. Test Statistic	Sig.	P
AMI-HF	–3,582	5,821	–,615	,538	1,000
AMI-Controls	–23,656	7,113	–3,326	,001	,003
HF-Controls	–20,074	7,435	–2,700	,007	,021

⋆ The correlation is significant at $p \leq 0.05$

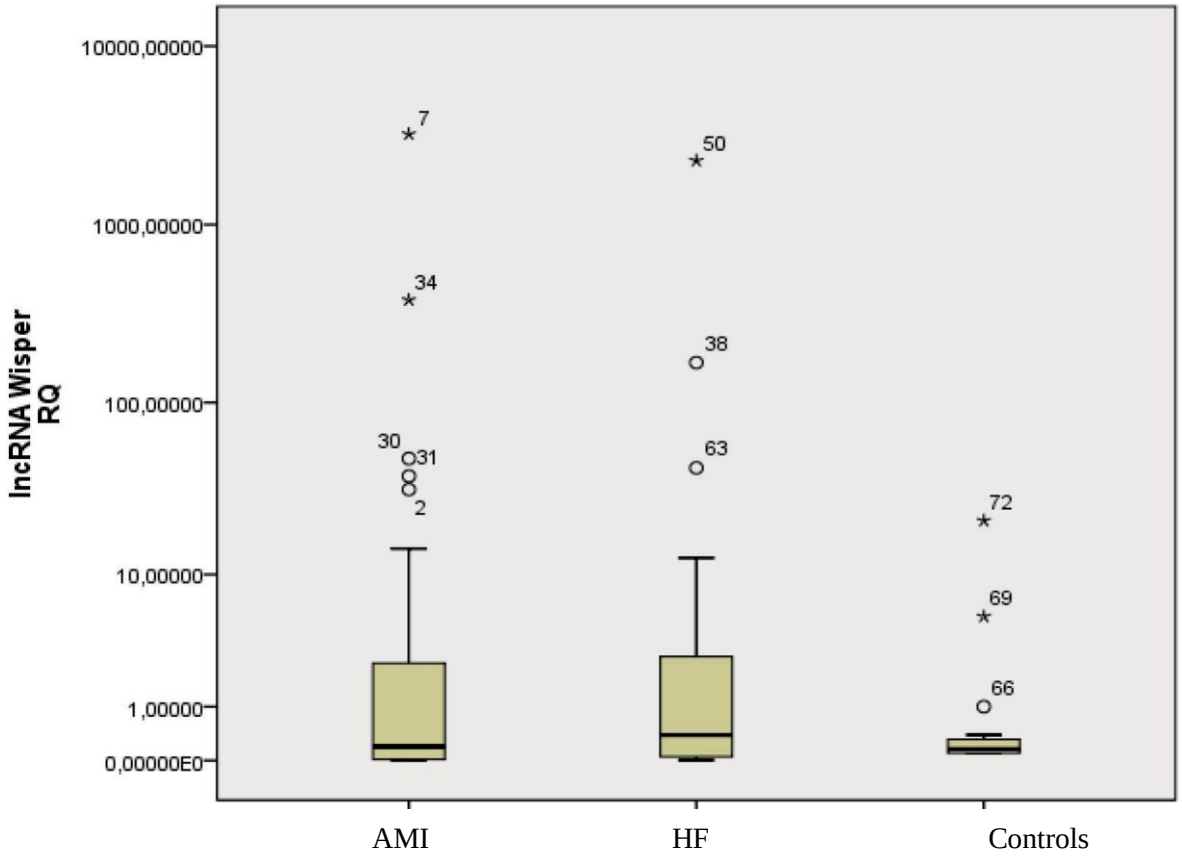


Figure 10. Plasma Expression of lncRNA Wisper in the Studied Groups

5. Plasma Expression of lncRNA Wisper in Patients with Heart Failure

In the course of the study, a ROC curve analysis was performed, according to which the plasma expression of lncRNA Wisper differentiates patients with Heart Failure from the control group with 80% sensitivity and 67% specificity. The diagnostic accuracy of the used marker was determined by obtaining the largest area under the curve – AUC = 0.783 (95% CI: 0.648 – 0.919, p = 0.002), at the optimal cut-off values (Fig. 11).

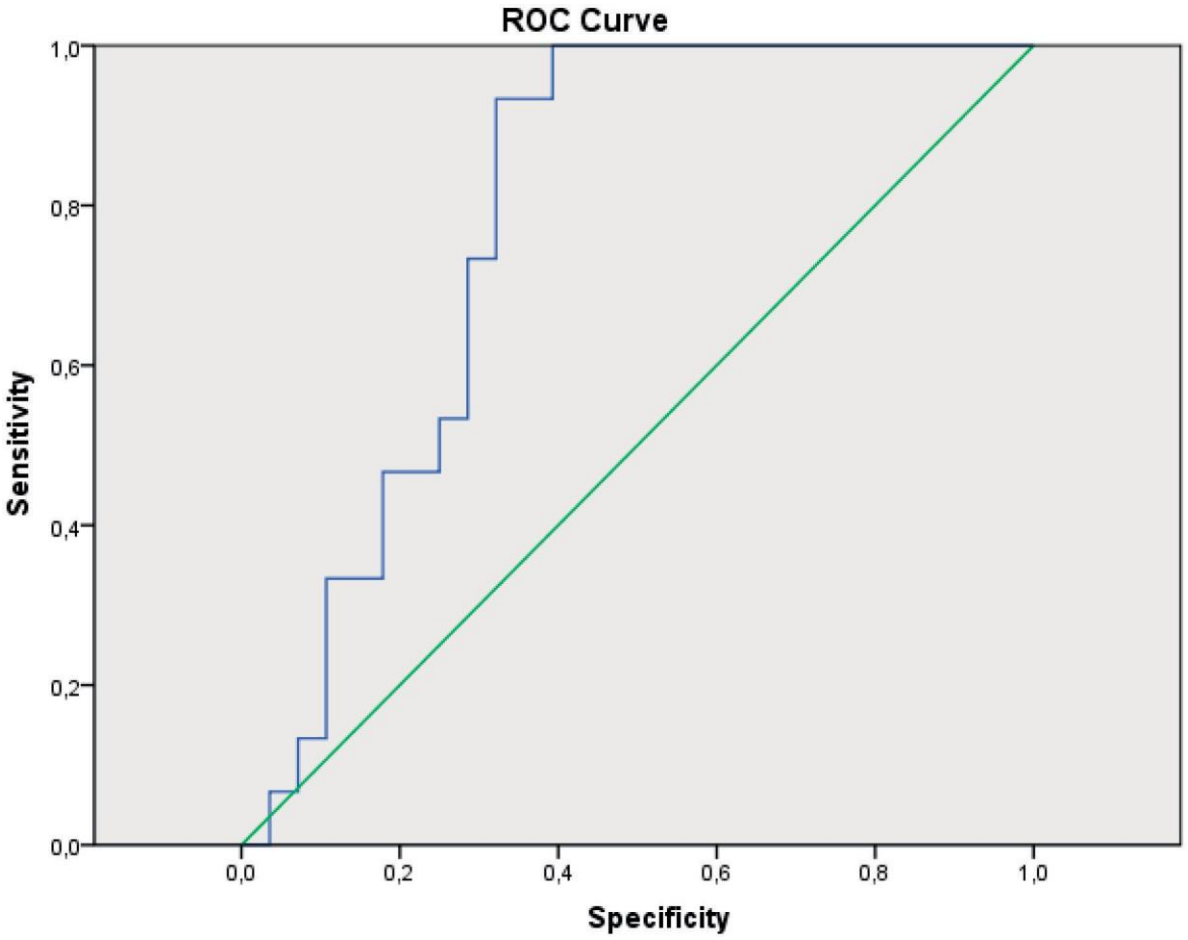


Figure 11. Plasma Levels of lncRNA Wisper Expression Differentiating Heart Failure Patients from the Control Group

1. By Chi-Square analysis, no difference was found in the plasma expression of lncRNA Wisper according to gender in the Heart Failure patient group (p = 0.358).

2. In a subsequent analysis of lncRNA Wisper plasma expression in HF patients, a significant but inversely proportional difference was found depending on patient age, with a correlation coefficient (rho –0.452) and (p = 0.016) (Table 4, Fig. 12).

Table 4. Correlation Analysis of lncRNA Wisper Expression by Age in the HF Group

			lncRNA Whisper	Years
Spearman's	lncRNA Whisper	Correlation Coefficient (rho)	1,000	–,452*
		P-value	.	,016
	Years	Correlation Coefficient	–,452*	1,000
		P-value	,016	

*. The correlation is significant at $p \leq 0.05$

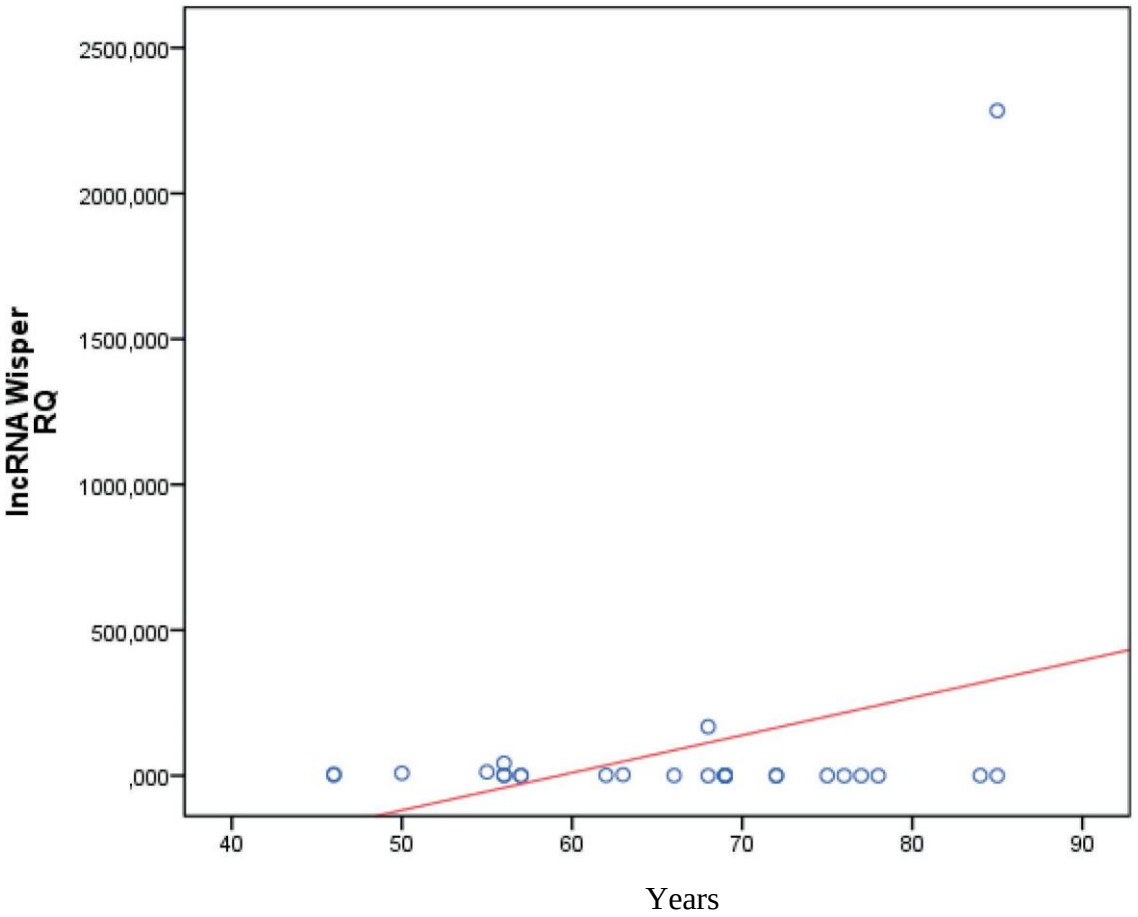


Figure 12. Correlation Analysis of lncRNA Wisper Expression by Age in HF Patients

Analysis of the relationship of lncRNA Wisper with ejection fraction in patients with HF

No correlation was found between lncRNA Wisper expression and ejection fraction (p = 0.903) (Table 5).

