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This dissertation was made at the Institute of Cardiovascular Disease, Dubrava University Hospital, Zagreb, Croatia and Clinic of Cardiology, University Clinical Center of Kosova, Prishtina

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Prof. Masar Gashi, MD, PhD

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To whom it may concern

It is my hope that this thesis will contribute to the future research on the function of apelin in pathways and regulation of cardiac metabolism, and to help in potential utility of apelin-12 measurement in STEMI patients as an additional risk stratification tool and in the future possibility for the usage as a therapy.

TABLE OF CONTENTS

I. LIST OF ABBREVIATIONS

ACE	Angiotensin converting enzyme
ACS	Acute coronary syndrome
ADP	Adenosine diphosphate
AMI	Acute myocardial infarction
ANP	Atrial natriureic peptide
APLN	Apelin
APJ	Apelin receptor
ASA	Acetylsalicylic acid
ATP	Adenosine triphosphate
AUC	Area under the curve
BNP	Brain-type natriuretic peptide
cGMP	Cyclic guanylate monophosphate
CK	Creatine kinase
CKM	Creatine kinase M-type
CKMB	Creatine kinase myocardial band
CRP	C-reactive protein
cTn	Cardiac troponin
CVD	Cardiovascular disease
DAG	Diacylglycerol

DBP	Diastolic blood pressure
ECG	Electrocardiogram
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
EF	Ejection fraction
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric-oxide synthase
EPC	Endothelial progenitor cell
ESCs	Embryonic stem cells
GC	Guanylyl cyclase
GPCR	G-protein coupled receptor
FMCBT	First-medical-contact-to-balloon time
HDL	High density lipoprotein
HIF-1	Hypoxia inducible factor 1
HRE	Hypoxia response element
IHD	Ischemic heart disease
IP3	Inositol trisphosphate
IR	Ischemia reperfusion
IRA	Infarct-related artery
LAD	Left descending coronary artery
LCx	Left circumflex coronary artery

LBBB	Left bundle branch block
LDL	Low density lipoprotein
LMA	Left main artery
LV	Left ventricular
MA	Modified apelin-12
MACE	Major adverse cardiac events
MAPK	Mitogen-activated protein kinase
MI	Myocardial infarction
MRI	Myocardial reperfusion injury
mPTP	Mitochondrial permeability transition pore
mtCKs	Mitochondrial sarcomeric creatine kinase
MEK1/2	Mitogen-activated protein kinase
NCX	Na+/Ca2+ exchanger
NHE	Sarcolemmal Na+/H+ exchanger
NO	Nitric oxide
NPR-A	Natriuretic peptide receptor A
NPs	Natriuretic peptides
NSTEMI	Non-ST-segment elevation myocardial infarction
NT-proBNP	N-terminal proBrain-type natriuretic peptide
NPs	Natriuretic peptides
NPR-A	Natriuretic peptide receptor A

O2	Oxygen
PAECs	Pulmonary arterial endothelial cells
PASMCs	Pulmonary arterial smooth muscle cells
PCr	Phosphocreatine
PH	Pulmonary hypertension
PI3K	Phosphoinositide-3-OH kinase
PIP2	Phosphatidyl inositol bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PLB	Phospholamban
pPCI	Primary percutaneous coronary intervention
RAS	Renin-angiotensin system
RCA	Right coronary artery
RISK	Reperfusion injury salvage kinase
ROC curve	Receiver operating characteristic curve
ROS/RNS	Reactive oxygen and nitrogen species
RyRs	Ryanodine receptors
SBP	Systolic blood pressure
SD	Standard deviation
SERCA	SR Ca2+ ATPase
Sp1	Site-1 protease

SR	Sarcoplasmic reticulum
SREBP	Sterol regulatory element binding protein
STEMI	ST-segment elevation myocardial infarction
SWMAs	Segmental wall motion abnormalities
TCFA	Thin cap fibroatheroma
TIMI	Thrombolysis in myocardial infarction
TXA2	Thromboxane A2
UFH	Unfractionated heparin
VP	Vasopressin
VSMC	Vascular smooth muscle cells

1. INTRODUCTION AND BACKGROUND FOR THE PROPOSED RESEARCH

Ischemic heart disease is the most common cause of death worldwide. Myocardial infarction is responsible for more than 15% of deaths each year. Acute myocardial infarction may result in ischemic, mechanical, arrhythmic, embolic, or inflammatory complications. Thus, the functions of the coronary care unit, fibrinolytic therapy, catheter-based reperfusion, and lipid-modifying therapy have substantially decreased the incidence of complications and mortality rate and have improved the outcomes in survivors of an acute myocardial infarction.

Generally, clinical diagnosis of acute myocardial infarction using traditional methods usually relies on a clinical history of chest pain, changes in electrocardiographic (ECG) data (in at least two contiguous leads) and biomarker levels. Many researchers have attempted to discover new biomarkers in recent years, particularly biomarkers that predict complications and outcomes of acute myocardial infarction. In particular, apelin-12 levels in patients with ST-segment elevation myocardial infarction (STEMI) represent a new tool not only for risk stratification but also for the therapy.

Apelin is a new biomarker which has positive effects in cardiovascular system not sufficiently explored in human. With this topic we tend to confirm the value of apelin in myocardial reperfusion in acute myocardial infarction with ST segment elevation in humans. These similar studies were done with animal models. Finding a relation of apelin to troponin (neither explored before in animals nor in humans) during acute myocardial infarction and occurrence of MACE will be a novel approach to the new prediction of outcomes after MI.

1.1. Definition of acute myocardial infarction

The definition of acute myocardial infarction (AMI) is based on evidence of myocardial injury that is usually confirmed by an increase in the cardiac troponin levels, indicating necrosis of the myocardial tissue.

ST-segment elevation myocardial infarction (STEMI) is a clinical syndrome defined by characteristic symptoms of myocardial ischemia associated with a persistent electrocardiographic ST elevation and the subsequent release of biomarkers of myocardial necrosis (1, 2).

1.2. Epidemiology

Cardiovascular disease (CVD) remains the most common cause of death worldwide and accounts for 45% of all deaths attributed to non-communicable diseases. Ischemic heart disease (IHD) accounts for 20% of all deaths in Europe, although substantial variations have been observed between countries. A substantially high mortality rate has been observed in patients with acute myocardial infarction (AMI), particularly patients with ST-segment elevation myocardial infarction (STEMI). Overall, the in-hospital mortality rate of patients with STEMI in the national registries of the ESC countries ranges between 4 and 12%, while the reported 1-year mortality rate among patients with STEMI in angiography registries is approximately 10%. Regarding gender, the burden of the ischaemic heart disease increases for women up to ten years later than men. This disease often occurs in men prior to the age of 60 years, but the number of women with this disease increases after age 60 and the incidence becomes similar for both genders (1, 3, 4).

1.3. Classification of myocardial infarction

Myocardial infarction is classified into various types based on pathological, clinical, and prognostic differences, along with different treatment strategies (1, 2). ECG data (changes in the ST-segment and T wave) are necessary and should be immediately acquired and interpreted. Two groups of patients are differentiated, depending on whether the electrocardiogram presents an ST elevation:

a) patients with chest pain and a persistent (>20 min) ST-segment elevation (STEMI) that is generally referred to as acute total occlusion and requires immediate treatment by reperfusion therapy, and

b) patients with chest pain but no persistent ST-segment elevation (NSTEMI) (5).

ST-segment elevation generally reflects an acute total coronary occlusion, and immediate treatment strategies, such as reperfusion therapy, will be sufficient for the rapid restoration of vessel patency to maximise myocardial salvage.

From the clinical perspective, myocardial Infarction (MI) is also classified into 5 types based on the Universal Definition of Myocardial Infarction: type 1 is a spontaneous myocardial infarction due to plaque rupture, type 2 is myocardial infarction due to an imbalance in the myocardial oxygen supply and demand, type 3 is related to sudden unexpected cardiac death, type 4a is associated with a percutaneous coronary intervention, type 4b is associated with documented stent thrombosis, and type 5 is associated with coronary artery bypass grafting (2).
The majority of patients with STEMI are classified as having experienced a type 1 myocardial infarction with evidence of a coronary thrombus.

1.4. Pathophysiology

Thrombus formation on a coronary atherosclerotic plaque is the predominant underlying mechanism of total coronary occlusion in patients with STEMI. A vulnerable plaque is described as an atherosclerotic lesion with a high risk of rupture, and the lesion consists of a large lipid-rich or necrotic core with an overlying thin ruptured fibrous cap-thin cap fibroatheroma (TCFA). Plaque rupture occurs as result of the fragmentation and loss of elastin in the fibrous cap and infiltration by macrophages (foam cells) (6, 7).
Plaque rupture, which is defined as the formation of a gap in the fibrous cap of a vulnerable plaque, exposes the necrotic core to the blood and circulating coagulation factors, resulting in the formation of a thrombus on the ruptured plaque.
Coronary occlusion results in the immediate cessation of oxidative phosphorylation and production of adenosine triphosphate (ATP), where anaerobic glycolysis becomes the main source of newly generated ATP. Anaerobic glycolysis is the transformation of glucose to lactate and results in its rapid accumulation in the ischemic myocardium.

The metabolic effects of myocardial ischemia are characterized by the efflux of intracellular K^+ to the interstitium and accumulation of K^+ in the extracellular space, followed by a reduction in the action potential. Additionally, intracellular Na^+ and Ca^{2+} levels are increased in the ischemic myocardium, promoting an arrhythmogenic environment.

Within seconds, these processes lead to severe systolic dysfunction of the myocardium. These effects are completely reversible if the duration of the ischemic insult is < 20 minutes, while a 20-30 minute**s** interval of severe ischemia is sufficient to induce irreversible changes in some cardiomyocytes of the subendocardial area. Cardiomyocyte death progresses from the subendocardium to the subepicardium, which is defined as a "wavefront of necrosis", as the duration of the ischemic event increases (8). The first ultrastructural changes are evident within the first several minutes due to a reduction in the glycogen concentration in the cell and the disruption of the function of relaxed myofibrils and sarcolemmas. The process of necrosis advances from the subendocardium to subepicardium, and this process is affected by possible collateral flow, oxygen consumption, etc. Therefore, the proper timing of the reperfusion therapy is crucial to reduce myocardial injury (8).

In patients with acute myocardial infarction, plasma levels of creatine kinase (CK) are increased due to a lack of oxygen. CK exists as different isoenzymes, of which CK-MB is a more specific marker of myocardial necrosis. CK-MB levels typically peak at 3 to 24 hours and time to return to baseline after three to four days (9).

The necrosis of ischemic myocytes is the most common mechanism of troponin release. The troponin complex consists of three isoforms of troponin subunits: troponin I, T, and C. Cardiac troponin I and T are specific biomarkers of myocardial injury that have been used for the early diagnosis of myocardial infarction and an evaluation of the prognosis. Troponin I levels peak at 3 to 24 hours and time to return to the normal range after five to ten days, while troponin T levels peak at 3 hours to two days and return to baseline levels after five to fourteen days (10, 11).

1.5. Infarct size, reperfusion injury and remodeling

The myocardial infarct size depends on the level of coronary occlusion, collateral circulation to the ischemic area and duration of total vessel occlusion. A more proximal occlusion of the coronary artery is associated with a larger ischemic region and larger completed infarct. The coronary collateral circulation is an alternative conduit for blood flow, limiting myocardial necrosis in patients with an acute coronary occlusion. The timely administration of reperfusion therapy is the most effective treatment for reducing the myocardial infarct size (IS) and decreasing the duration of ischemic injury (12). Viable myocardium is observed even several hours after the coronary occlusion, and thus well-designed and appropriate reperfusion therapy is a key factor contributing to myocardial salvage. The size of the infarct, collateral blood flow, microvascular dysfunction, and other factors play important roles in determining the final infract size and the possible occurrence of complications (12).

Reperfusion of an occluded coronary artery is required to prevent infarction of the ischemic myocardium, but reperfusion can trigger myocardial injury, a phenomenon called myocardial reperfusion injury (MRI). The pathogenesis of ischemia reperfusion injury consists of the generation of reactive oxygen and nitrogen species (ROS/RNS), reduced availability of nitric oxide (NO), Ca^{2+} overload, and mitochondrial permeability transition pore (mPTP) opening (13). In the clinic, the types of reperfusion injury include reperfusion arrhythmias, myocardial stunning, microvascular obstruction, intramyocardial haemorrhage, and lethal myocardial reperfusion injury. Based on the Thrombolysis in Myocardial Infarction (TIMI) flow grade, MRI is characterized by a TIMI flow ≤ 2, also called coronary "no-reflow" (14). Therefore, even when timely coronary revascularization is performed through the administration of thrombolytic therapy or a primary percutaneous coronary intervention (PPCI), the process can induce reperfusion injury. During this process, cardiomyocyte death occurs through several pathophysiological mechanisms and contributes up to 50% of the final myocardial infarct size (14).

After an acute myocardial infarction, the left ventricle undergoes a series of changes in shape, size, and thickness, which is referred to as ventricular remodeling. Ventricular remodeling precedes the development of clinically evident major adverse cardiac events

(MACE) by months to years. Left ventricular remodeling results from multifactorial mechanisms. Cardiomyocyte necrosis triggers intracellular signalling pathways that modulate the processes of dilatation, hypertrophy and formation of fibrous tissue. Although infarcted areas are thin, non-infarcted areas undergo hypertrophy, leading to changes in the left ventricular shape followed by increased wall tension and progressive ventricular dilatation. In the presence of myocardial wall stress, increased blood levels of natriuretic peptide B (BNP) and atrial natriuretic peptide (ANP) are associated with increased diuresis and vasodilation (15, 16). Remodeling also serves as a prognostic marker to identify patients at risk of developing complications. Patients with STEMI may present evidence of restrictive filling due to diastolic remodeling induced by myocardial ischaemia, necrosis and microvascular dysfunction. Diastolic remodeling is associated with left ventricular systolic function. Furthermore, left ventricular remodeling is strongly correlated with mortality and systolic dysfunction (15). This finding has also been confirmed by studies identifying an association between a large infarct size, echocardiographic changes (diastolic dysfunction) and left ventricular dilatation (16).
The apelin-APJ axis may be upregulated in patients with good left ventricular remodeling or downregulated in patients with cardiac troponin degradation, and the release of cardiac natriuretic hormones inhibits the pathophysiological mechanisms responsible for ventricular remodeling (17-19). The roles of hypoxia in the release of apelin, ischaemia reperfusion injury, and the actions of apelin in cardiac contractility remain unclear.

1.6. Apelin, troponin, creatine kinase-MB, natriuretic peptide and the cardiovascular system

1.6.1. Apelin

The human apelin receptor (APJ, gene symbol APLNR) is an orphan G protein-coupled receptor (GPCR). The gene encoding the APJ receptor is located on chromosome 11q2 and encodes a G-protein coupled receptor (GPCR) that contains seven hydrophobic transmembrane domains, with sites for phosphorylation, palmitoylation and glycosylation. The process of internalization of the APJ receptor is regulated by amino acids in both the

N-terminal (e.g., Asp23 and Glu20) and C-terminal portions. Apelin is an endogenous high-affinity ligand for the APJ receptor, constituting a novel signalling pathway (20, 21). Human APJ receptors are expressed in the central nervous system, cardiovascular tissues, lung, stomach, intestine, spleen, kidney, placenta and ovary. In cardiovascular tissues, APJ is expressed on a number of cell types, including ventricular cardiomyocytes, vascular smooth muscle cells (VSMCs) and intramyocardial endothelial cells (22).

The gene encoding human apelin (APLN gene) is located on chromosome Xq25-26.1. In response to hypoxia, the APLN gene encodes a 77-amino acid preproprotein (preproapelin) that is cleaved by endopeptidases into a mature apelin peptide, apelin 36, or a family of shorter peptides (apelin-17, -13, and -12), the latter of which also exists as pyroglutamyl form (23). Pyr-apelin 13 and apelin 17 are the predominant isoforms in plasma, and pyr-apelin 13 has been detected as the primary isoform in human heart; pyr-apelin 13 also exhibits the highest affinity for the apelin receptor (24).

Angiotensin-converting enzyme 2 (ACE2) is a negative regulator of the renin-angiotensin system (RAS) that catalyses the conversion of Angiotensin II to Angiotensin 1-7. Apelin is a second catalytic substrate for ACE2 and functions as an inotropic and cardioprotective peptide. ACE2 establishes a mode of crosstalk between apelin and RAS in controlling cardiac contractility and heart failure.

Apelin 13 is a substrate of angiotensin-converting enzyme 2 (ACE2), a pleiotropic monocarboxypeptidase that is capable of catalysing the cleavage of a diverse range of peptide substrates. Thus, ACE2 represents a major negative regulator of apelin in the heart and vasculature (25). ACE2 mediates the degradation of pyr-apelin 13 through the cleavage of its C-terminal phenylalanine, producing pyr-apelin 12 and apelin 12 with cardioprotective effects. Although both the short and long forms of apelin possess similar functions, they differ in their tissue distribution, potency, and receptor binding affinities. Apelin-12 is the shortest form that retains activity, suggesting that the C-terminal 12 amino acids are essential for receptor binding and for the full agonistic activity of the apelinergic system (Fig. 1).

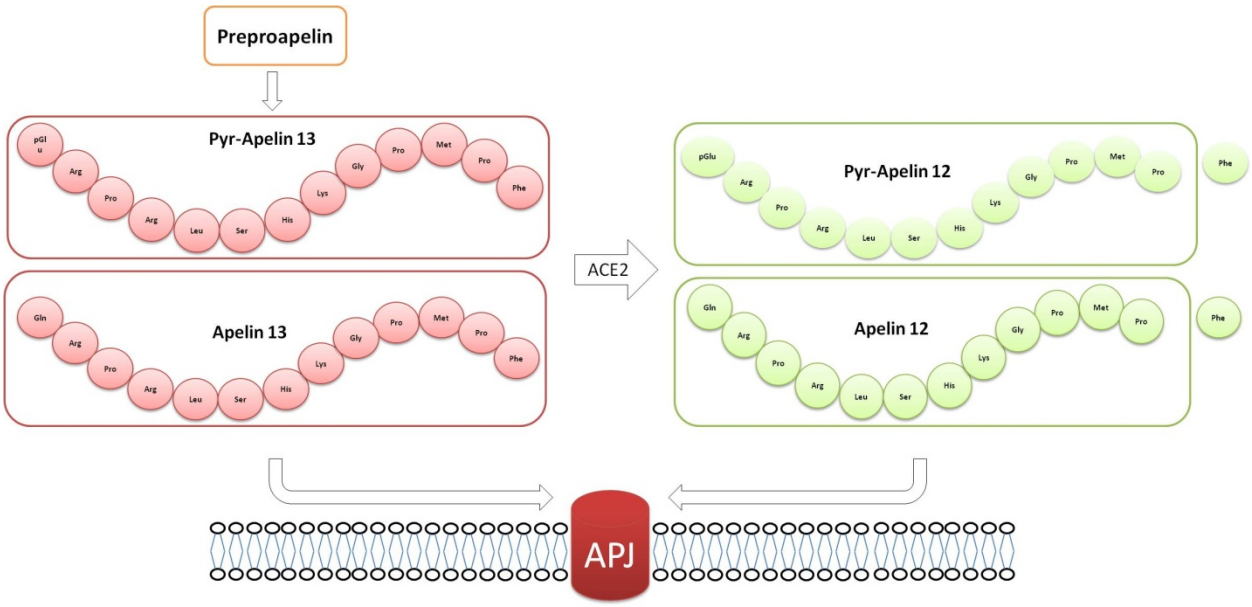


Figure 1. The apelin/apelin receptor system. The 77 amino acids pre-pro-apelin is cleaved to generate fragments of various size. ACE potentiates the degradation of pyrapelin 13 and apelin-13 through cleavage of their C-terminal phenylalanine, subsequently producing pyr-apelin 12 and apelin-12. ACE: angiotensin-converting enzyme.

1.6.2. Cardiovascular roles of apelin/APJ

The apelin peptide exerts both central and peripheral cardiovascular effects. During myocardial injury, the cells have been reported to migrate to the injured site and play a crucial role in regenerating the myocardium. This finding provides additional insights into the role of apelin as a mediator of myocardial cell differentiation that recruits progenitor cells to assist with myocardial regeneration, as shown in studies using mouse models.

The apelinergic system is involved in cardiac development and promotes the directed differentiation of embryonic stem cells (ESCs) into cells of the cardiac lineage. The effects of apelin on promoting the differentiation of ESCs into cardiomyocytes represent a promising therapy in myocardial regeneration. Despite advances in medical, percutaneous, and surgical interventions, myocardial infarction may be followed by myocardial injury and contractile dysfunction, resulting in heart failure due to the limited

mitotic capacity of terminally differentiated cardiomyocytes (26). On the other hand, tube formation in blood vessels results from lumen formation and cord hollowing, the latter of which involves apelin.

The proliferation and assembly of endothelial cells (ECs), which are regulated by apelin, result in the formation of a tube surrounded by smooth muscle cells and pericytes to maintain the structural stability (27).

The positive effects of apelin on the cardiovascular system include the regulation of the cardiac contractile function, vascular tone, and fluid balance (28-30). Apelin plays a role in controlling diuresis, pituitary hormone release, cardiomyocyte apoptosis and inflammation (31-34).

Apelin exerts directs effects on cardiomyocyte contractility and electrophysiology. The effect of apelin on cardiac contractility is mediated by a phospholipase C and protein kinase C-dependent pathway following the stimulation of sarcolemmal Na^+/H^+ exchanger (NHE), leading to intracellular alkalization and increased myofilament sensitivity to intracellular Ca^{2+}. These events also stimulate the reverse Na^+/Ca^{2+} transport, and increased intracellular Ca^{2+} concentrations will lead to a sustained increased in cardiac contractility. Protein kinase C also modulates sarcoplasmic reticulum (SR) Ca^{2+} levels, which play a significant role in regulating cardiac contractility. APJs are located in the intercalated disc regions that support the role of apelin in modulating intercellular communication by increasing conduction velocity and the frequency of spontaneous activation (35-37).

Apelin and APJ are expressed in the vessels and may be involved in the size-sensing mechanism. Apelin promotes the activation of endothelial nitric oxide synthase (eNOS) and increases the release of nitric oxide (NO), resulting in an increased level of cyclic guanosine monophosphate (cGMP) and the subsequent relaxation of the vascular musculature (38). Apelin peptides also may act directly on vascular smooth muscle APJ receptors and induce vasoconstriction, but NO production blocks this effect in the presence of a functioning endothelium.

The expression of the apelin gene is regulated by hypoxia-inducible factor-1 (HIF-1). HIF-1 is a heterodimeric protein composed of HIF-1α and HIF-1β subunits. In response to

hypoxia, HIF-1α escapes oxygen-dependent proline hydroxylation and degradation, accumulates, translocates to the nucleus, and heterodimerizes with HIF-1β. This complex interacts with the hypoxia response element (HRE) of the target gene and promotes the assembly of the transcriptional machinery. The level of apelin expression and secretion in cardiomyocytes corresponds to the level of activated HIF. Myocytes exposed to hypoxia (24 h, 2% O_2) display HIF activation and nuclear translocation, followed by increased levels of apelin secretion. Thus, during hypoxia and after HIF activation, cardiomyocytes process the apelin storage forms into secreted forms. The apelin storage forms are mainly located in the sarcoplasmic network around the nucleus, similar to the atrial natriuretic peptide (ANP). Different apelin peptides vary in their potency to mediate the different biological effects of apelin. Cardiomyocytes secrete peptides that appear to range in size between apelin-16 and apelin-12 (39).