Table 5. Correlation analysis of the relationship between lncRNA Wisper Expression and Ejection Fraction

		lncRNA_Wisper RQ	EF %
lncRNA_Wisper RQ	Pearson correlation	1	–,024
	P		,903
EF %	Pearson correlation	–,024	1
	P	,903	

Plasma Expression of lncRNA Wisper in the Heart Failure Patient Group with Comorbidities (Diabetes Mellitus, Dyslipidemia, Chronic Kidney Disease)

In the course of the study, an analysis was performed of the relationship between plasma expression of lncRNA Wisper and the most common comorbidities in HF patients.

In approximately one-third of the studied patients (39.3%), HF is accompanied by DM. No significant correlation was found between the lncRNA Wisper expression level and the presence of DM (p = 0.312).

In one-quarter of the studied HF patients, dyslipidemia is present, and no significant difference was found between the lncRNA Wisper expression level and the presence of dyslipidemia (p = 0.730).

Accompanying CKD was found in 17.9% HF patients. There is no significant difference in lncRNA Wisper expression and the presence of CKD (p = 0.267). The data are presented in Table 6 and Figure 13 (A, B, C).

Table 6. Distribution of lncRNA Wisper by Comorbidities in HF Patients

Mann-Whitney – comorbidities	value	P
DM	72,000a	,358
Dyslipidemia	67,000a	,730
CKD	39,000a	,267

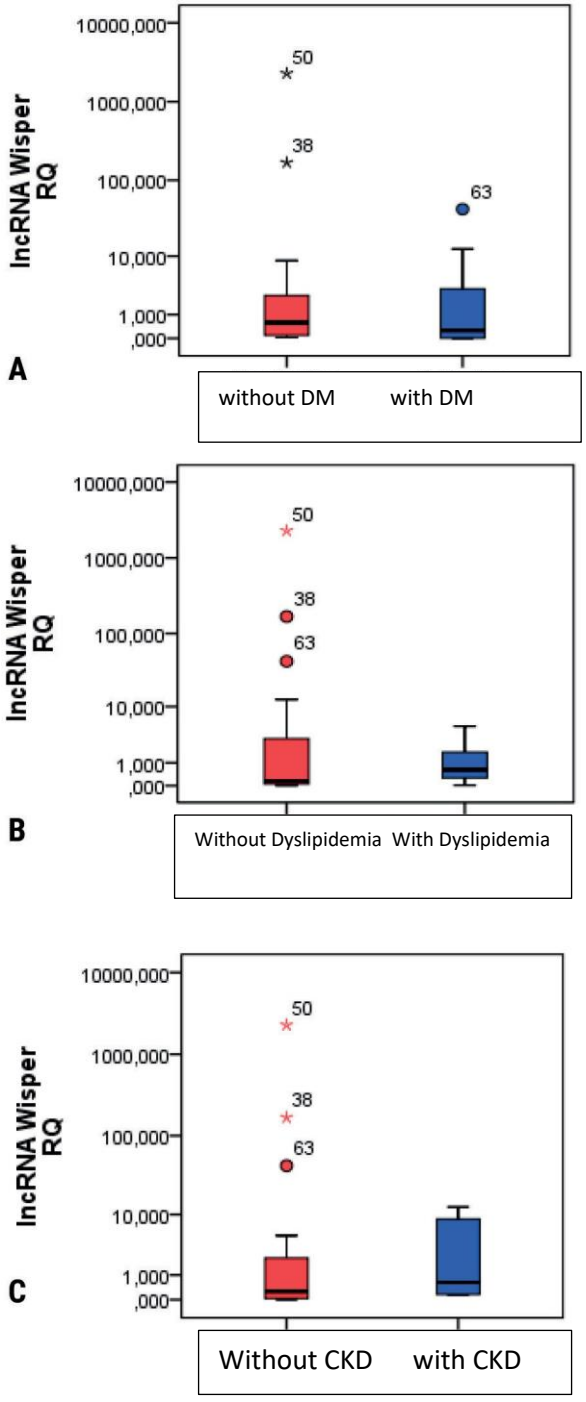


Figure 13. Box Plot Analysis Showing the Relationship between Plasma Expression of lncRNA Wisper and Comorbidities in HF Patients

6. Plasma expression of lncRNA Wisper in patients with acute myocardial infarction (STEMI)

The expression levels of lncRNA Wisper were significantly higher in the AMI patient group (mean 101.663 ± 87.119) compared to the control group (mean 10.209 ± 3.597).

Plasma expression levels of lncRNA Wisper discriminated ST-segment elevation myocardial infarction (STEMI) patients from the control group with a sensitivity of 80% and specificity of 71%. The diagnostic accuracy of the biomarkers was determined by obtaining the largest area under the curve – AUC = 0.780 (95% CI: 0.658 – 0.903, p = 0.002), at the respective cut-off values (Figure 14).

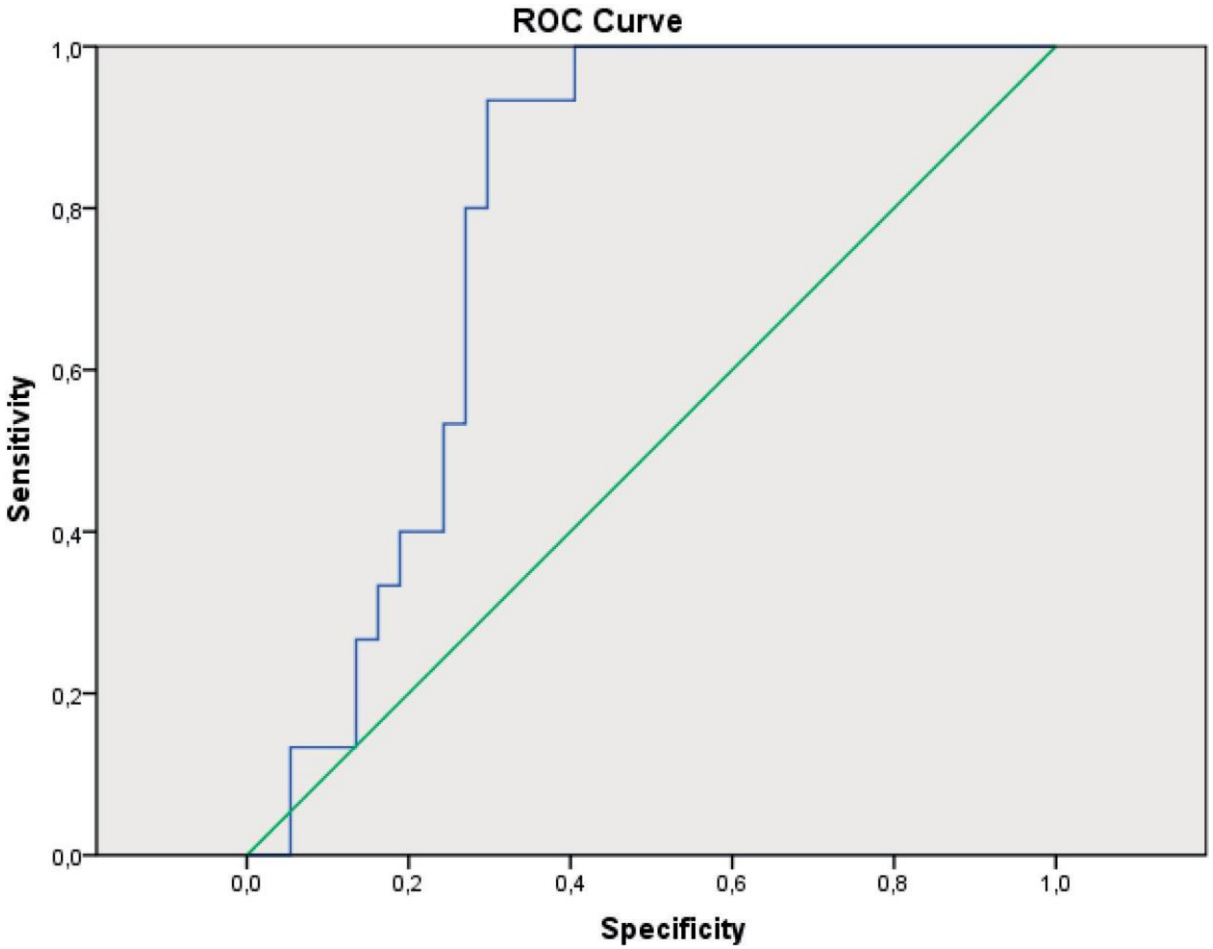


Figure 14. Plasma Expression Levels of lncRNA Wisper Differentiating Acute Myocardial Infarction Patients from the Control Group

1. By Chi-Square analysis, no statistically significant difference was observed between male and female subjects regarding plasma expression of lncRNA Wisper (P = 0.469)
2. Upon analysis of plasma lncRNA Wisper expression stratified by patient age in the ST-segment elevation myocardial infarction (STEMI) cohort, no significant correlation was identified (p = 0.612) (Table 7).

Table 7. Analysis of lncRNA Wisper Expression in AMI Patients according to their age

			lncRNA Wisper	**Years**
Spearman's rho	lncRNA Wisper	Correlation Coefficient	1,000	,086
		P	.	,612
	Years	Correlation Coefficient	,086	1,000
		P	,612	.

Plasma Expression of lncRNA Wisper in Acute Myocardial Infarction Patients with Comorbidities (Diabetes Mellitus, Dyslipidemia, Chronic Kidney Disease)

For the purposes of the study, an analysis was conducted to investigate the relationship between lncRNA Wisper and patients' comorbidities.

Approximately one-third of patients (37.8%) with ST-segment elevation myocardial infarction (STEMI) had DM. No significant correlation was observed between lncRNA Wisper expression levels and accompanying diabetes mellitus (p = 0.47), indicating that diabetes mellitus does not alter lncRNA Wisper expression in AMI patients.

A substantial proportion of patients in the AMI group presented with dyslipidemia (75.7%). Upon data analysis, no significant difference was found in plasma lncRNA Wisper levels between patients with and without accompanying dyslipidemia (p = 0.5).

A part of AMI patients included in the study had CKD (21.6%). In-depth analysis of the results did not demonstrate a significant difference in lncRNA Wisper expression among acute myocardial infarction patients between subgroups with and without chronic kidney disease (p = 0.51). The results are presented in Table 8 and Figure 15 (A, B, C).

.

Table 8. Distribution of lncRNA Wisper According to Comorbidities in AMI Patients

Mann-Whitney – comorbidities	value	P
MD	142,000a	,474
Dyslipidemia	114,000a	,503
CKD	113,500a	,514

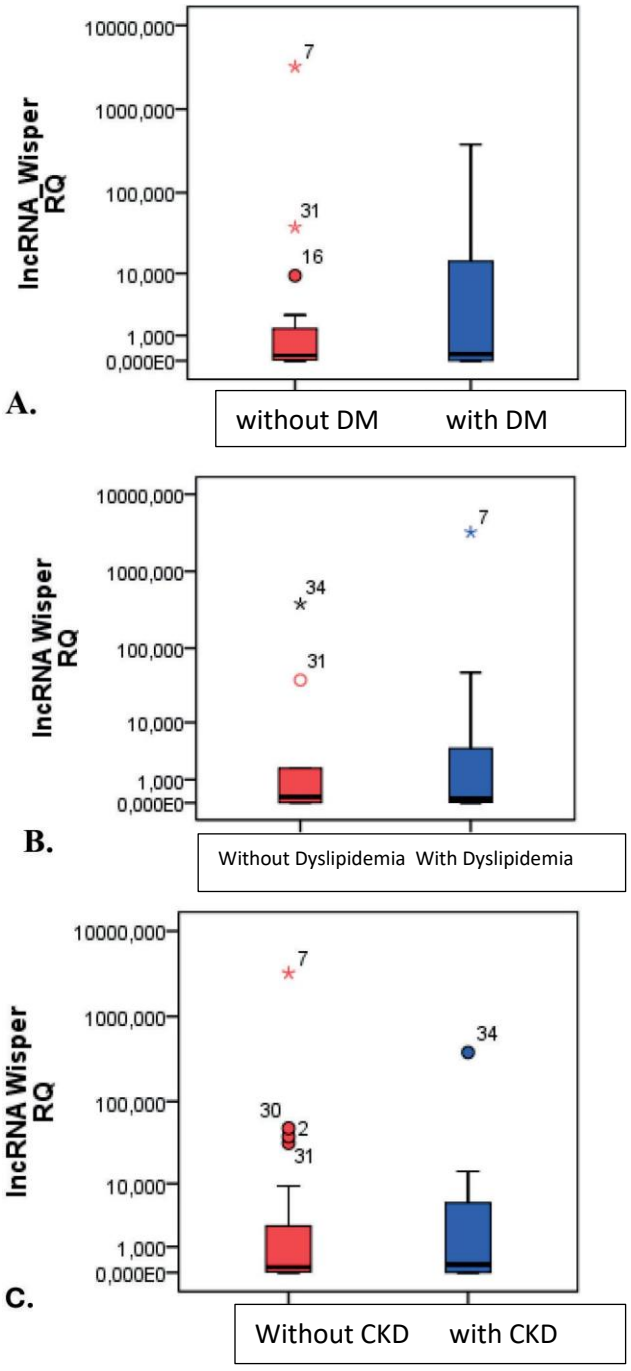


Figure 15.

Box Plot Analysis Demonstrating the Relationship Between Plasma Expression of lncRNA Wisper and Comorbidities in AMI Patients

3. Comparative Analysis of Plasma Expression of **lncRNA Wisper** with Laboratory Parameters and Ejection Fraction in Acute Myocardial Infarction Patients

LncRNA Wisper and HMGB1

In the conducted study, high mobility group box 1 (HMGB1) levels were measured in AMI patients. The observed significantly elevated HMGB1 values in AMI patients fully correspond to established literature data and its proven value as a biomarker for tissue necrosis ($p = 0.07$). The data are presented in Table 9 and visualized in Figure 16.

Table 9. Mean HMGB1 Values in Acute Myocardial Infarction Patients Compared to the Control Group

Groups:	HMGB	Std. Deviation	Std. Error Mean
HMGB1 MI	,720162	,4374448	,0719155
Controls	,678933	,4238309	,1094327

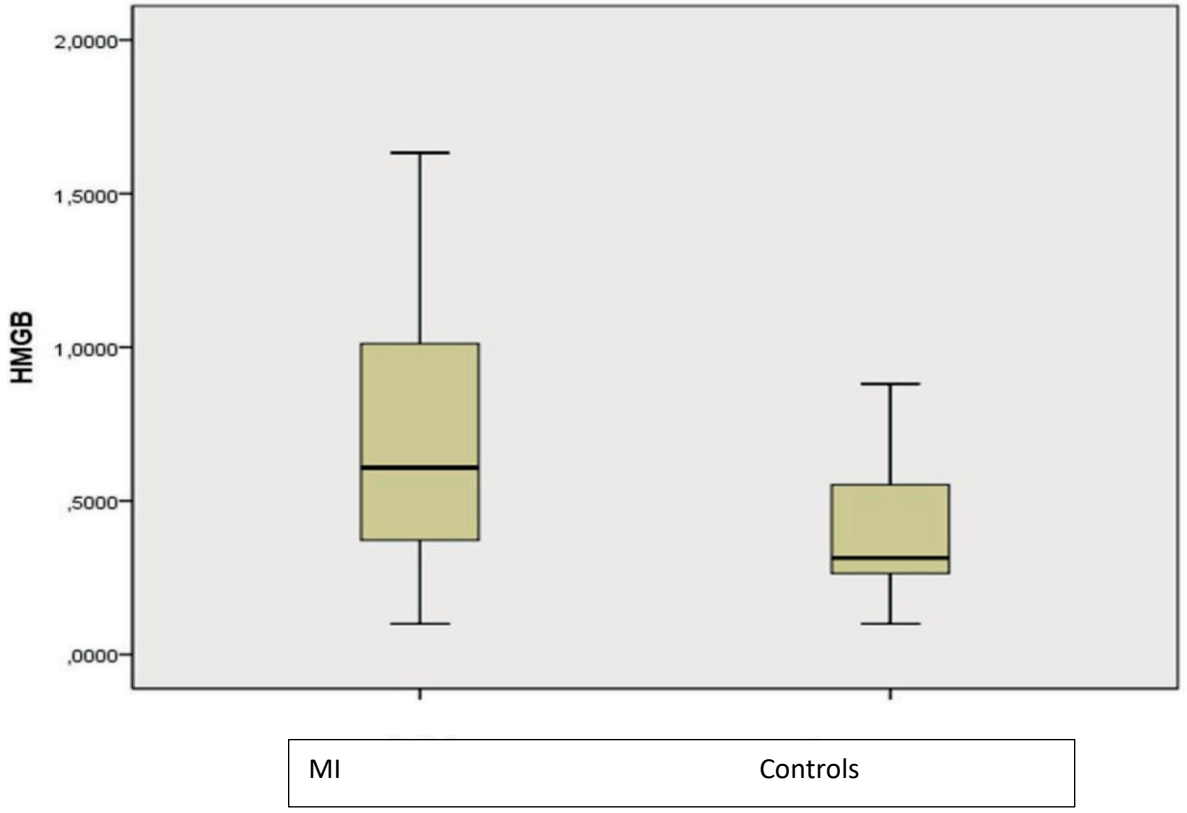


Figure 16. Mean HMGB1 Values in AMI Patients Compared to the Control Group

In the myocardial infarction patient group, a significant correlation was observed between plasma expression levels of lncRNA Wisper and blood HMGB1 levels, respectively (rho = 0.385, p = 0.019). The results are presented in tabular and graphical formats (Table 10, Figure 17).

Table 10. Comparative Analysis of lncRNA Wisper and HMGB1 Expression in AMI Patients

			lncRNA Wisper	**HMGB1**
Spearman's rho	lncRNA_Wisper	Correlation Coefficient	1,000	,385*
		P-value	.	,019
	HMGB1	Correlation Coefficient	,385*	1,000
		P-value	,019	.

*. The correlation is significant at $p \leq 0.05$

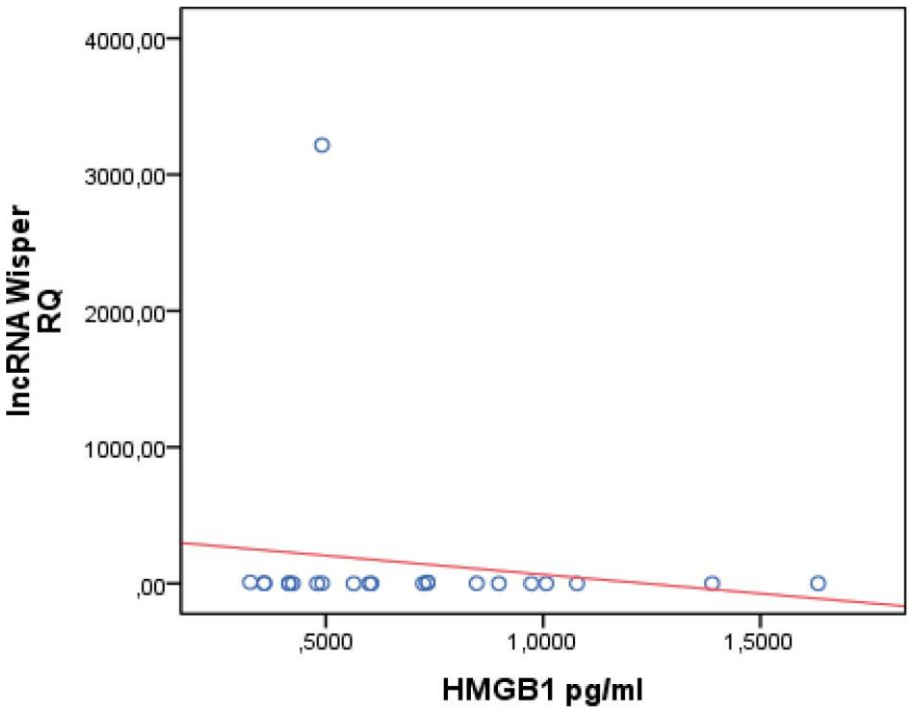


Figure 17. Correlation Distribution of Mean Values Between lncRNA Wisper and HMGB1

LncRNA Wisper and Troponin

Within the study, the relationship between lncRNA Wisper expression levels, analyzed in blood plasma up to 12 hours from AMI symptom onset, and troponin levels was evaluated. Specifically, troponin was examined up to 12 hours from AMI symptom onset ($p = 0.615$), at peak troponin levels ($p = 0.799$), and between 24-48 hours from AMI symptom onset ($p = 0.622$). No direct correlation was established between troponin dynamics and plasma lncRNA Wisper expression in AMI patients (Table 11, Figure 18 A, B, C).

Table 11. Correlation Analysis of the Relationship Between Plasma lncRNA Wisper Expression and Troponin Levels in Dynamics

lncRNA_ Wisper RQ	**Troponin up to the 12th hour**	**Troponin max**	**Troponin 24-48th hour**		
lncRNA_ Wisper	Correlation Coefficient (rho)	1,000	-,086	,043	–,084
	P-value	.	,615	,799	,622
	Number	37	37	37	37
Troponin up to the 12th hour	Correlation Coefficient-rho	–,086	1,000	,392*	,398*
	P-value	,615	.	,017	,015
	Number	37	37	37	37
Troponin max	Correlation Coefficient	,043	,392*	1,000	,742**
	P-value	,799	,017	.	,000
	Number	37	37	37	37
Troponin 24-48th hour	Correlation Coefficient	–,084	,398*	,742**	1,000
	P-value	,622	,015	,000	.