1.6.3. Apelin in cardiovascular disease

Apelin expression is increased in response to acute hypoxia and cardiac infarction. In rats with acute myocardial infarction, hypoxia induces apelin gene expression through the hypoxia-inducible factor (HIF) pathway to increase the level of apelin, which might improve cardiac function. Therefore, in the myocardium subjected to a hypoxic insult, apelin secretion is targeted to acutely increase cardiac contractility through the mechanism proposed above by activating the Na^+/H+ exchanger (NHE), followed by the intracellular alkalinization and sensitization of cardiac myofilaments to the intracellular Ca^{2+} concentration, as well as an indirect increase in the intracellular Ca^{2+} concentration via the Na^+/Ca^{2+} exchanger (39). Additionally, apelin modulates sarcoplasmic reticulum Ca^{2+} concentrations through protein kinase C to protect the ischemic myocardium (39, 40).

The most effective early treatment for patients with STEMI is timely coronary revascularization using a primary percutaneous coronary intervention or thrombolytic therapy. However, reperfusion therapy can trigger reperfusion injury by causing further cardiomyocyte death through the generation of ROS/RNS, reduced availability of NO, Ca^{2+} overload, and mPTP opening (13). An increase in apelin/APJ signalling activates

components of the reperfusion injury salvage kinase (RISK) pathway, such as phosphatidylinositol-3-OH kinase (PI3K), Akt/protein kinase B and p44/42 mitogen-activated protein kinase (MAPK), protecting the myocardium from ischemia-reperfusion injury (41, 42). The phosphorylation of intermediates in the pathways listed above has been confirmed to be increased by the administration of apelin within five minutes of reperfusion following the period of lethal ischaemia (42). The apelin/APJ signalling pathway has been identified as a potentially important mediator of the pathophysiology of heart failure. As a mediator of cardiovascular control by increasing cardiac contractility and reducing the cardiac load, apelin may also be used to treat patients with ischemic heart failure (23, 43-47). Researchers have not clearly determined whether the increase in apelin-APJ signalling observed in rats at six weeks after myocardial infarction is due to ischaemia or the onset of heart failure. Elevated levels of apelin have been detected in early stages of heart failure and decrease with more advanced disease (23). Based on important changes in the left ventricular shape, elastance, and contractility, the apelin-APJ system plays an important role in cardiovascular control. These findings, together with changes in the circulating apelin levels in patients with moderate left ventricular dysfunction, contribute to positioning apelin as a “valuable peptide” for the heart (44).

Hypertension is characterized by increased arterial blood pressure that is a potential risk factor for many cardiovascular events, such as coronary artery disease, stroke, and peripheral vascular disease. In rats with hypertension, levels of the apelin and APJ mRNAs are reduced, which correlates with a lower plasma apelin level. The expression of the APJ receptor protein is decreased in cardiomyocytes isolated from the left ventricular tissue of hypertensive rats with heart failure. The hypotensive effect of apelin might be mediated by the Akt/eNOS pathway and overcomes a direct vasoconstrictor effect on vascular smooth muscle (48-51).

Apelin inhibits platelet activation and aggregation induced by thrombin and collagen through a mechanism dependent on integrin αIIbβ3, calcium mobilization via PI3K, and TXA2 synthesis. Therefore, apelin plays a critical role in regulating platelet activation and might serve as a novel promising therapy target for thrombotic disease in the future (52).

Atherosclerosis is a chronic lipid metabolism disorder characterized by the accumulation of plaques inside the arterial wall and the presence of foam cells, leading to vascular disease. Apelin decreases lipid accumulation in foam cells and induces autophagy by activating the PI3K/Beclin-1 pathway (53). The level of apelin is decreased in human atherosclerotic coronary arteries, and coronary collateral formation in patients with stable angina correlates with higher plasma apelin levels (54).

Apelin is inversely correlated with the LDL cholesterol level, indicating its role in atherosclerosis. Regulation of the apelin/APJ pathway by site-1 protease (Sp1) transcription factor cleaving endoplasmic reticulum loop of sterol regulatory element binding protein (SREBP) is reported to result from decreased intracellular cholesterol levels (55-58).

Apelin also plays an important role in the mechanism of atherosclerosis. A reduction in LDL-cholesterol levels results in increased apelin levels (58).

Pulmonary hypertension (PH) is characterized by reduced apelin expression in both pulmonary arterial endothelial cells (PAECs) and microvascular endothelial cells. An increase in the activity of apelin will be a therapeutically viable option because the deletion of apelin aggravates vascular remodeling associated with exacerbated pulmonary hypertension by decreasing the activation of endothelial nitric oxide synthase (eNOS) (59). Apelin is also expressed in the pulmonary vasculature; therefore, an exogenous apelin treatment contributes to inhibiting the proliferation and migration of pulmonary arterial smooth muscle cells (PASMCs) by regulating the level of autophagy, which is essential for the arterial remodeling process observed during pulmonary hypertension (60).

Apelin also plays a role in fluid balance. The APJ mRNA has been detected in the brain and is critical for the control of fluid homeostasis (61). Apelin reduces the plasma vasopressin (VP) level and increases diuresis in the in the kidney by increasing the renal microcirculation (62).

1.6.4. Troponin

The sarcomere is the fundamental contractile unit of the heart and is comprised of thick filaments (myosin) and thin filaments. The sarcomere length ranges from approximately 1.6 to 2.2 u in human hearts. The myosin protein has a molecular weight of approximately 470 kDa. Myosin consists of two heavy chains, each with two associated light chains. Each heavy chain consists of a globular head region and a long α-helical tail. The α-helical tails of two heavy chains twist around each other in a coiled-coil structure to form a dimer, and two light chains associate with the neck of each head region to form the complete myosin molecule. The myosin heads are the site of the myosin ATPase, an enzyme that hydrolyses ATP to form the actin and myosin cross bridge. These heads interact with a binding site on actin.

The thin filaments are composed of three different proteins: actin, tropomyosin, and troponin. Actin has two forms. G-actin is a monomeric globular form and F-actin is filamentous polymer of G-actin. The F-actin filaments are arranged in a helical structure associated with tropomyosin to which the troponin complex is attached. The troponin complex is composed of three subunits: troponin-T (37 kDa), which attaches to tropomyosin; troponin-C (20 kDa), which serves as a binding site for Ca^{2+} during excitation-contraction coupling; and troponin I (24 kDa), which inhibits the binding of myosin to actin.

Each troponin isoform is encoded by a separate gene, and thus the specific functions of cTn are fundamentally attributed to its protein structure. The sequences of cTnI and cTnT differ from their skeletal counterparts. The cTnI protein contains 210 AA residues, 31 of which have been utilized for assay development since they form an N-terminal extension that is not present in skeletal troponin (63).

Myofibrillar contraction is activated by depolarization and then modulated by the interaction of Ca^{2+} with specific regulatory sites on the contractile apparatus of striate muscle. This regulatory site is the troponin complex, a tadpole-shape heterotrimer immobilized on the thin filament, which acts in an allosteric manner to regulate the Ca^{2+}-dependent interaction of actin and myosin filaments. When Ca^{++} binds to troponin-C, a conformational change occurs in the troponin complex such that troponin I moves away

from the myosin binding site on actin, thereby making it assessable to the myosin head. When Ca^{++} is removed from troponin-C, the troponin complex resumes its inactivated position, thereby inhibiting myosin-actin binding.

This interaction of the myosin heads with actin filaments when sufficient calcium arrives from the sarcoplasmic reticulum (SR) is called crossbridge cycling (10, 64).

Cardiac troponin (cTn) is released into the circulation following an acute myocardial infarction (AMI) and other types of acute myocardial injury. Thus, myocardial injury is detected when blood levels of sensitive and specific biomarkers, such as cTn or the MB fraction of creatine kinase (CKMB), are increased. As components of the contractile apparatus of myocardial cells, cardiac troponin I and T are expressed almost exclusively in the heart.

Myofibrils are repeating units of Ca^{++}-activated and ATP-consuming sarcomeres. Therefore, if Ca^{++} leaks into the cardiomyocyte cytoplasm, the sarcomeres contract and quickly consume all the ATP, resulting in necrosis and the release of cTn along with other cellular contents. Troponin is released from the myofibril due to proteolytic degradation in the myocardium mediated by three enzymes: 1) calpain 1, a Ca^{2+}-dependent cysteine protease; 2) caspase, a cysteine protease involved in mediating apoptosis; and 3) matrix metalloproteinase-2, a zinc-dependent endopeptidase. These enzymes are also present in the blood and form a complex with cTnI. Normal cardiac function relies on the expression of all three troponin subunits. The N- and C-terminal regions of cTnI are the most susceptible to proteolysis. The central region of cTnI is the Ca^{2+}-dependent TnC binding domain and is the most stable; therefore, this region is targeted by most cTnI assays (10).

Although increased levels of these biomarkers in the blood reflect injury leading to necrosis of myocardial cells, they do not indicate the underlying mechanism (65). Various possibilities have been suggested to account for the release of structural proteins from the myocardium, including the normal turnover of myocardial cells, apoptosis, cellular release of troponin degradation products, increased cellular wall permeability, formation and release of membranous blebs, and myocyte necrosis (66).

The high myocardial specificity and clinical sensitivity of cTnI/T as a marker of myocardial injury is well accepted. However, claims of specificity for any particular disease are untenable. Neither cTnI nor TnT are exclusively released due to myocardial infarction (MI), and they are released as a result of ischemic, non-ischemic and extra-cardiac conditions.

Numerous acute and chronic conditions result in elevated cTn levels in the absence of acute coronary syndrome (ACS). Therefore, the increased levels of cTn observed in the absence of ACS are likely to be attributed to multiple factors, including myocardial ischemia, increased wall tension and ventricular strain, direct myocyte trauma, excess catecholamines, and possibly impaired renal clearance.

Regardless of the pathobiology, myocardial necrosis due to myocardial ischaemia is designated as MI. Following acute myocardial infarction, the levels of troponins predict MACE (67-69). The use of a sensitive troponin I assay improves early diagnosis (70), and these patients are more likely to undergo angiography (71, 72).

1.6.5. Creatin kinase-MB

Creatine kinase (CK) catalyses the exchange of a phosphate between ADP and creatine. In excitable tissues, such as the brain, skeletal muscle and heart, CK exerts a crucial effect on energy transduction from the mitochondria to the sites of ATP utilization.

In cardiomyocytes, creatine kinase (CK) is expressed as three isoenzymes: creatine kinase M-type, creatine kinase B-type and mitochondrial sarcomeric creatine kinase (mtCKs) (73). Adenosine triphosphate (ATP), a primary source of energy in cardiac cells, is generally produced from a variety substrates (fatty acids and glucose) in the mitochondria through oxidative phosphorylation. Creatine kinase M-type (CKM) interacts with ATPases to regenerate ATP according to the actual energy requirements (74). The high energy phosphoryl bonds of ATP are available at the sites of utilisation and require a phosphagen system that consists of the reversible interaction of creatine and ATP under the control of cytosolic creatine kinase M-type (CKM): Creatine + ATP ↔ Phosphocreatine + ADP + H^+ (75, 76). The phosphocreatine (PCr)/CK system generates

a large amount of ATP, and it is particularly important in situations with a high metabolic demand when the rate of ATP use exceeds its capacity for generation by other metabolic pathways. In patients with a myocardial infarction, ATP and phosphocreatine (PCr) levels are rapidly depleted due to a lack of oxygen, resulting in tissue damage and increased CK-MB levels. The MB isoenzymes of CK are also present in small amounts in the small intestine, tongue, diaphragm, uterus, and prostate.