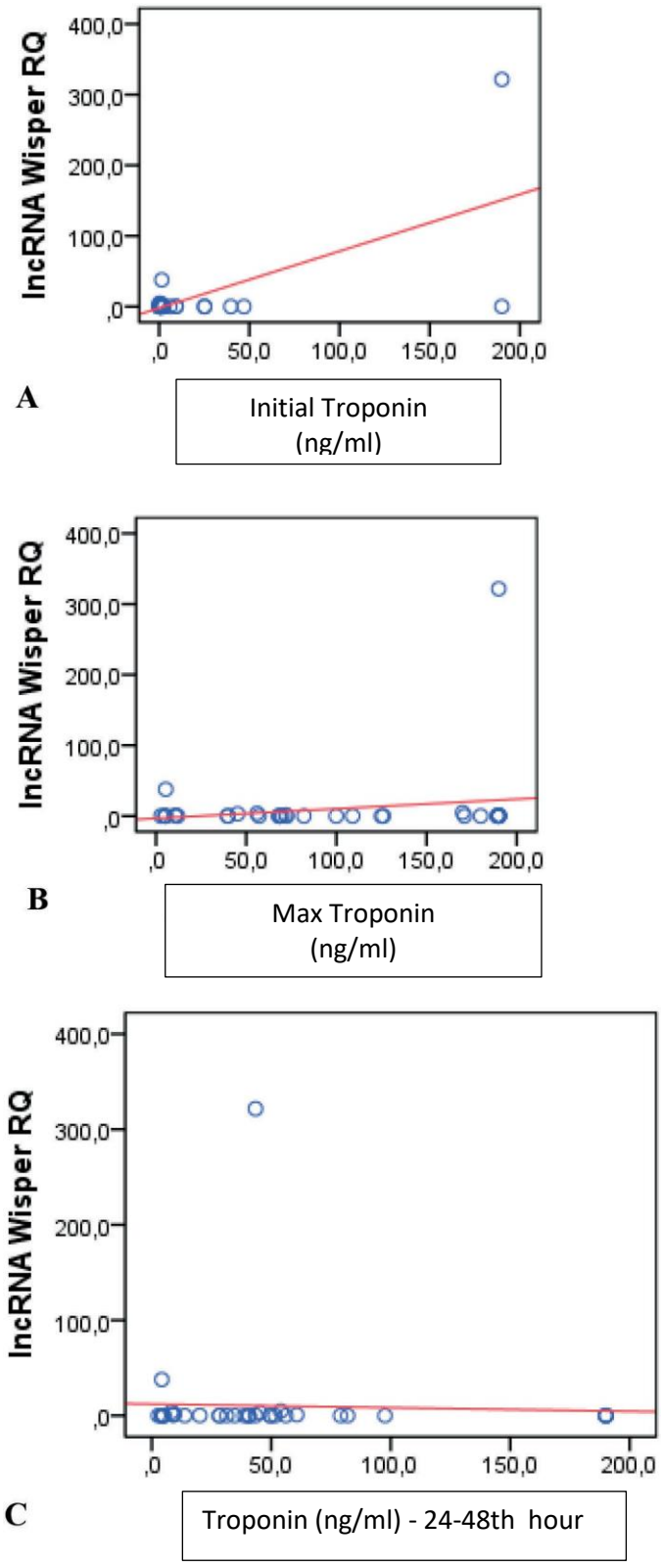


Figure 18. Correlation Analysis of lncRNA Wisper Expression Levels and Troponin Blood Levels

LncRNAWisper и RIPK3

Within the study, the relationship between plasma expression of lncRNA Wisper and the necroptosis marker RIPK3 was evaluated.

An inverse (rho –0.164), yet non-significant correlation was observed between plasma lncRNA Wisper expression and blood RIPK3 levels (p = 0.425) up to 12 hours from symptom onset. There is a tendency for high lncRNA Wisper levels to differentiate patients with low RIPK3 values in the AMI group (Figure 19).

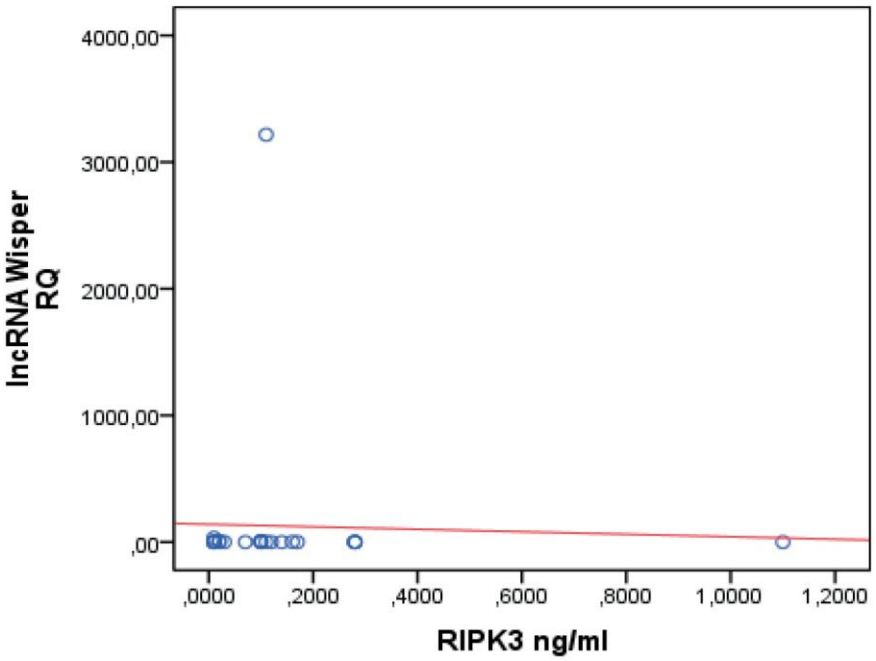


Figure 19. Correlation Analysis of lncRNA Wisper Expression Levels and RIPK3

LncRNA Wisper and EF %

Using the Spearman correlation test, no correlation was found between plasma expression of lncRNA Wisper and ejection fraction (EF%) (p = 0.967) among AMI patients (Figure 20).

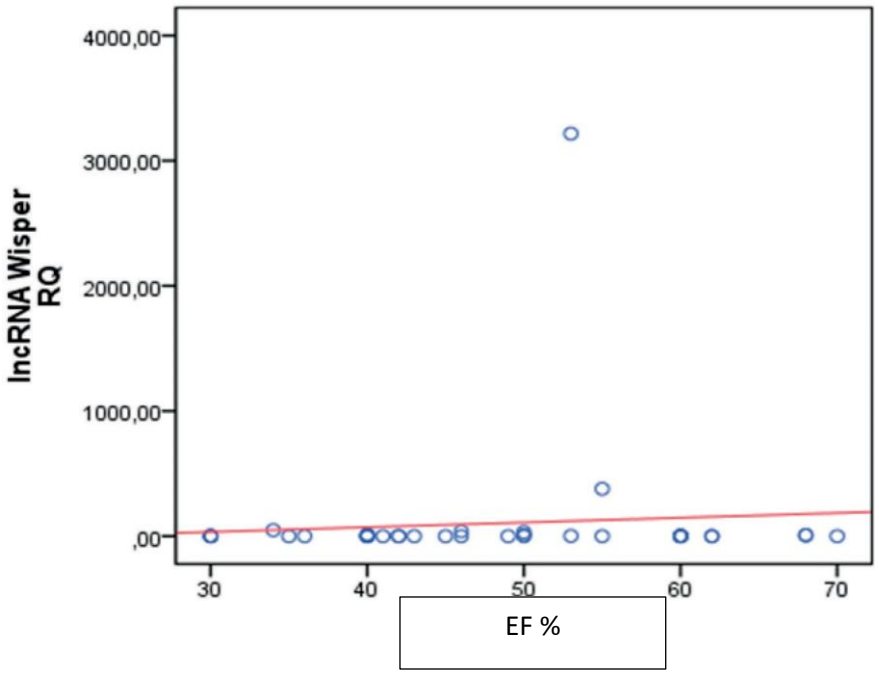


Figure 20. Correlation analysis of LncRNA Wisper expression levels and EF (%)

7. Plasma Expression of lncRNA NRF of the Studied Patient Groups

The Shapiro-Wilk test for normality revealed a non-uniform distribution of lncRNA NRF expression among the studied groups (p = 0.000) (Table 12).

Table 12. Distribution of lncRNA NRF Expression in AMI and HF Patients Compared to the Control Group

		statistic	df	Sig.
lncRNA_NRF	AMI	,430	22	,000
	HF	,342	20	,000
	Controls	,622	15	,000

To determine the role and clinical significance of circulating lncRNA NRF in cardiac diseases, plasma levels were analysed in patients with heart failure and acute myocardial infarction. The obtained results clearly confirm that plasma expression of lncRNA NRF is elevated in both HF and AMI. The highest mean value was observed in the AMI group (mean = 901.3 ± 2454.36), followed by the HF group (mean = 117.6 ± 364.3), with the lowest levels found in the control group (mean = 0.52 ± 0.73) (Table 13).

Table 13. Mean Values of lncRNA NRF in AMI, HF Patients and the Control Group

Групи	Mean	Std. Deviation
AMI	901,2663439	2454,361291
HF	117,5759681	364,1633650
Control	,5244469053	,7253748996

The Kruskal-Wallis analysis conducted among the three studied groups revealed a significant difference in the measured mean plasma expression levels of lncRNA NRF ($p = 0.013$). The results are presented in both tabular and graphical formats (Table 14, Fig. 21).

Table 14. Kruskal-Wallis Analysis of lncRNA NRF Expression Among the Three Studied Groups (AMI, HF, Control Group)

Total N	57
Test Statistic	8,721a
Degree Of Freedom	2
P	,013

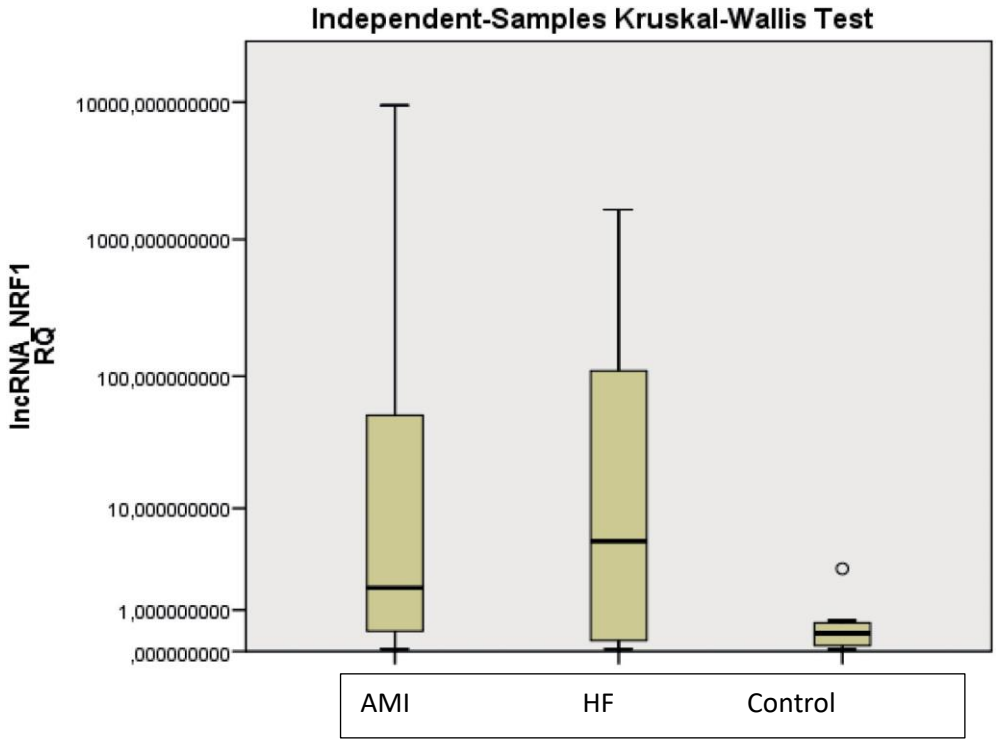


Figure 21. Box Plot Analysis Illustrating Plasma Expression of lncRNA NRF in the AMI, HF, and Control Groups

A subgroup analysis compared the mean plasma expression levels of the respective RNA between the different groups. The expression level of lncRNA NRF was significantly higher in the AMI group compared to the control group (p = 0.012). There was a non-significant correlation in plasma expression levels of lncRNA NRF between the HF group and the control group (p = 0.077). No significant difference in the expression of the studied RNA was observed when comparing the HF and AMI groups (p = 1.0).