1.6.6. Natriuretic peptide

Brain-type natriuretic peptide (BNP) is one of the three members of the natriuretic peptide family that is predominantly expressed in the adult heart. BNP is synthesized as a 134-amino acid preproBNP precursor, which is cleaved to the 108-amino acid proBNP through the removal of a 26-amino acid signal peptide. The processing of proBNP forms an N-terminal proBNP (NT-proBNP) fragment composed of amino acid residues 1–76 and a C-terminal active BNP composed of amino acid residues 77–108 (78, 79).

Natriuretic peptides (NPs) are secreted in response to left ventricular filling pressure and wall stress; the former are encoded by genes located on chromosome 1 (80-82). In response to myocardial ischemia, an elevated level of BNP is induced by hypoxia inducible factor 1 (HIF-1) (83, 84).

Soluble guanylyl cyclase (GC) receptors mediate the effects of NPs on the target tissue. An increase in cyclic guanylate monophosphate (cGMP) levels is observed after the activation of both GC-A and B receptors (85). The effects of BNP are mediated by natriuretic peptide receptor A (NPR-A), a guanylyl cyclase-coupled receptor, causing a reduction in the cytosolic calcium concentration (86, 87). Activation of guanylyl cyclase-A by NPs protects against acute heart failure and attenuates chronic cardiac ring by inhibiting the renin-angiotensin system (RAS) (88). BNP is a predictor of MACE after myocardial infarction (89), and patients with larger infarcts may present an additional peak at 5 days (90). Therefore, an increase in the BNP concentration identifies patients at risk of adverse left ventricular remodeling and MACE (91-93).

1.7. Relationships between apelin, troponin, creatine kinase-MB, and natriuretic peptide

1.7.1. Relationship between apelin and troponin

After apelin binds to its receptor, phospholipase C (PLC) is activated and generates inositol trisphosphate (IP3) and diacylglycerol (DAG) from phosphatidyl inositol bisphosphate (PIP2). Diacylglycerol activates protein kinase C (PKC), increasing the activity of the sarcolemmal Na^+/H^+ exchanger (NHE). This process increases the pH, which indirectly increases the intracellular Ca^{2+} concentration through the reverse Na^+/Ca^{2+} exchanger (NCX).

Sarcoendoplasmic reticulum Ca^{2+} handling plays a significant role in regulating cardiac contractility. Ca^{2+} uptake into the SR is mediated by the sarcoendoplasmic reticulum Ca^{2+}-ATPase isoform 2 (SERCA2), which is regulated by phospholamban (PLB). PLB inhibits SERCA2, leading to a reduced rate of Ca^{2+} uptake into the SR and a slower rate of relaxation. Phosphorylation of PLB causes the dissociation of PLB from SERCA2, allowing faster rates of SR Ca^{2+} uptake and relaxation and enhanced contractility through increased SR Ca^{2+} loading. SR Ca^{2+} release is regulated by the SR Ca^{2+} release channel, which binds ryanodine and is therefore referred to as the ryanodine receptor.

Apelin also increases the intracellular Ca^{2+} concentration by activating the calcium release channels associated with ryanodine receptors (RyRs) and protein kinase C, which decreases the phosphorylation of phospholamban (PLB), reducing the function of the SR Ca^{2+} ATPase (SERCA) (94, 95).

Intracellular alkalinzation resulting from sarcolemmal NHE activation increases the Ca^{2+} sensitivity of myofilaments and potentiates the positive inotropic effect of apelin.

Through protein kinase C, apelin activates its sites on troponin I, thereby regulating Ca^{2+} sensitivity and ATPase activity in the myocardium. The phosphorylation of one or more of these sites on cTnI plays a central role in suppressing myofilament ATPase activity and increasing myofilament Ca^{2+} sensitivity. The increase in Ca^{2+} sensitivity may also be associated with a slowing of Ca^{2+} dissociation from troponin C and the prolongation of attached crossbridges during the force-generating or work-performing cycle (Fig. 2) (96, 97).

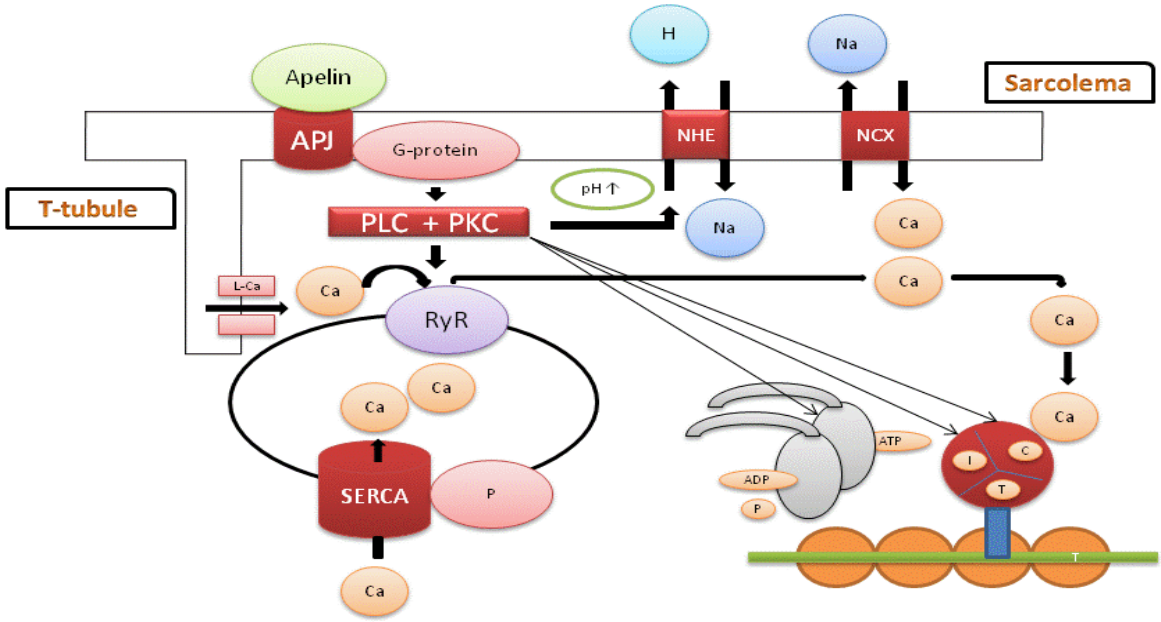


Figure 2. Influence of apelin on troponin.

Protein kinase C activation during ischaemia/infarction is thought to be a cardioprotective mechanism because this protein is a regulatory kinase that phosphorylates a number of target proteins involved in excitation-contraction coupling and myocardial contractility, particularly the site-specific phosphorylation of cTnI to regulate myofilament Ca^{2+} sensitivity and ATPase activity (98).

Several structures contribute to Ca^{2+} homeostasis in cardiomyocytes, such as Ca^{2+} channels, Ca^{2+} pumps (ATPases), and ionic exchangers. Notably, cTnI is the inhibitor within the trimeric troponin complex, which, together with cTnC and cTnT, controls the position of tropomyosin on the thin actin filaments in response to Ca^{2+}.

Dysregulation of cellular Ca^{2+} homeostasis, often in the form of Ca^{2+} overload, activates protease I (calpain I), leading to the proteolytic degradation of cTnI (40).

Calpains are a family of Ca^{2+}-dependent cysteine proteases that are involved in various physiological proteolytic events. Calpains produce a limited proteolysis of specific substrates, and they are implicated as modulators of a wide variety of biological phenomena, including proliferation, cell migration, differentiation, protein homeostasis and cell signalling. In terms of the calpain structure, 15 isoforms have been reported. The main isoforms are the ubiquitous μ-calpain (calpain-1) and m-calpain (calpain-2), which require micromolar and millimolar levels of Ca^{2+}, respectively. Calpain exists in the cytosol as an inactive enzyme and translocates to membranes in response to an increase in the cellular Ca^{2+} level (99, 100).

Inappropriate calpain activation occurs under pathological conditions in which Ca^{2+} homeostasis is disrupted, as has been observed during ischemia/reperfusion, and contributes to myocardial injury through the proteolysis of a wide variety of proteins. A number of structural (titin, alpha-fodrin, desmin and alpha-actinin) and regulatory (cTnI and cTnT) proteins within the contractile apparatus are potential targets of calpain-1 (101). Calpains have large subunit (80 kDA) and small subunit (30 kDA) that bind Ca^{2+}, resulting in the activation of calpain. Calpain activity is also regulated by autoproteolysis and calpastatin, suggesting that calpains are components of a regulatory proteolytic system (102).

The selective cleavage of 17 amino acid residues at the C-terminus of TnI is the primary effect of calpain on cTnI proteolysis in ischemia/reperfusion injury, resulting in cardiac dysfunction.

Thus, during ischaemia/infarction, levels of phosphorylated TnI, Ca^{2+} sensitivity and ATPase activity are decreased in the myocardium, and subsequently the elevated Ca^{2+} levels activate protease I (calpain I), which may lead to the proteolytic degradation of troponins (Fig. 3)(103-106).

Concurrently, the levels of apelin and APJ are increased to limit myocardial injury.

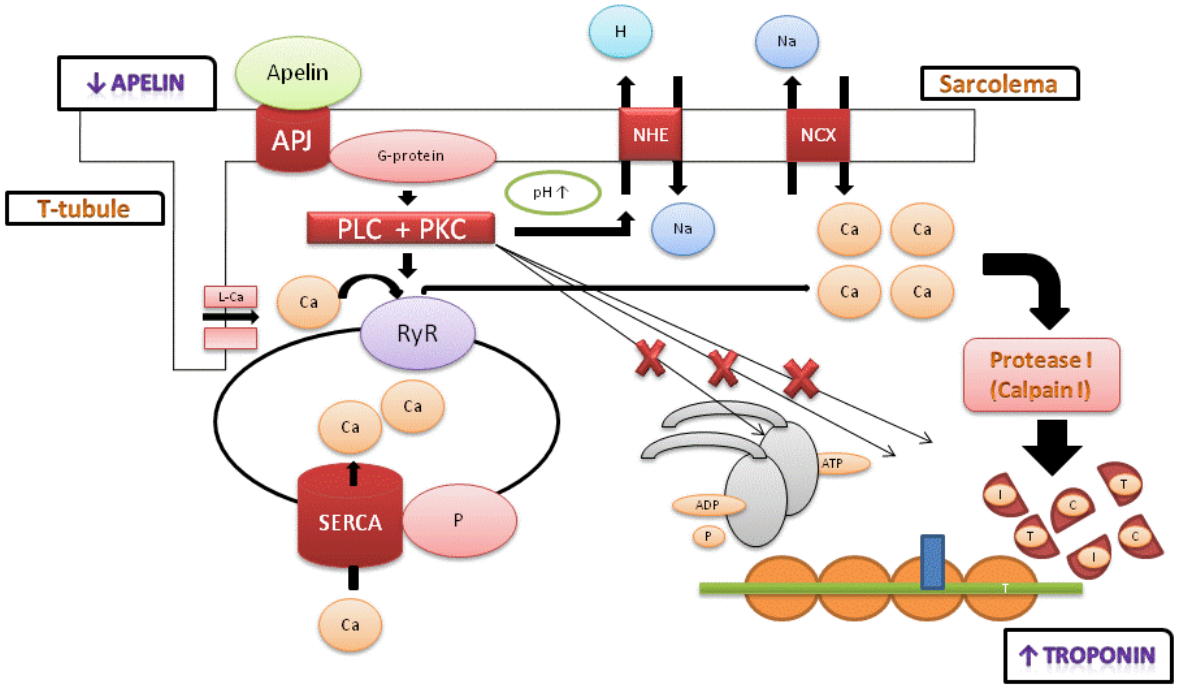


Figure 3. Apelin and troponin during ischemia/myocardial infarction.