Table 15. Comparison of Plasma Expression of lncRNA NRF in AMI, HF Patients and the Control Group

Groups	Test Statistic	Std. Error	Std. Test Statistic	Sig.	P
Control- HF	12,650	5,669	2,231	,026	,077
Control -AMI	15,964	5,557	2,873	,004	,012
HF-AMI	3,314	5,128	,646	,518	1,000

8. Plasma Expression of lncRNA NRF in Patients with Heart Failure

According to the data from the ROC analysis, the plasma expression levels of lncRNA NRF differentiate HF patients with 73% sensitivity and 55% specificity compared to the control group. The diagnostic accuracy of the biomarkers was determined by obtaining the largest area under the curve – AUC = 0.783 (95% CI: 0.532 – 0.881, p = 0.039) at the optimal cut-off values (Fig. 22).

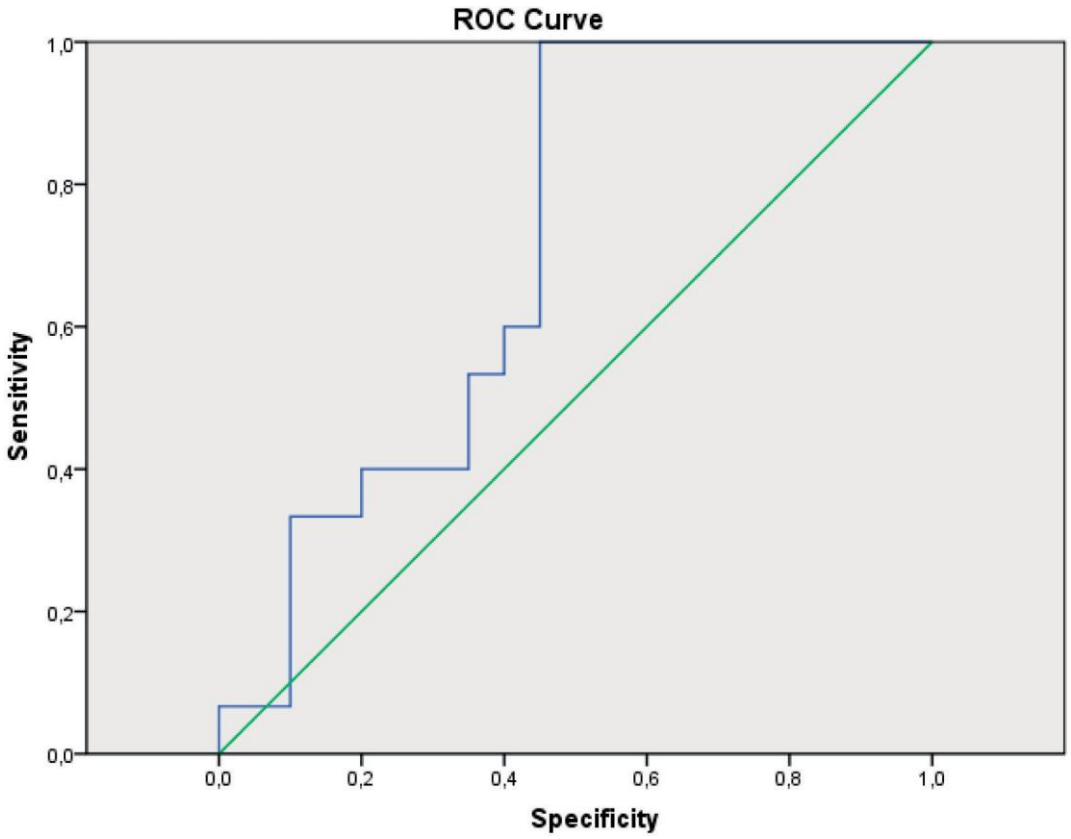


Figure 22 Plasma Expression Levels of lncRNA NRF Differentiating Patients with Heart Failure from the Control Group

– By Chi-Square analysis, no difference was observed in plasma expression of lncRNA NRF between males and females (p = 0.33);
– The comparative analysis of plasma expression of lncRNA NRF in patients with heart failure showed no significant difference related to age (P = 0.13) (Table 16).

Table 16. Analysis of lncRNA NRF Expression by Age in the Heart Failure Group

			Years	**lncRNA NRF**
Spearman‘s rho	Years	Correlation Coefficient	1,000	,350
		P	.	,130
	lncRNA NRF	Correlation Coefficient	,350	1,000
		P	,130	.

– The study also analyses the relationship between lncRNA NRF and ejection fraction (%) in HF patients.

It was found that the expression of lncRNA NRF does not differ significantly (p = 0.729) in relation to ejection fraction (%) in patients with heart failure (Table 17).

Table 17. Correlation Analysis of the Relationship Between lncRNA NRF and Ejection Fraction (%)

		lncRNA NRF RQ	**ФИ%**
lncRNA NRF RQ	Pearson Correlation	1	-,083
	P		,729
EF %	Pearson Correlation	-,083	1
	P	,729	

▶ **Plasma Expression of lncRNA NRF in the Group of Patients with Heart Failure and Comorbidities (Diabetes Mellitus, Dyslipidemia, Chronic Kidney Disease)**

Approximately one third of HF patients (39.3%) have DM, with no significant correlation found between plasma expression of lncRNA NRF and diabetes mellitus (p = 0.45).

In 25% of HF patients, where dyslipidemia was observed, no significant difference in the level of lncRNA NRF expression was found (p = 0.503).

Regarding CKD, observed in 17.9% of HF patients, no significant difference was found between plasma expression of lncRNA NRF and the presence of chronic kidney disease (p = 0.33). The results are presented in Table 18 and Figure 23 (A, B, C).

Table 18. Distribution of lncRNA NRF by Comorbidities in AMI Patients

Mann-Whitney – Comorbidities	**Value**	**P**
DM	15,000a	,454
Dyslipidemia	23,000a	,503
CKD	44,000a	,331

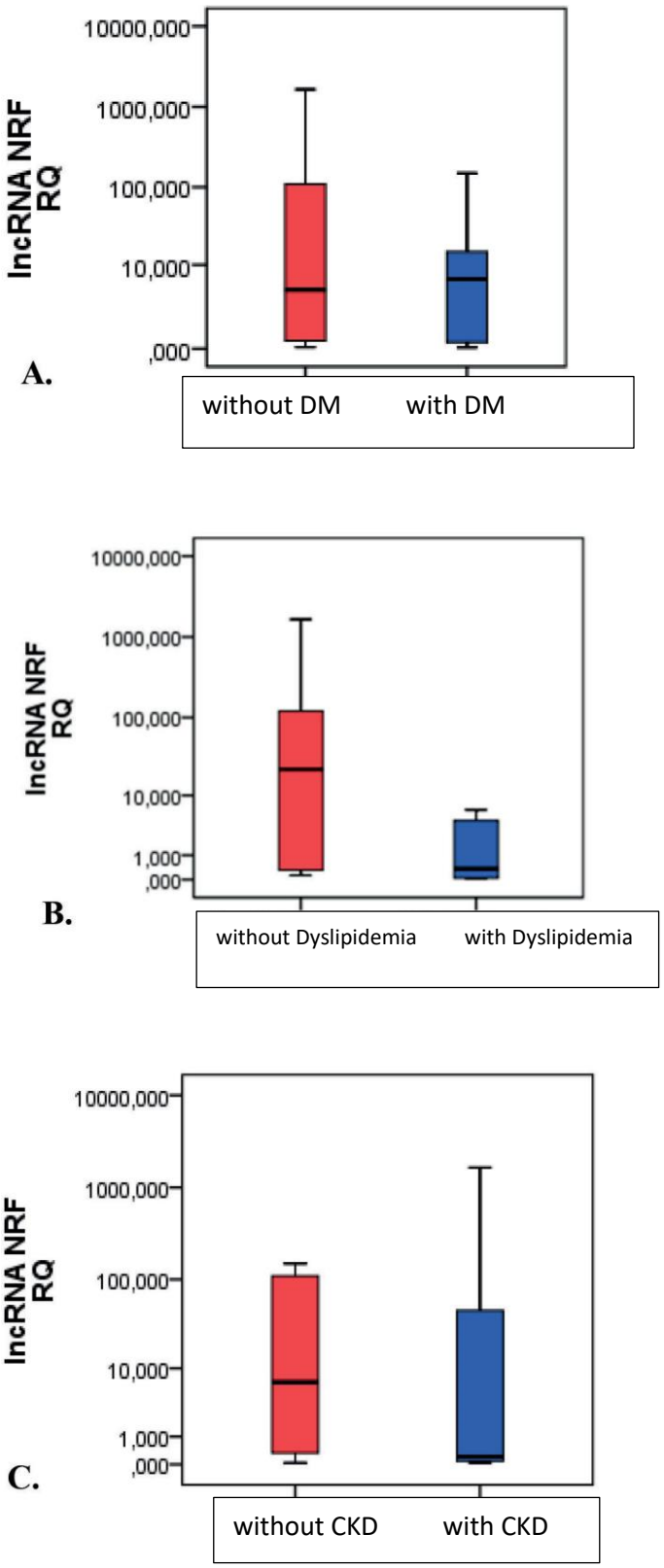


Figure 23. Box Plot Analysis Showing the Relationship Between Plasma Expression of lncRNA NRF and Comorbidities in Patients with Heart Failure

9. Plasma Expression of lncRNA NRF in Patients with Acute Myocardial Infarction (STEMI)

According to the ROC analysis, the plasma expression levels of lncRNA NRF differentiate patients with STEMI from the control group with a sensitivity of 73% and specificity of 68%. The diagnostic accuracy of the biomarkers was determined by obtaining the largest area under the curve – AUC = 0.794 (95% CI: 0.650 – 0.938, p = 0.003) (Fig. 24) at the optimal cut-off values.

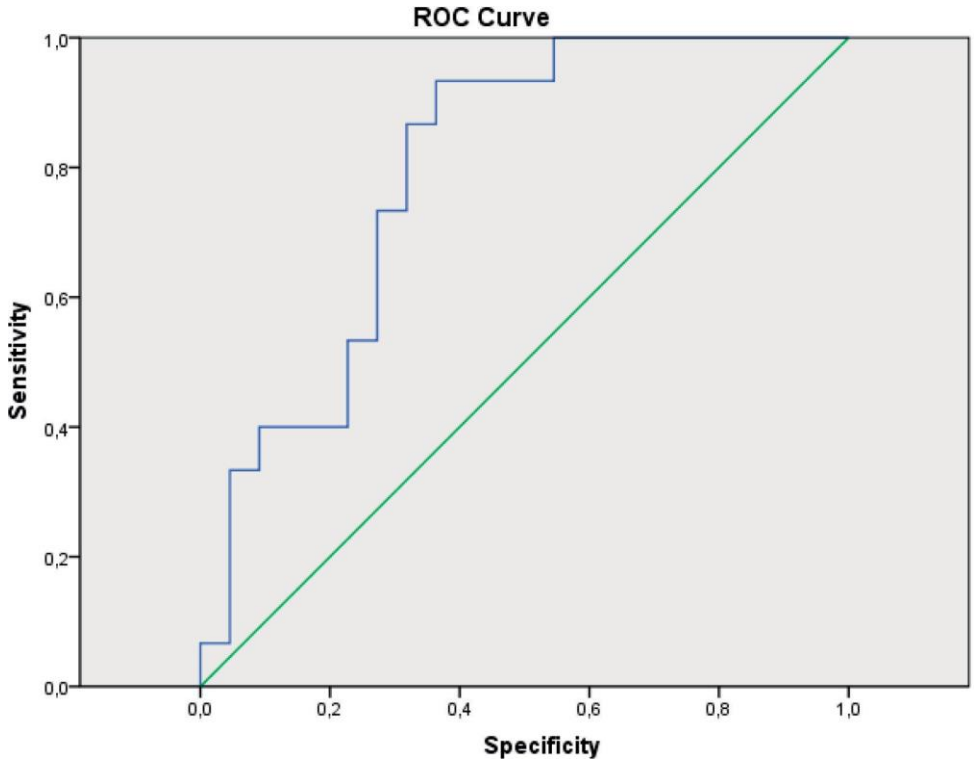


Figure 24. Plasma Expression Levels of lncRNA NRF Differentiating Patients with Myocardial Infarction from the Control Group

–There is no difference in the expression of lncRNA NRF according to gender (p = 0.216) (Table 19).