1.7.2. Relationship between apelin and creatine kinase-MB

Cardiomyocytes are highly oxidative cells in which the mitochondria are located at sites of high ATP demand, and together with myofilaments and the sarcoplasmic reticulum (SR), these cells create intracellular energetic units around sarcomeres (107). In cardiomyocytes, ischemia and hypoxia represent acute crises of energy generation, followed by increased activity of creatine kinase (CK) that catalyses the exchange of a phosphate between ADP and creatine. Creatine kinase (CK) is structurally associated with sarcoplasmic reticulum (SR) membranes and is capable of linking energy production and utilization through phosphocreatine (PCr) to rephosphorylate all of the ADP produced by ATP-ases (108). One of the enzymes that is activated by the binding of apelin to its

receptor is protein kinase C, through which apelin activates its sites on troponin I, thereby regulating Ca^{2+} sensitivity and ATPase activity in the myocardium (Figure 4) (96, 97).

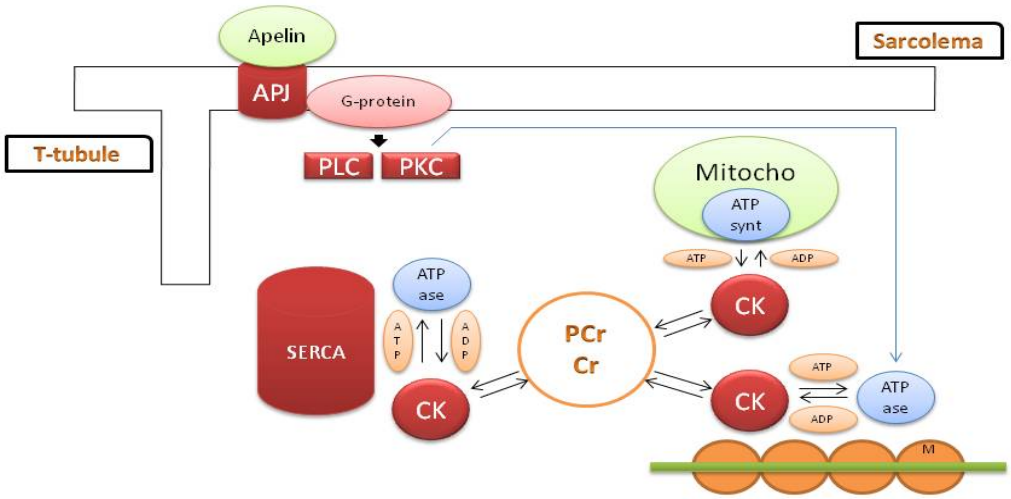


Figure 4. Association between apelin and creatine kinase.

1.7.3. Relationship between apelin and NT-proBNP

Persistent ischaemia and reperfusion may lead to contractile dysfunction. During hypoxia (24 h, 2% O_2) apelin gene expression and secretion are increased through the activation of hypoxia-inducible factor (HIF) (39). Hypoxia requires a functional mitochondrial electron transport chain to inhibit prolyl hydroxylases and promote HIF stabilization (109). Following anoxia, HIF is stabilized due to the lack of a functional mitochondrial respiratory chain, and thus the apelin gene is not expressed and apelin levels are not increased (110). In the absence of apelin, Ca^{2+} desensitization of the actin-myosin system, excess Ca^{2+} concentrations, calpain I activation, troponin degradation, ventricular dysfunction, and BNP secretion are observed (90).

2. HYPOTHESIS

During the acute phase of STEMI, high level of apelin-12 has a protective effect on reperfusion injury and lowers troponin I serum levels, whereas during the non-acute phase of myocardial infarction high level of apelin-12 lowers the rate of major adverse cardiac events (MACE).

3. AIMS AND PURPOSE OF THE RESEARCH

3.1. AIMS OF THE RESEARCH

The general aim of this study was to evaluate apelin-12 levels in acute and non-acute phase of ST-segment elevation myocardial infarction (STEMI) and its correlation to the rate of major adverse cardiac events (MACE).

Specific aims were:

a. To analyze the protective effect of apelin-12 from reperfusion injury during acute pahes of STEMI.
b. To assess correlation between apelin-12 levels during acute phase of STEMI and number of coronary artery stenoses.
c. To analyze the impact of apelin-12 levels on troponin I levels during acute STEMI phase.
d. To analyze the association between apelin-12 levels and creatine kinase-MB levels.
e. To analyze the impact of apelin-12 level on release of N-terminal pro-brain natriuretic peptide.

3.2. PURPOSE OF THE RESEARCH

Purpose of the research is confirmation of positive effects of the apelin-APJ axis in patients with acute ST segment elevation myocardial infarction. In the future, apelin-12 may be used in patients with reperfusion injury and in patients with increased numbers of stenotic vessels to enable coronary artery collateral development, especially in patients who are not candidates for percutaneous or surgical revascularization.

4. MATERIALS AND METHODS

4.1. Study population

In this dual-centre, prospective observational study, one hundred consecutive patients with ST-segment elevation acute myocardial infarction who presented or were referred during the period of one year (March 2013 to March 2014) to the Coronary Care Unit of Dubrava University Hospital-Zagreb and the University Clinical Center of Kosova-Pristina were included.

Patients meeting the following criteria were included in our study:

1) continuous chest pain lasting > 30 min,
2) an electrocardiogram (ECG) with ST-segment elevation (measured at the J-point) ≥ 2.5 mm in men < 40 years, ≥ 2 mm in men ≥ 40 years, or ≥ 1.5 mm in women in leads V2-V3 and/or ≥ 1mm in other leads [in the absence of left ventricular (LV) hypertrophy or left bundle branch block (LBBB)],
3) elevation of myocardial specific biomarkers' serum levels: troponin I and the MB fraction of creatine kinase (CK-MB),
4) undergoing reperfusion therapy (1, 111).

Patients with comorbidities during hospitalization that influence level of troponins were excluded:

1. Injury related to supply/demand imbalance of myocardial ischaemia: aortic dissection and severe anemia.
2. Injury not releated to myocardial ischaemia: myocarditis and chemotherapy (cardiotoxic agents, e.g. anthracyclines, herceptin).
3. Multifactorial or indeterminate myocardial injury: severe pulmonary embolism, sepsis, renal failure and severe acute neurological disease (stroke, subarachnoid haemorrhage) (112, 2).

All patients had undergone a periodical evaluation of the development of MACE which included: death of any reason, myocardial reinfarction, acute ischemic/heamorrhagic stroke, hospitalization due to angina pectoris worsening, chronic heart failure) in a follow-up period up to one year.

4.2. Reperfusion therapy

Primary percutaneous coronary intervention (pPCI) was performed in STEMI patients with first-medical-contact-to-balloon time (FMCBT) ≤120 minutes (113). In settings when primary PCI could not be performed in a timely fashion, pharmacoinvasive strategy-fibrinolysis combined with rescue PCI or routine early PCI strategy was performed.

Primary PCI as emergent procedure with stent was performed in the infarct-related artery (IRA) without previous fibrinolytic treatment. Before this procedure, all patients received the following protocol: 150-300 mg of acetylsalicylic acid (ASA), 600 mg of clopidogrel and 70-100 U/kg of unfractionated heparin (UFH). Additional treatments with glycoprotein IIb/IIIa inhibitors or intracoronary treatments such as vasodilatators were left to the discretion of the treating cardiologist.

Pharmacoinvasive strategy was performed by administering fibrinolysis in STEMI patients with first-medical-contact-to-balloon time (FMCBT) > 120 minutes (114). Rescue PCI was indicated in the case of failed fibrinolysis (ST-segment resolution < 50% within 60-90min of fibrinolytic administration, presence of haemodynamic or electrical instability, worsening ischaemia or persistent chest pain) (115). Routine early PCI strategy was indicated after successful fibrinolysis (preferably 2-24 h after fibrinolysis).

Contra-indications to fibrinolytic therapy (1) were considered as:

1. Absolute:
    a. Previous intracranial haemorrhage or stroke unknown origin at anytime
    b. Ischaemic stroke in the preceding 6 months
    c. Central nervous system damage or neoplasms or arteriovenous malformation
    d. Recent major trauma/surgery/head injury (within the preceding month)
    e. Gastrointestinal bleeding within the past month

f. Known bleeding disorders (excluding menses)
g. Aortic dissection
h. Non-compressible punctures in the past 24 hours (e.g. liver biopsy, lumbar puncture)

2. Relative:
    a. Transient ischaemic attack in the preceding 6 months
    b. Oral anticoagulant therapy
    c. Pregnancy or within 1 week postpartum
    d. Refractory hypertension (SBP >180mmHg and/or DBP >110mmHg)
    e. Advance liver disease
    f. Infective endocarditis
    g. Active peptice ulcer
    h. Prolonged or traumatic resuscitation

During pharmacoinvasive strategy adjunctive antiplatelet and anticoagulant therapies were used in doses: 150-300 mg of acetylsalicylic acid (ASA), 300 mg of clopidogrel and 60 U/kg of unfractionated heparin (UFH). Additional treatments were left to the discretion of the treating cardiologist.

4.3. Coronary angiography

Based on coronary angiography, the number of diseased vessels, culprit lesions and stenoses were determined. The culprit lesion was defined as the lesion with the highest degree of stenosis or with angiographic signs of endoluminal thrombi and/or plaque rupture. Stenoses ≥ 50% of the lumen of the left main artery (LMA) or ≥ 70% of the lumen of major epicardial vessels were considered significant. Coronary thrombus was defined as an intraluminal filling defect or an area of contrast staining within the coronary lesion. Plaque rupture was defined by the presence of a small crater or visible intimal flap. Based on the Thrombolysis in Myocardial Infarction (TIMI) flow grade, MRI is characterized by a TIMI flow ≤ 2, also called coronary "no-reflow" (14). With TIMI flow < 2, thrombotic

coronary occlusions were defined on the basis of morphology and lack of collateral vessels (116).

4.4. Echocardiography

All patients underwent an echocardiographic examination while were draped and prepared for reperfusion therapy. The wall motion alterations were defined with the 17-segment model (6 basal, 6 midventricular, 5 apical) (117). All segments of the left ventricle were scored with the usual method: 1-normokinesis, 2-hypokinesis, 3-akinesis, 4-dyskinesis and 5-aneurysm, thorugh the short axis projections and apical 2-, 3- and 4-chamber vizualisation. Left ventricular segments were assigned to five different territories supplied by coronary arteries (118). The territory supplied by the left descending coronary artery (LAD) includes segments 1, 6, 7, 12, 13, 16, and 17. The territory supplied by the right coronary artery (RCA) includes segments 2, 3, and 9. The territory supplied by the LAD or left circumflex coronary artery (LCx) includes segments 5, 11 and 15. The territory supplied by the RCA or LAD includes segments 8 and 14. The territory supplied by the RCA or LCx includes segments 4 and 10 (118). LV ejection fraction was assessment by modified Simpson's method of discs, acquiring LV volumes from apical 4- and 2-chamber views (117, 119).

4.5. Myocardial reperfusion injury

Timely myocardial reperfusion after an acute myocardial infarction is the most effective strategy for salvaging the myocardium and improving clinical outcomes. However, this process of restoring blood flow to the ischemic myocardium can itself induce further cardiomyocyte death, a phenomenon known as myocardial reperfusion injury (120). Myocardial reperfusion injury was defined by a 12-lead electrocardiography (ECG) 1 h after successful recanalization of the infarct related artery as > 30% persistent ST-segment elevation and by coronary angiography as thrombolysis in myocardial infarction (TIMI) grade ≤ 2 (14).

4.6. Laboratory data

The levels of apelin-12, creatine kinase (CK), the MB fraction of creatine kinase (CKMB), troponin I, NT-proBNP, CRP, triglycerides, LDL and HDL cholesterol, and other routine laboratory parameters were measured.

In particular, we evaluated the levels of apelin-12 and troponin I on the first and seventh days after reperfusion therapy in all patients.

Blood samples for the measurement of routine laboratory parameters were collected at admission. On the first and seventh day after reperfusion therapy, blood samples were collected into lavender Vacutainer tubes that contain EDTA and can collect up to 7 ml of blood/tube. The blood was transferred from the lavender vacutainer tubes to centrifuge tubes containing aprotinin (0.6TIU/ml of blood) and then centrifuged at 1600 x g for 15 minutes at 4°C. The serum was aliquoted and stored at -80 degrees Celsius to prevent degradation. Serum apelin-12 and troponin I concentrations were measured with enzyme-linked immunosorbent assay (ELISA) method; creatine kinase (CK) and the MB fraction of creatine kinase (CKMB) were measured by immunological inhibition method; and measurement of NT-proBNP is done with electro-chemiluminiscence method.

Apelin- 12 (Catalog Nr. EK-057-23, Phoenix Pharmaceuticals, Belmont, CA, USA), troponin I (Catalog Nr. EK-311-05, Phoenix Pharmaceuticals, Belmont, CA, USA) (Abbot Diagnostics, Wiesbaden, Germany), CK (Catalog Nr. 04524977190 Roche Diagnostics, GmbH, Mannheim, Germany), CK-MB (Catalog Nr. 05168562190, Roche Diagnostics, GmbH, Mannheim, Germany) and NT-proBNP (Catalog Nr. 04842464190, Roche Diagnostics, GmbH, Mannheim, Germany) assay kits were used for 100 patients according to the manufacturer's instructions.

4.7. Statistical analysis

All statistical analyses were performed using IBM-SPSS for Windows software, version 21 (SPSS Inc., Chicago, IL).

Continuous variables are presented as the mean ± standard deviation or as the median (range), whereas categorical variables are presented as percentages and absolute numbers.

Continuous variables were compared with the Wilcoxon test (paired samples) with a prespecified statistical significance of $p<0.001$. The Kruskal-Wallis test was used to evaluate the variability of apelin values on the first day based on segmental wall motion abnormalities (SWMAs) and apelin values on the seventh day based on different numbers of stenotic coronary arteries.

Based on the Mann-Whitney test was analyzed the relationship between apelin-12 and the final TIMI grade flow in the acute phase of myocardial infarction. In the adjusted logistic regression analysis, apelin-12 was correlated with TIMI flow considering other risk factors: age, gender, hypertension, dyslipidemia and diabetes mellitus.

Spearman's correlation was used to analyse the degree of association between apelin-12 and troponin I. Based on the regression analysis of the relationship between apelin-12 and troponin I was predicted one variable from the other.

Association between apelin and MACE after a follow-up period of 12 months. ROC curve analyses were used to determine a cut-off value of apelin-12 for an association with MACE. Using Kaplan-Meier estimates, we evaluated the association between apelin and MACE after a follow-up period of 12 months. The log-rank is used to compare the survival distributions of two samples.

Association of apelin-12 with creatine kinase MB depending reperfusion injury in patients with ST-segment elevation myocardial infarction. Comparisson of laboratory variables between patients with different TIMI flow (TIMI flow ≤ 2 and 3) was performed using Mann-Whitney test.

Association between apelin-12 and N-terminal pro-brain natriuretic peptide depending reperfusion injury in patients with ST-segment elevation myocardial infarction. Comparisson of laboratory variables between patients with different TIMI flow (TIMI flow ≤ 2 and 3) was performed using Mann-Whitney test. The degree of association between variables was analyzed with Pearson correlation.

4.8. Ethical consideration

The study was approved by the institutional Ethics Committee of Dubrava University Hospital-Zagreb and the University Clinical Center of Kosova-Pristina.

Written informed consent were obtained from all patients before inclusion in the study.

5. RESULTS

5.1. Baseline characteristics

Baseline characteristics of the study population are displayed in Table 1. The mean age was 60.52±11.50 years old with most presented male gender (60.00%) also presented in Graph 1. In our study population we analyzed the presence of risk factors (Graph 2). The study population had highest incidence of the following risk factors: high blood pressure, followed by dyslipidemia and smoking, and the diabetes mellitus and positive family history where found less frequent. In terms of coronary angiographic findings, as shown in Graph 3, the culprit artery was as follows: the left anterior descending artery (LAD) in 48%, right coronary artery (RCA) in 40% and circumflex artery (LCx) in 12%), whereas in terms of vessel disease, 40% of patients presented with one-vessel disease, 31% with two-vessel disease and 29% with triple-vessel disease (Graph 4).

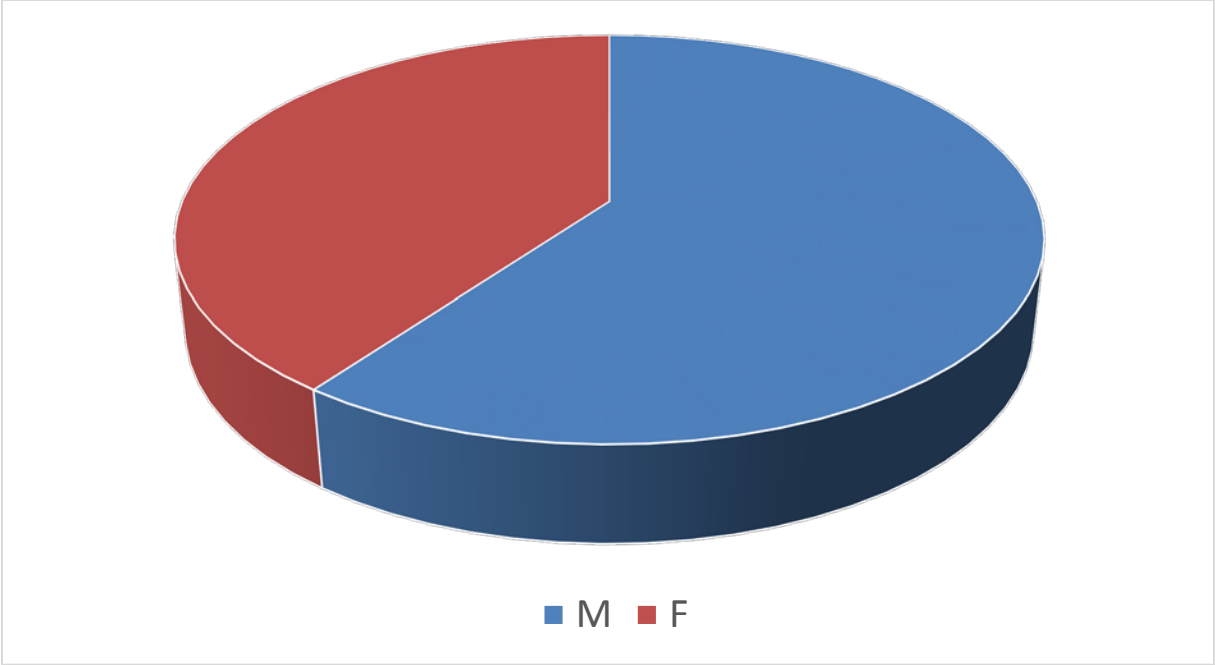
Table 1. Baseline characteristics of patients

Age (year), mean (±SD)	60.52±11.50
Gender (male), n (%)	60 (60.00%)
Medical history	
Hypertension, n (%)	59 (59.00%)
Diabetes mellitus, n (%)	19 (19.00%)
Dyslipidemia, n (%)	32 (32.00%)
Smoking, n (%)	32 (32.00%)
Family history of cardiovascular disease, n (%)	20 (20.00%)
Killip class > 1, n (%)	13 (13.00%)
Ejection fraction, mean (±SD)	50.34±10.20
Laboratory values	
Haemolobin (g/dL), mean (±SD)	13.54±1.39
Creatinine (umol/L), median (range)	92.90 (67.21-124.34)

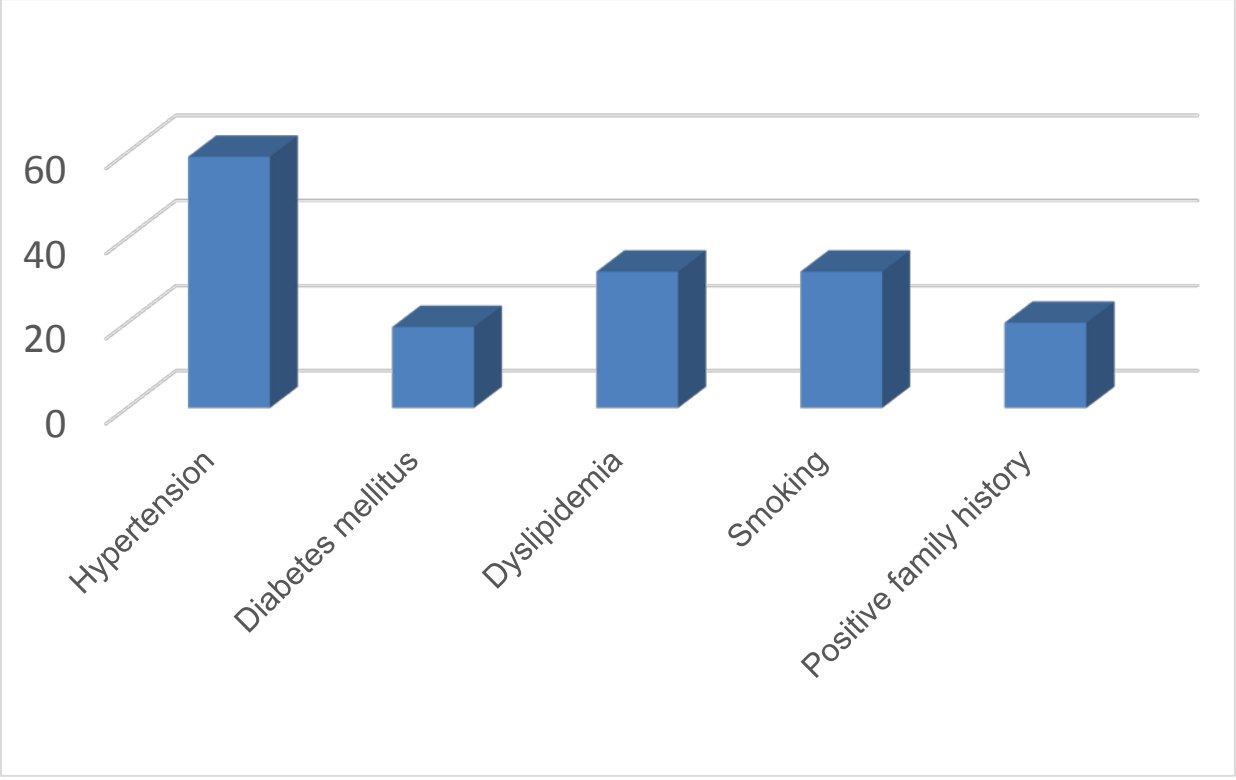
Creatine kinase-MB (IU/L), median (range)	163.5 (15.0-929.0)
Creatine kinase (IU/L), median (range)	1414.0 (42.0-7550.0)
Apelin 12 on the first day (ng/mL), median (range)	2.98 (0.45-15.25)
Apelin 12 on the seventh day (ng/mL), median (range)	2.33 (0.26-10.90)
Troponin I on the first day (ng/mL), mean (±SD)	54.80±60.99
Troponin I on the seventh day (ng/mL), mean (±SD)	12.43±24.48
Anterior infarction, n (%)	48 (48.00%)
Coronary angiographic findings	
Culprit lesion, n (%)	
RCA	40 (40.00%)
LAD	48 (48.00%)
LCx	12 (12.00%)
Vessel disease, n (%)	
1	40 (40.00%)
2	31 (31.00%)
3	29 (29.00%)
Final TIMI grade flow	
3	76 (76.00%)
2	24 (24.00%)