Table 19. Expression Level of lncRNA NRF According to Gender

		lncRNA NRF	Gender
lncRNA NRF	Correlation	1	,275
	P		,216
Gender	Correlation	,275	1
	P	,216	

– Regarding age and the expression of lncRNA NRF, no significant correlation was observed (p = 0.56) in patients with STEMI (Table 20).

Table 20. Analysis of lncRNA NRF Expression by Age in the AMI Group

			Years	**lncRNA NRF**
Spearman‘s rho	Years	Correlation Coefficient	1,000	,131
		P	.	,560
	lncRNA NRF	Correlation Coefficient	,131	1,000
		P	,560	.

1. **Plasma Expression of lncRNA NRF in the Group of Patients with Acute Myocardial Infarction and Comorbidities (Diabetes Mellitus, Dyslipidemia, Chronic Kidney Disease)**

Approximately one-third of patients with STEMI (37.8%) are diagnosed with DM. However, no significant difference was found between the plasma expression of lncRNA NRF and the presence of diabetes (p = 0.761).

In patients within the STEMI group diagnosed with dyslipidemia (25%), no direct association was observed between the plasma expression of lncRNA NRF and dyslipidemia (p = 0.043).

When analysing CKD patients, who represent 21.6% of the STEMI group, no significant difference was found between the plasma expression level of lncRNA NRF and CKD (p = 0.345). The results are presented in Table 21 and Figure 25 (A, B, C).

Table 21. Distribution of lncRNA NRF by Comorbidities in AMI Patients

Mann-Whitney – Comorbidities	**value**	**P**
DM	45,500a	,761
Dyslipidemia	17,500a	,043
CKD	22,000a	,345

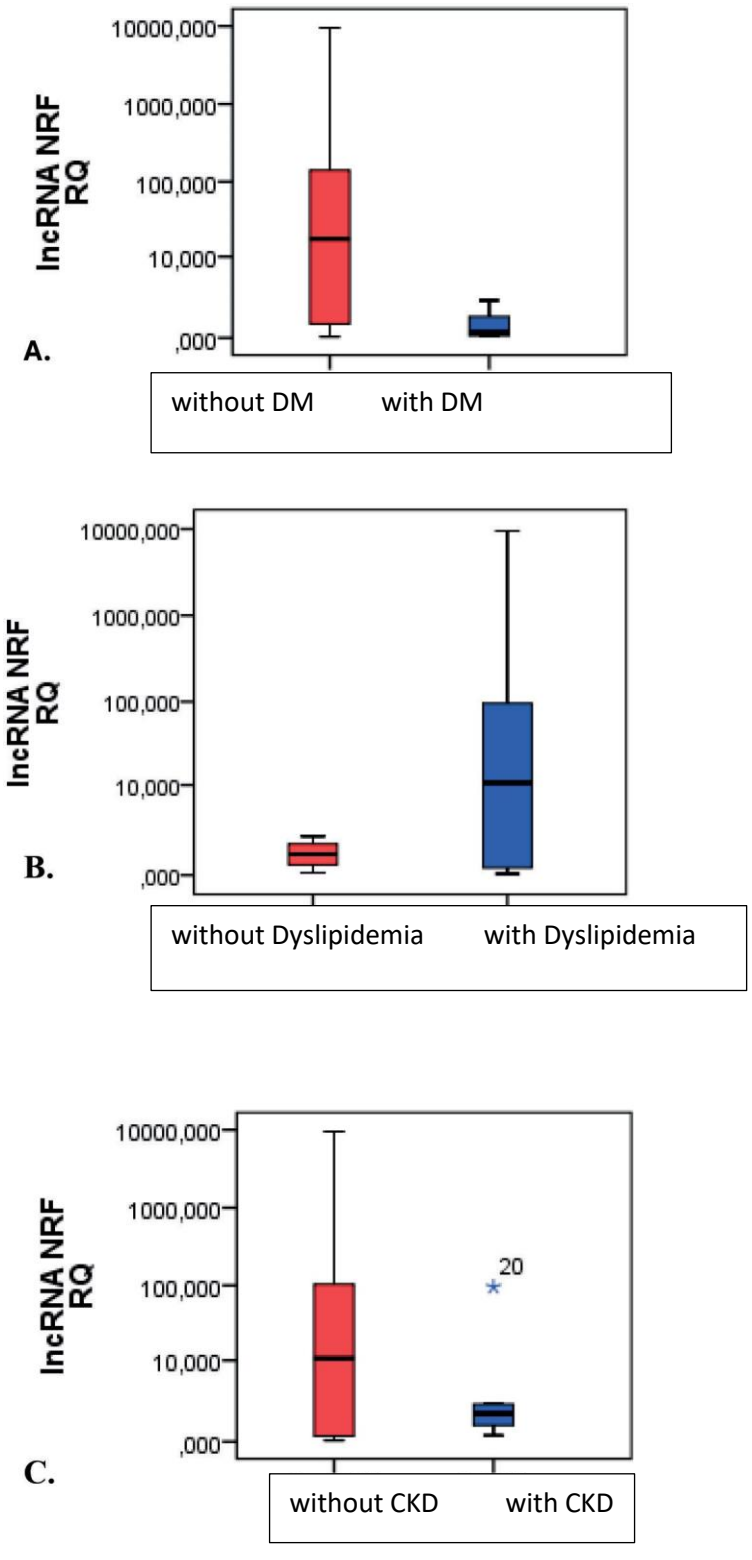


Figure 25. Box plot analysis showing the relationship between plasma expression of lncRNA NRF and comorbidities in AMI patients

- **Comparative analysis of plasma expression of lncRNA NRF with laboratory markers and ejection fraction in AMI patients.**

LncRNA NRF and Troponin

For the purposes of the study, the relationship between the levels of expression of lncRNA NRF, analysed in blood plasma within 12 hours from the onset of symptoms of AMI, and the blood levels of troponin within 12 hours from symptom onset was investigated. The analysis included initial troponin levels (p = 0.5), maximum measured troponin (p = 0.68), and troponin levels at 24–48 hours from symptom onset (p = 0.43), as shown in Figure 26 – A, B, C. No direct correlation was found between the dynamics of troponin measurements and the plasma expression of lncRNA NRF in AMI patients.

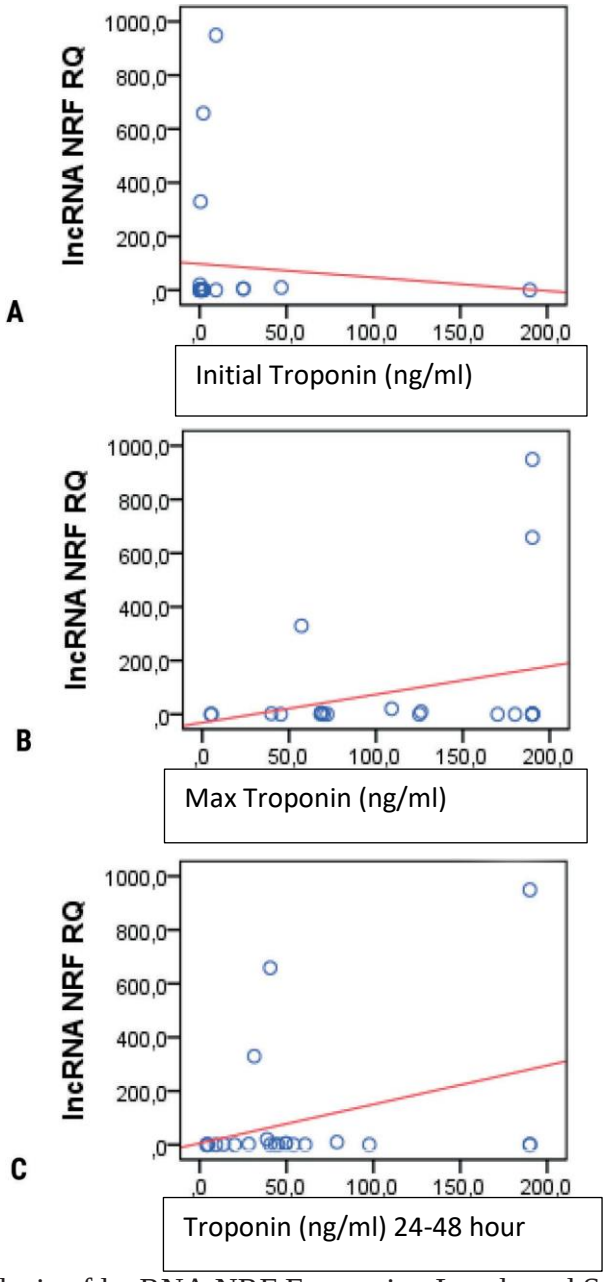


Figure 26. Correlation Analysis of lncRNA NRF Expression Levels and Serum Troponin Concentrations

LncRNA NRF and HMGB1, RIPK3

Upon subsequent analysis utilizing the Spearman correlation test, no statistically significant correlation was detected between the expression levels of lncRNA NRF and the blood serum concentrations of the necrosis marker HMGB1 (p = 0.215) and the necroptosis marker RIPK3 (p = 0.691), as demonstrated in Figures 27A and 27B.

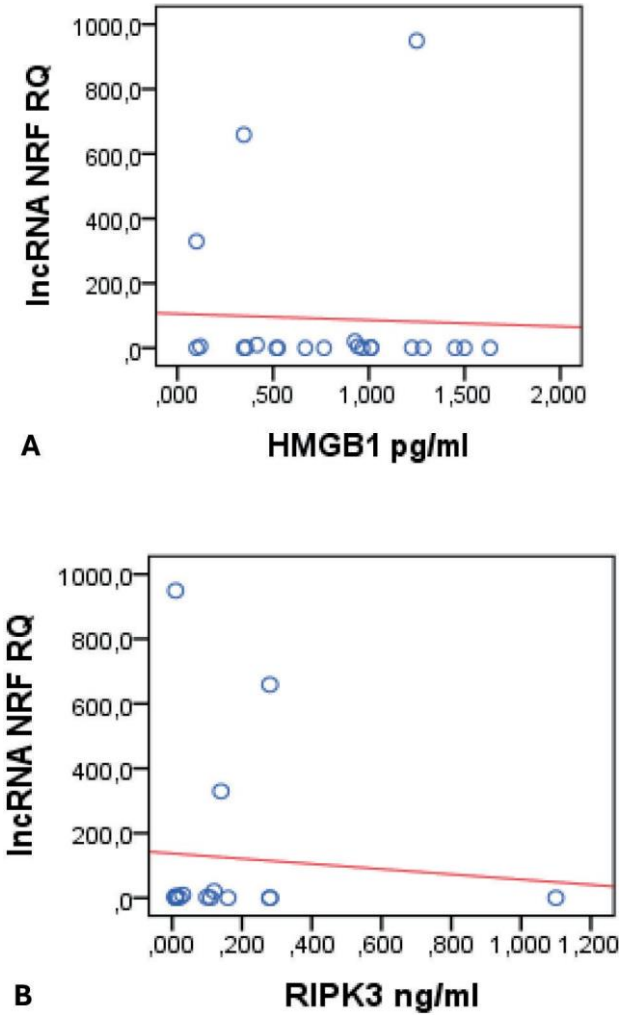


Figure 27. Correlation Analysis of lncRNA NRF Expression Levels with HMGB1 and RIPK3 Markers

LncRNA NRF and EF %

Based on the Spearman correlation test, no correlation was found between plasma expression of lncRNA NRF and ejection fraction - EF% (p = 0.213) in AMI patients (fig. 28).

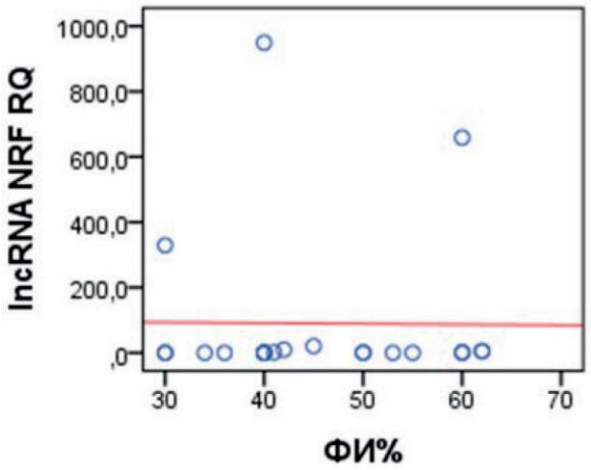


Figure 28. Correlation Analysis of lncRNA NRF Expression Levels and EF%

IV. DISCUSSION

1. Expression of lncRNA Wisper in Heart Failure

The development of heart failure (HF) is a multi-stage process that progressively deteriorates quality of life. High morbidity and mortality are associated with significant healthcare costs (Savarese et al., 2023). Over the past decades, substantial advances have been achieved in both HF prevention and treatment (Shen et al., 2017). Despite this, global mortality due to HF continues to rise (Ponikowski et al., 2014).

HF progression is associated with the emergence of maladaptive structural changes in the heart. Cardiomyocyte hypertrophy and fibrotic tissue development reduce contractility. The neurohormonal response to reduced cardiac volume is activation of the Renin-Angiotensin-Aldosterone System (RAAS) and the sympathetic nervous system. All these factors lead to vasoconstriction, fluid retention, and HF progression (Yap et al., 2010). Understanding the pathophysiological aspects of HF development could, through in-depth analysis, establish new, specific treatment targets. Long-chain non-coding RNAs (lncRNAs) may play a role in defining such potential targets (Boeckel et al., 2019).

LncRNAs represent one of the most remarkable but less studied transcriptomes in the heart. During the processes of cardiomyocyte growth, differentiation, and maturation, a large set of long-chain RNA molecules have been described (Beermann et al., 2018; Devaux et al., 2015; He et al., 2016).

Various lncRNAs have been described in heart failure dynamics, which control processes such as cardiac hypertrophy, apoptosis, and fibrosis (Han et al., 2023).

Although the exact origin and function of lncRNAs in circulation are unknown, they are found to be variably expressed, with stable blood levels in patients with various cardiovascular diseases and healthy individuals. This property determines their potential use as suitable biomarkers for cardiovascular disease diagnosis (Busch et al., 2016). During myocardial injury, they are released into circulation, similar to proteins (Araszkiewicz et al., 2014).

In a 2017 study, Micheletti et al. investigated lncRNA Wisper in cardiac fibroblasts of mouse models with experimentally induced myocardial infarction (Micheletti et al., 2017). Additionally, in the same study, lncRNA Wisper was described in the cardiac tissue of 26 patients with aortic stenosis, which is associated with excessive fibrotic tissue accumulation. It was reported that lncRNA Wisper expression correlates with the development of cardiac fibrosis in both mouse models and humans (Micheletti et al., 2017).

In our study, we analyzed the potential role of lncRNA Wisper as a possible diagnostic marker for patients with heart failure.

For this purpose, we compared the expression level of lncRNA Wisper in the plasma of patients with heart failure and a control group, finding significantly higher expression levels of lncRNA Wisper in patients with heart failure compared to the control group ($p = 0.021$). Plasma expression levels of lncRNA Wisper differentiated heart failure patients from the control group with 80% sensitivity and 67% specificity, with AUC = 0.783 (95% CI: 0.648–0.919), $p = 0.002$. These results demonstrate the potential role of the investigated RNA as a sensitive marker for distinguishing heart failure patients from the control group.

Cardiac fibrosis is considered one of the primary pathomorphological processes in heart failure development (Frangogiannis and Kovacic, 2020).

In a study (Puls et al., 2020), myocardial fibrosis was found to be associated with lower ejection fraction, assessed in patients with aortic stenosis. Myocardial fibrosis is significantly more pronounced in patients with EF below 50%.

Cardiac fibrosis correlates positively with the degree of pathological left ventricular remodeling and clinical heart failure (HF) manifestation. It is more significantly expressed in patients with higher New York Heart Association (NYHA) functional classification of heart failure (Puls et al., 2020).

In our study, we investigated HF patients and low ejection fraction (EF <40%), with likely more pronounced myocardial fibrosis. This hypothesis aligns with global literature data regarding the relationship between lncRNA Wisper and fibrosis degree (Micheletti et al., 2017). We hypothesize that higher lncRNA Wisper expression levels are associated with excessive fibrosis development, as this LncRNA is known to regulate fibroblast proliferation and growth (Micheletti et al., 2017).

Based on this hypothesis and obtained results, the current study demonstrates the potential prognostic significance of lncRNA Wisper as a marker for fibrosis development in HF.

In our research, no correlation was found between lncRNA Wisper expression levels and ejection fraction ($p = 0.68$). We attribute this probable outcome to the studied patient population. All investigated HF patients had low ejection fraction (EF < 40%). In almost all patients participating in our study, the etiology of HF was a previous MI with left ventricular remodelling, developed fibrosis, and suppressed systolic function.

Our study found that comorbidities and conditions such as DM, dyslipidemia, and CKD do not affect lncRNA Wisper expression in the plasma of the studied patients. Specifically, for diabetes mellitus ($p = 0.358$), dyslipidemia ($p = 0.358$), and chronic kidney disease ($p = 0.358$). Considering these are the most common comorbid conditions, this result supports the value of lncRNA Wisper as a potential biomarker for myocardial fibrosis, independent of other comorbidities.

Of particular interest was the comparison of plasma lncRNA Wisper expression between males and females. It is known that left ventricular remodelling processes in women proceed more favourably, and they develop clinical HF manifestations later. In our obtained results, no difference was found based on the gender of the studied patients ($p = 0.469$). This may be due to the fact that all patients included in the study already have advanced HF and developed myocardial fibrosis. On the other hand, these data again confirm the value of lncRNA Wisper as a biomarker whose results will not be modulated by gender.

The results of plasma expression levels of lncRNA Wisper according to patient age were surprising to us. A strong, but inverse correlation was established ($p = 0.016$) with a correlation coefficient (rho – 0.452). The data indicate that older patients, in whom myocardial fibrosis is expected to be more pronounced, express lncRNA Wisper to a lesser extent compared to younger patients. Interpreting this fact is challenging, especially in the absence of literature data. This is likely due to already developed fibrosis or age-related cellular changes.

According to available literature, the expression level of lncRNA Wisper in human plasma has not been previously investigated in medicine. The only human study reported increased lncRNA Wisper expression in cardiac tissue among patients with aortic stenosis, which leads to left ventricular dysfunction and fibrotic tissue accumulation. The expression of this long-chain RNA correlated with increased collagen synthesis, which is observed during fibrosis development (Micheletti et al., 2017). In this context, additional studies with a large number of patients are necessary to comprehensively investigate the role of lncRNA Wisper in myocardial fibrosis pathogenesis, which could potentially open new therapeutic opportunities.

For clinical practice application, the fact that lncRNA Wisper is expressed in blood plasma is of significant importance, meaning samples can be easily obtained without invasive methods. It would be interesting to compare its sensitivity and specificity with imaging methods such as routine echocardiography and the increasingly popular magnetic resonance imaging (MRI). lncRNA Wisper may have high value as a marker for early detection of remodelling and fibrosis processes, before they can be diagnosed through imaging methods.

2. LncWisper Expression in Myocardial Infarction

Despite undeniable advances in myocardial infarction treatment in the era of interventional cardiology, this disease remains a leading cause of disability and mortality in developed countries (Thiene and Basso, 2010).

The high incidence and socioeconomic consequences draw attention not only to early diagnosis and treatment but also to long-term consequences of experienced myocardial infarction (Li et al., 2020).

Several studies have evaluated the expression of various lncRNAs in blood and their potential value as prognostic biomarkers in acute myocardial infarction (AMI) (Wang et al., 2019).

For instance, the study by Wang et al. in 2019 demonstrated increased expression of three long-chain RNAs – lncRNA H19, MIAT, MALAT1 – in patients with AMI (Wang et al., 2019).

The biomarker potential of five additional circulating RNAs, specifically aHIF, ANRIL, KCNQLOT1, MIAT, and MALAT1, was evaluated in the blood of patients who survived AMI (Vausort et al., 2014).

In our study, we investigated lncRNA Wisper expression levels in the blood plasma of patients with AMI. To date, there are no literature data on a similar study with lncRNA Wisper. We found significantly higher lncRNA Wisper values among the AMI patient group compared to the control

group. Plasma lncRNA Wisper expression differentiated STEMI patients from the control group with considerable sensitivity of 80% and specificity of 71%, p = 0.002.

The obtained results demonstrate that lncRNA Wisper increases during the first 12 hours from the AMI onset. Although it may be a biomarker for myocardial necrosis, it appears not to follow the dynamics of plasma troponin levels. The characteristic change in troponin levels, its correlation with the area of myocardial necrosis, as well as the reliability and accessibility of its investigation make it an irreplaceable biomarker in AMI.

At first glance, LncRNA Wisper does not appear to have advantages for the diagnosis of acute myocardial infarction, but it may have value in another aspect. There is evidence that lncRNA Wisper is a regulator of fibrosis and plays a role in the activation of fibroblasts (Micheletti et al., 2017). If we assume that lncRNA Wisper initiates these processes at such an early stage of AMI, it is possible that it could serve as a predictor for the development of fibrosis and its degree during the chronic phase of myocardial infarction, with corresponding clinical manifestations.

Despite the limited patient group in which the study was conducted and the need for a larger and more homogeneous group, current results suggest that Wisper increases during the first 12 hours from the onset of STEMI.

In our study, we observed an interesting significant correlation (p = 0.019) between the plasma expression of lncRNA Wisper and blood levels of HMGB1.