CVD: *cardiovascular disease.*

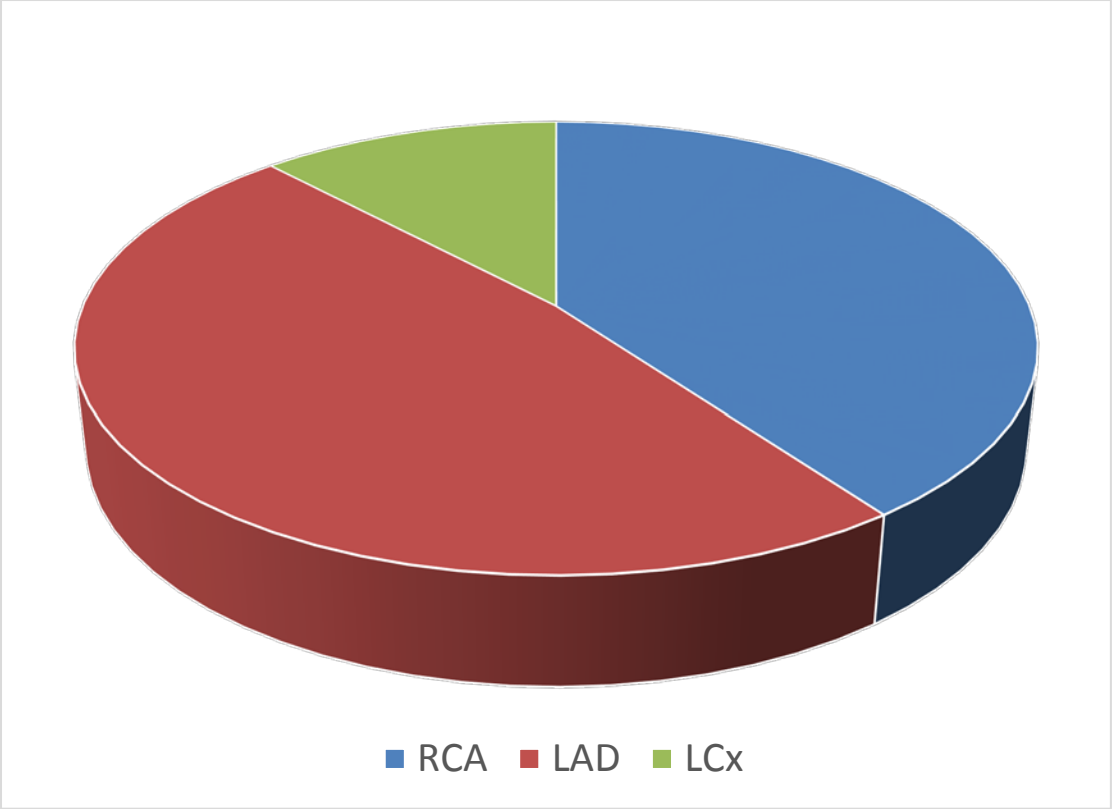
RCA, *right coronary artery*; LAD, *left coronary artery*; LCx, *left circumflex artery.*



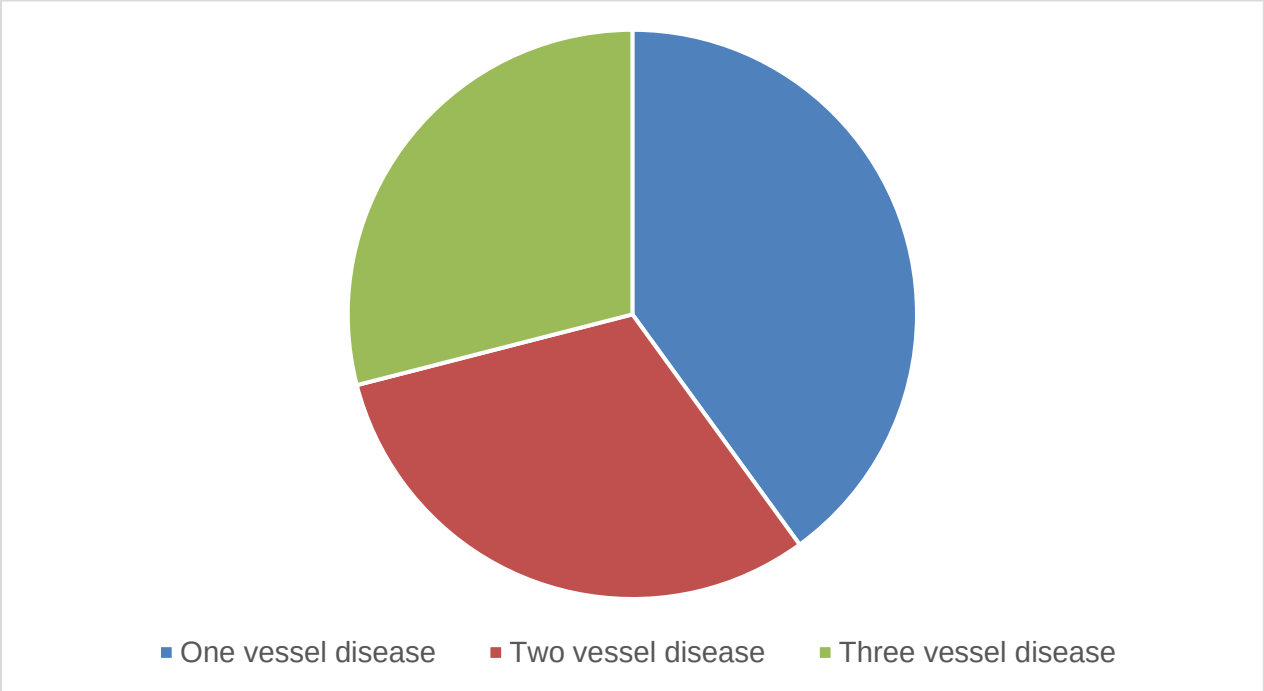
Graph 1. Overall Study Popullation by Gender



Graph 2. Presence of Risk Factors in our Study Popullation

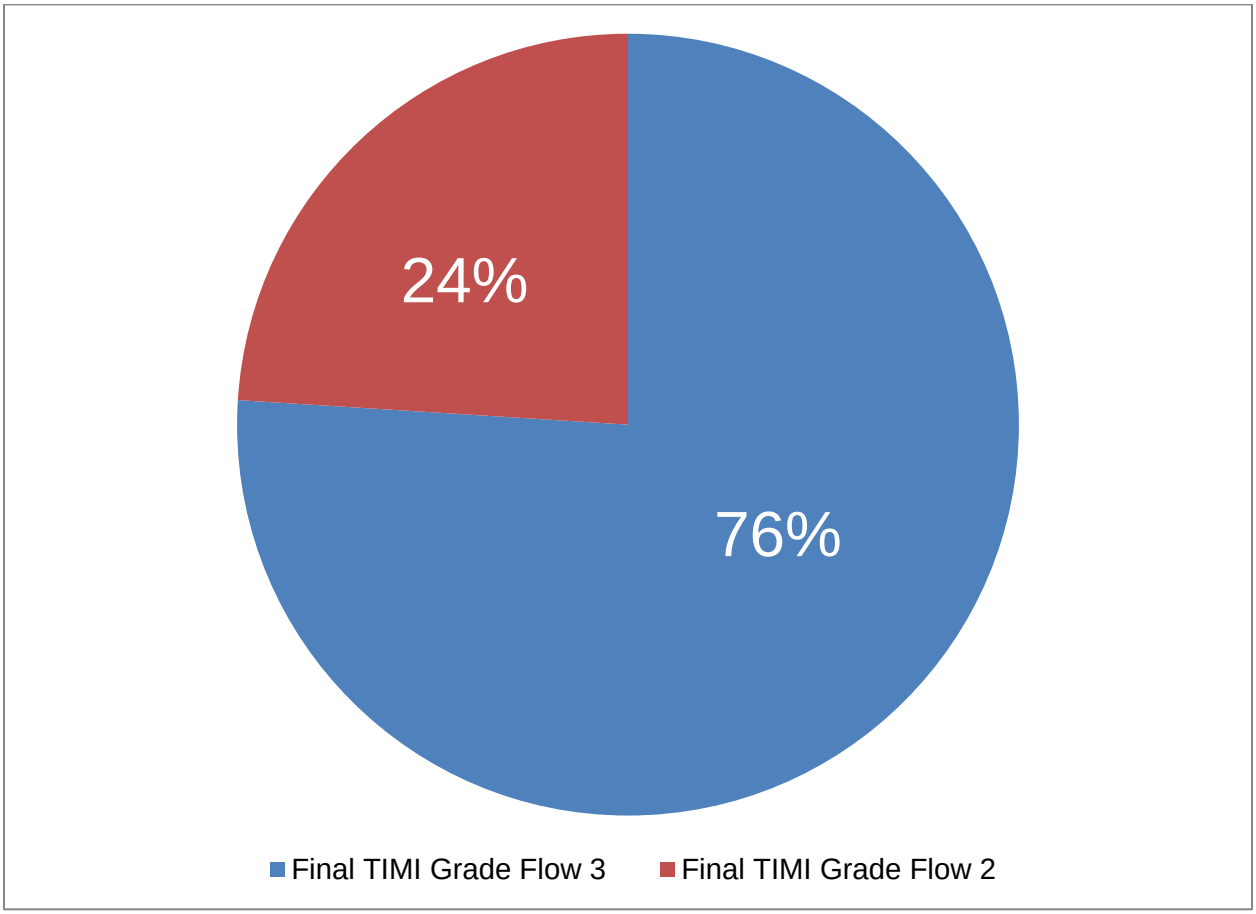


Graph 3. Culprit lesion- Coronaro angiographic findings



Graph 4. Coronaro angiographic findings

The coronary angiography findings according to TIMI grade flow as in Table 1, we presented also in Graph 5 and there is obvious that 76.00% of our study population had Final TIMI grade 3.



Graph 5. Final TIMI Grade Flow- Coronaro angiographic findings

5.2. Acute and non-acute phases of myocardial infarction and the level of apelin

On the first day of the acute phase of myocardial infarction, the median of the apelin-12 level was 2.98 (0.45-15.25), and on the seventh day of the non-acute phase, it was 2.33 (0.26-10.90). The Wilcoxon test revealed a statistically significant difference (p=0.006) in apelin values between the first and seventh day. Variability was observed in the apelin values on first day (Kruskal-Wallis test) based on segmental wall motion abnormalities (SWMAs) (p=0.046), and on the seventh day based on the different numbers of coronary lesions and stenoses, which exhibited a statistically significant difference (p<0.001) (Table 2). When we alayzed the Apelin 12 level in the first and seventh day for each separate parameter, it resulted that concerning age, gender, and risk factors analyzed, the apelin 12 level was always higher in the first day (comparing with the seventh day) and the difference between values in each day showed mainly non significance (Table 2). Also, there was a significant association between apelin-12 and ejection fraction (EF), with a p value <0.029. Figure 5 shows the high values of apelin-12 on the 1st day based on segmental wall motion abnormalities (SWMAs), while Figure 6 shows the low value of apelin-12 on the 7th day based on the number of stenoses.