HMGB1 is a DNA-binding protein that plays a role in various intracellular and extracellular processes. The involvement of HMGB1 in inflammatory and regenerative responses has been discussed (Klune et al., 2008). HMGB1 is passively released by necrotic cells or actively by cells involved in the inflammatory response, leading to an increased production of pro-inflammatory mediators and free radicals. On the other hand, HMGB1 induces hypertrophy and apoptosis in cardiomyocytes, stimulates the activity of cardiac fibroblasts, and promotes the proliferation and differentiation of cardiac stem cells. Therefore, in addition to harmful effects on cardiomyocytes, this molecule also possesses certain regenerative capabilities for cardiomyocytes (Raucci et al., 2019).

This hypothesis is further confirmed by clinical studies. For instance, several studies report a potential link between HMGB1 and myocardial ischemia (Germani et al., 2007). All of them demonstrate that elevated levels of HMGB1 in myocardial ischemia are associated with worse prognosis. In a study by Goldstein et al. (2006), blood levels of HMGB1 in 9 patients with acute coronary syndrome were significantly higher than those in a control group of volunteers. Another study (Andrassy et al., 2008) reports high plasma levels of HMGB1 in patients with STEMI during hospitalization.

In the study by Kohno et al. (2009), which included 35 STEMI patients, HMGB1 levels in the blood were temporarily elevated, peaking at 12 hours within the first 7 days following hospitalization. On the other hand, the administration of HMGB1 following a myocardial infarction induced by permanent ligation of the coronary artery improves cardiac function by stimulating tissue regeneration. HMGB1 reduces contractility, induces hypertrophy and apoptosis in cardiomyocytes, stimulates the activity of cardiac fibroblasts, and promotes the proliferation and differentiation of cardiac stem cells. Interestingly, maintaining appropriate nuclear levels of HMGB1 protects cardiomyocytes from apoptosis by preventing oxidative stress on DNA (Kohno et al., 2009).

In cell cultures, a connection between HMGB1 and LncRNAs has been reported. Some of these RNAs evidently control the synthesis of HMGB1 (Wang and Gan, 2022). The consequences of this control and its clinical significance remain unclear due to the dual role of HMGB1.

In cell cultures, a connection between HMGB1 and LncRNAs has been reported. Some of these RNAs evidently control the synthesis of HMGB1 (Wang and Gan, 2022). The consequences of this control and its clinical significance remain unclear due to the dual role of HMGB1.

Enhancing regeneration or suppressing the inflammatory response along the LncRNA Wisper-HMGB1 axis is a potential future therapeutic target.

As in the group of patients with HF, in those with STEMI, the levels of expression of lncRNA Wisper are also unaffected by gender, age, and comorbidities such as DM, CKD, and dyslipidemia. This makes lncRNA Wisper a reliable candidate for a potential biomarker.

Based on the obtained results and the analysis of the literature, we can summarize that lncRNA Wisper has increased expression in the plasma of patients with myocardial fibrosis and necrosis. Further in-depth studies are required to validate its value as a prognostic biomarker CVDs.

3. Expression of lncRNA NRF in Heart Failure

The analysis of plasma levels of lncRNA NRF in HF patients shows a marked tendency for an increase, but does not reach a statistically significant difference compared to the control group ($p = 0.077$). A possible reason for this is the well-known fact that different types of cardiovascular diseases (CVDs) are associated with different forms of cell death. For instance, in myocardial infarction, necrosis predominates (Guo et al., 2022), and it is known from the literature that lncRNA NRF directly participates in the realization of necrosis (Huang, 2018). In chronic heart failure, the leading form of cell death is apoptosis (van Empel et al., 2005).

In a study by Yan, L., et al. (Yan et al., 2020), published in 2020, higher levels of expression of lncRNA NRF were found in patients with heart failure following an acute myocardial infarction

(AMI), compared to heart failure of non-ischemic origin. In this case, the increased expression of this RNA is associated with excessive necrosis that occurred during the AMI (Yan et al., 2020).

As with lncRNA Wisper, factors such as age, gender, and comorbidities (DM, CKD and dyslipidemia) do not influence the plasma expression of lncRNA NRF.

4. Expression of lncRNA NRF in Myocardial Infarction

The current study also evaluated the expression of lncRNA NRF in the plasma of patients with STEMI. The highest levels of plasma expression of lncRNA NRF were observed in the group of patients with STEMI ($p = 0.012$), with no correlation between the patients with HF and AMI, as well as a weak but non-significant correlation between the patients with HF and the control group.

The plasma levels of expression of lncRNA NRF differentiate STEMI patients from the healthy control group with 73% sensitivity and 68% specificity ($p = 0.003$). Thus, according to the analysis, the increased expression of lncRNA NRF is observed only in the STEMI group.

LncRNA NRF has been described as an RNA that increases the level of programmed necrosis in cardiomyocytes subjected to ischemic injury (Wang et al., 2016).

Programmed necrosis is a type of cell death that predominates when apoptotic cells are inactive (Cho et al., 2010; Lamkanfi et al., 2007). Several studies suggest that programmed necrosis is one of the main forms of cell death during acute cardiomyocyte injury (Bryant et al., 1998; Kingma and Yellon, 1992; Lissoni et al., 1992).

The probable involvement of lncRNA NRF in promoting this type of cell death in the context of AMI has been confirmed in the study by Wang, K., et al. (2016), which examined cardiomyocytes from mouse models (Wang et al., 2016). Programmed necrosis or necroptosis is mediated by specific receptor kinases: serine-threonine protein kinase 1 and 2 (respectively RIPK1 and RIPK3) (Cho et al., 2009; Guerra et al., 1999). As a participant in this complex regulatory network, lncRNA NRF interacts with miR 873, which is also a regulator of necrosis, influencing the miR 873 / RIPK1 RIPK3 axis with an overall effect of promoting necrosis. Increased expression of lncRNA NRF correlates with the size of the infarction zone and the degree of necrosis observed in cardiac tissue from experimentally induced AMI in mouse models. Conversely, suppression of lncRNA NRF expression inhibits necrosis and reduces the infarction zone size (Wang et al., 2016).

These results are indicative of the predictive role of lncRNA NRF in the evolution of necroptosis.

In 2020, an interesting study by Yan, L., et al. (Yan et al., 2020) was published, conducted in humans. In this study, the authors compared the expression of lncRNA NRF in the blood of patients with AMI, who develop HF, and those with AMI but without HF. The level of expression of

lncRNA NRF was significantly higher in the group of patients with pronounced HF (based on clinical symptoms and LVEF). The reason for this is discussed as the more extensive infarction zone, associated with more excessive necrosis. The same study also points out the higher levels of expression of lncRNA NRF in the blood of patients with acute coronary syndrome compared to patients with chronic coronary artery disease (Yan et al., 2020).
The analysis of our study aligns with the previously published studies. The levels of plasma expression of lncRNA NRF are significantly higher in patients with AMI. This result correlates with the known data on the involvement of lncRNA NRF in the regulation of necroptosis as a key pathophysiological process in AMI (Guo et al., 2022).

According to our results, no direct dependence was found between the expression levels of lncRNA NRF and the presence of DM, dyslipidemia, or CKD.

A limiting factor of the study is the small patient population and the lack of follow-up on the expression of lncRNA NRF after undergoing PCI.

V. CONCLUSIONS AND CONTRIBUTIONS

1. Conclusions

Based on the conducted scientific study, we can draw the following conclusions:

1. Plasma expression of lncRNA Wisper is significantly increased in patients with heart failure NYHA class III-IV compared to the control group ($p = 0.021$)
2. Plasma expression of lncRNA Wisper is significantly increased in patients with STEMI up to 12 hours after the onset of the infarction, compared to the control group ($p = 0.03$).
3. Age is a factor that influences the plasma expression of lncRNA Wisper in patients with heart failure. As age increases, the plasma expression of lncRNA Wisper significantly decreases ($p = 0.016$).
4. Plasma expression of lncRNA Wisper correlates significantly with blood levels of HMGB1 in patients with STEMI ($p = 0.019$), but does not follow the dynamics of Troponin I in these patients, nor that of the necroptosis marker RIPK3.
5. Plasma expression of lncRNA NRF is significantly increased in patients with STEMI up to 12 hours after the onset of the infarction, compared to the control group ($p = 0.012$). However, in patients with heart failure, plasma expression of lncRNA NRF does not reach a significant difference compared to the control group ($p = 0.077$).
6. Factors such as gender and comorbidities (DM, dyslipidemia, CKD) do not influence

the plasma expression of the two studied long non-coding RNAs (lncRNA Wisper and lncRNA NRF).

2. Contributions

Contributions with scientific-theoretical character

1. . For the first time in Bulgaria, the expression of lncRNA Wisper and lncRNA NRF is investigated in human plasma of patients with acute myocardial infarction and heart failure, and the results are compared with a control group.
2. It has been established that the plasma expression of lncRNA Wisper increases in heart failure as well as in acute myocardial infarction.
3. It was revealed that age influences the plasma expression of lncRNA Wisper.
4. For the first time in Bulgaria, the potential predictive value of lncRNA Wisper for the development of fibrosis in patients with heart failure is reported.
5. For the first time in Bulgaria, the presence of a significant correlation between the plasma expression of lncRNA Wisper and HMGB1 is reported.
6. The increased expression of lncRNA NRF in patients with STEMI, compared to the control group, is analyzed.

Contributions of a Scientific and Practical Nature:

1. The value of lncRNA Wisper as a biomarker for myocardial fibrosis is reported, which is not influenced by additional factors such as gender and the most common comorbidities associated with heart diseases
2. The potential of lncRNA NRF as a biomarker for acute myocardial necrosis is established.
3. The identified patterns provide a foundation for future studies tracking the examined process

SUMMARY

Recent studies reveal the role of Lnc RNAs in the physiology and pathophysiology of the complex processes within the myocardial cell. Their regulatory functions at various epigenetic levels and their connection to the genesis of heart diseases uncover new, unexplored opportunities for diagnosis and treatment.

Our study analyses the plasma expression of two relatively new and under-researched long non-coding RNA molecules – lncRNA Wisper and lncRNA NRF, in socially significant diseases such as acute myocardial infarction and heart failure.

We found that the plasma expression of the examined Lnc RNAs not only changes in heart diseases but also exhibits specific characteristics. LncRNA Wisper, which is associated with the activation of fibroblasts, is a likely early predictor of fibrosis and is significantly elevated in patients with AMI and those with HF.

LncRNA NRF, which regulates necrosis, is expressed to a significantly greater extent in patients with STEMI and acute myocardial injury compared to those with heart failure.

For both examined Lnc RNAs, it was confirmed that their expression is not influenced by gender, age, or certain comorbidities.

The aberrant expression of lncRNA Wisper and lncRNA NRF in the blood plasma of patients with cardiovascular diseases reveals the potential significance of these long non-coding RNA molecules as markers of acute or chronic myocardial damage.

These results offer opportunities for more in-depth and specific studies that could demonstrate their value as biomarkers in clinical practice. Additional information from studies with a larger number of patients and standardized research methods would reveal the potential of the examined lncRNAs Wisper and NRF as prognostic markers.

This could improve not only the prognosis of CVD but also provide an opportunity for the creation of new therapeutic strategies.

SCIENTIFIC PUBLICATIONS ON THE SUBJECT

1. Doneva Y, V Valkov, Y Kashlov, G. Mihaylova, A Angelov, M Radanova (2021). CIRCULAR RNA – miRNA MEDIATED INTERACTION IN MYOCARDIALINFARCTION. Journal of IMAB, vol. 27, issue2.

2. Doneva Y (2023). Long Non-Coding RNAs – CHARACTERISTICS AND ROLE IN CARDIOVASCULAR DISEASES. CARDIOVASCULAR DISEASES Cardiovascular Diseases, Vol. 54, No. 1, 7–15 ref. 63 ref.