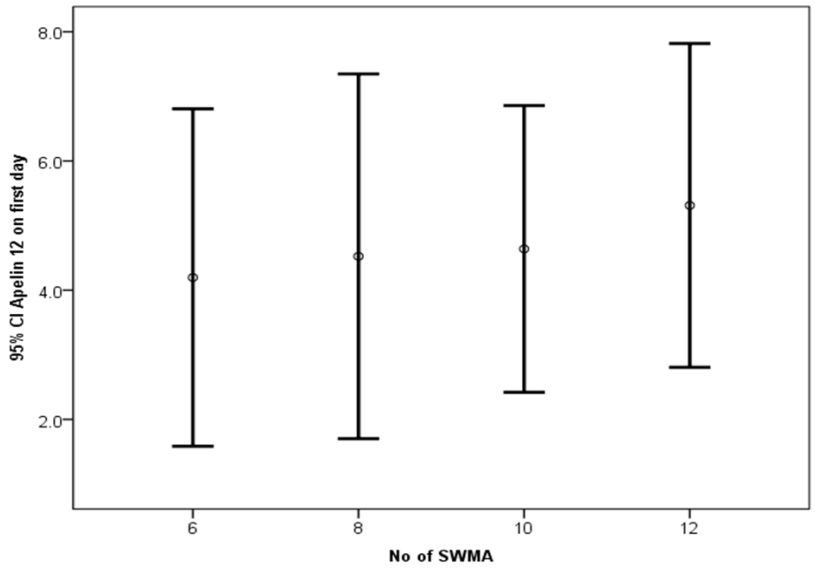


Figure 5. Multiple comparisons graph showing the proportional value of apelin-12 on the 1st day based on the number of SWMAs.

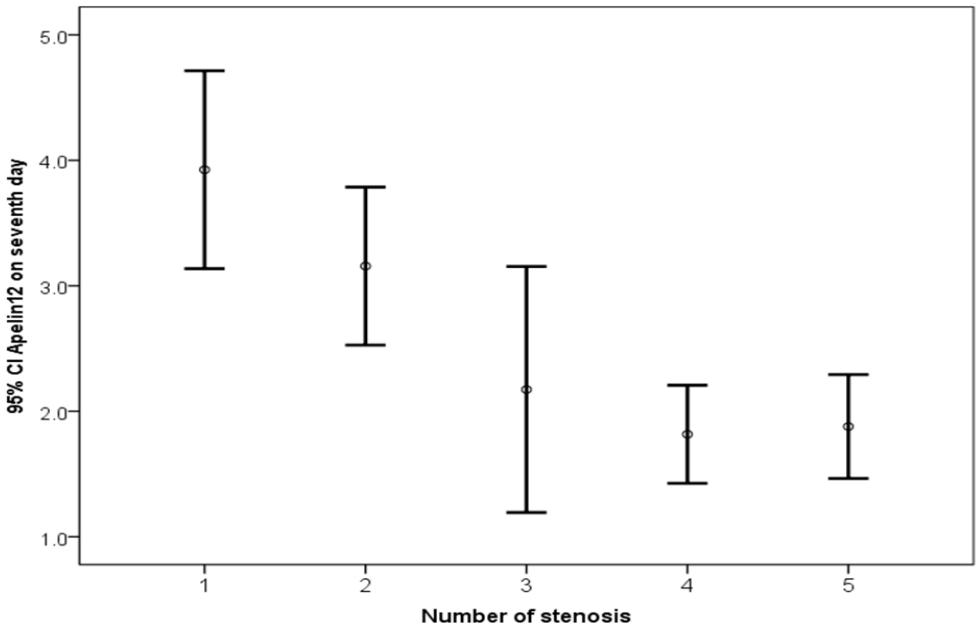


Figure 6. Multiple comparisons graph showing the proportional value of apelin-12 on the 7th day based on the number of stenoses.

5.3. Reperfusion injury and apelin

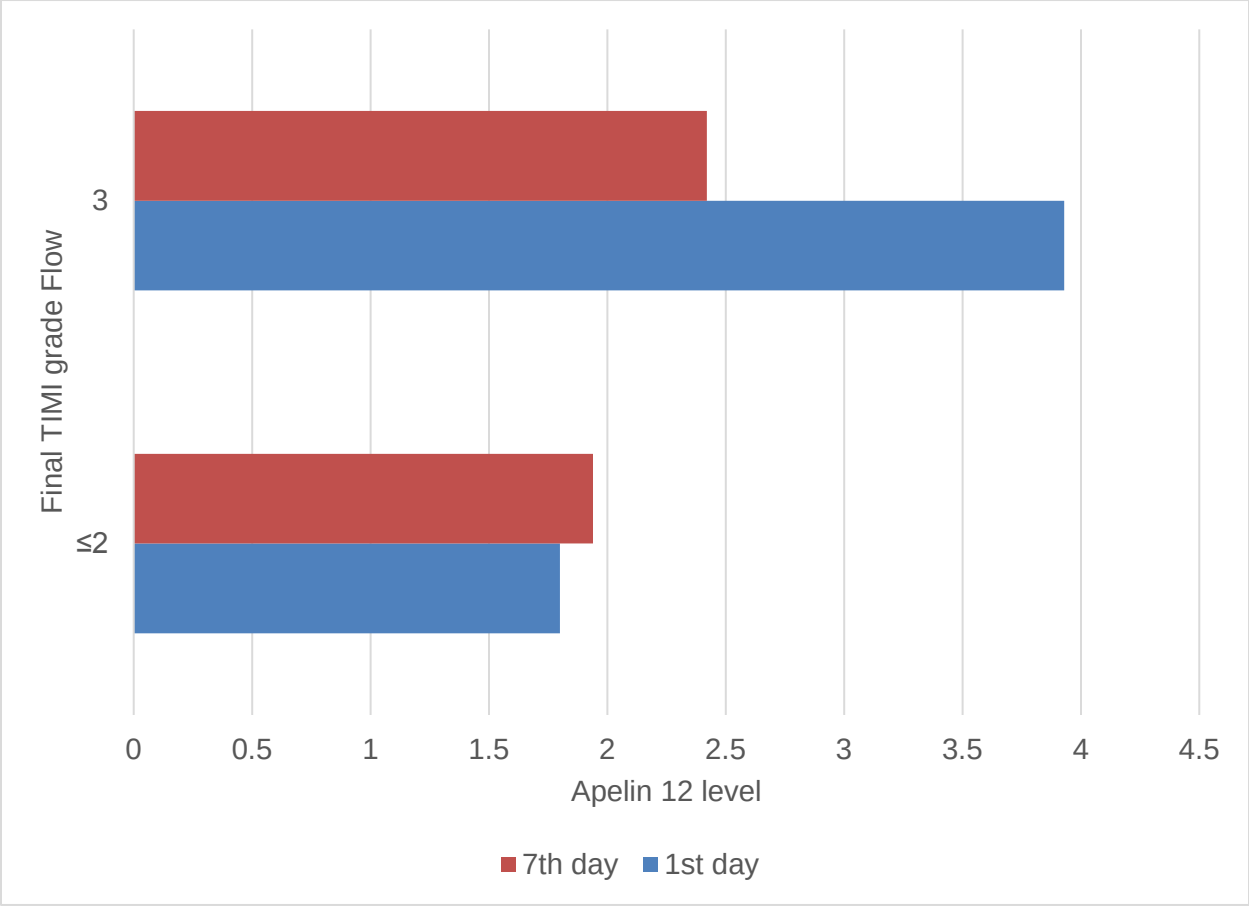
Myocardial ischemia-reperfusion injury after reperfusion therapy was present in 24.00% of patients according to the angiographic criteria. Based on the Mann-Whitney test, the relationship between apelin-12 and the final TIMI grade flow (patients without reperfusion injury with thrombolysis in myocardial infarction (TIMI) flow grade 3 and in patients with reperfusion injury with TIMI flow grade ≤ 2) in the acute phase of myocardial infarction was statistically significant (p=0.001) (Table 2 and Graph 6). In the adjusted logistic regression analysis, apelin-12 was correlated with TIMI flow independent of other risk factors (age, gender, hypertension, dyslipidemia and diabetes mellitus) with a relative risk of 1.36 (95% confidence interval 1.07-1.72) p=0.012.

Table 2. Main characteristics and apelin-12 levels. *Kruskal-Wallis test, and #Mann-Whitney test

	Apelin-12 first day	**p**	**Apelin-12 seventh day**	**p**
No of SWMA median (range)				
5, 6	5.47 (1.80-5.90)	0.046	2.33 (2.30-4.14)	0.47
7, 8	5.60 (0.46-7.08)		2.97 (1.87-5.28)	
9, 10	2.40 (1.30-13.15)		2.10 (0.49-6.98)	
11, 12	4.00 (0.0.56-15.25)		1.95 (0.26-3.53)	
No of stenosis median (range)				
1	3.37 (0.45-11.05)	0.082	3.31 (0.83-10.9)	<0.001
2	4.85 (0.60-13.15)		2.74 (0.47-6.98)	
3	1.80 (0.46-12.00)		1.70 (0.26-9.25)	
4	1.93 (1.00-6.96)		2.04 (0.49-2.4)	
5	2.80 (1.14-8.39)		1.8 (1.18-2.68)	
Final TIMI grade flow median (range)				
≤2	1.80 (0.46-9.25)	0.001	1.94 (0.26-8.88)	0.01
3	3.93 (0.45-15.25)		2.42 (0.49-10.90)	
Age median (range)				
≥65	3.15 (0.45-15.25)	0.66	2.20 (1.44-10.90)	0.96
<65	2.92 (0.51-13.50)		2.36 (0.26-8.88)	
Gender median (range)				
M	2.35 (0.45-13.50)	0.38	2.33 (0.26-10.90)	0.92
F	3.65 (0.50-15.25)		2.35 (0.55-9.75)	

Hypertension median (range)				
Yes	2.8 (0.45-15.25)	0.61	2.10 (0.26-10.90)	0.059
No	3.17 (0.50-12.00)		2.55 (0.83-9.75)	
Diabetes mellitus median (range)				
Yes	3.57 (0.56-9.33)	0.91	1.90 (0.55-4.63)	0.027
No	2.80 (0.45-15.25)		2.40 (0.26-10.90)	
Dyslipidemia median (range)				
Yes	3.55 (0.45-12.00)	0.77	2.34 (0.55-10.90)	0.46
No	2.73 (0.46-15.25)		2.33 (0.26-9.75)	
BMI median (range)				
>30	3.81 (0.45-11.05)	0.41	2.40 (0.47-10.90)	0.8
≤30	2.60 (0.46-15.25)		2.31 (0.26-9.75)	
Smoking median (range)				
Yes	3.70 (0.45-13.15)	0.33	2.33 (0.47-10.90)	0.75
No	2.15 (0.46-15.25)		2.35 (0.26-9.75)	

SWMAs: *segmental wall motion abnormalities*; and TIMI: *thrombolysis in myocardial infarction*.



Graph 6. Level of Apelin 12 in seperate days according to Final TIMI grade Flow

5.4. Relationship between apelin and troponin

The degree of association between apelin-12 and troponin I in the first day of the acute phase of STEMI was analysed with Spearman's correlation=-0.40 ($p < 0.001$). Based on the regression analysis of the relationship between apelin-12 and troponin I, one variable could be predicted from the other (Figure 7).

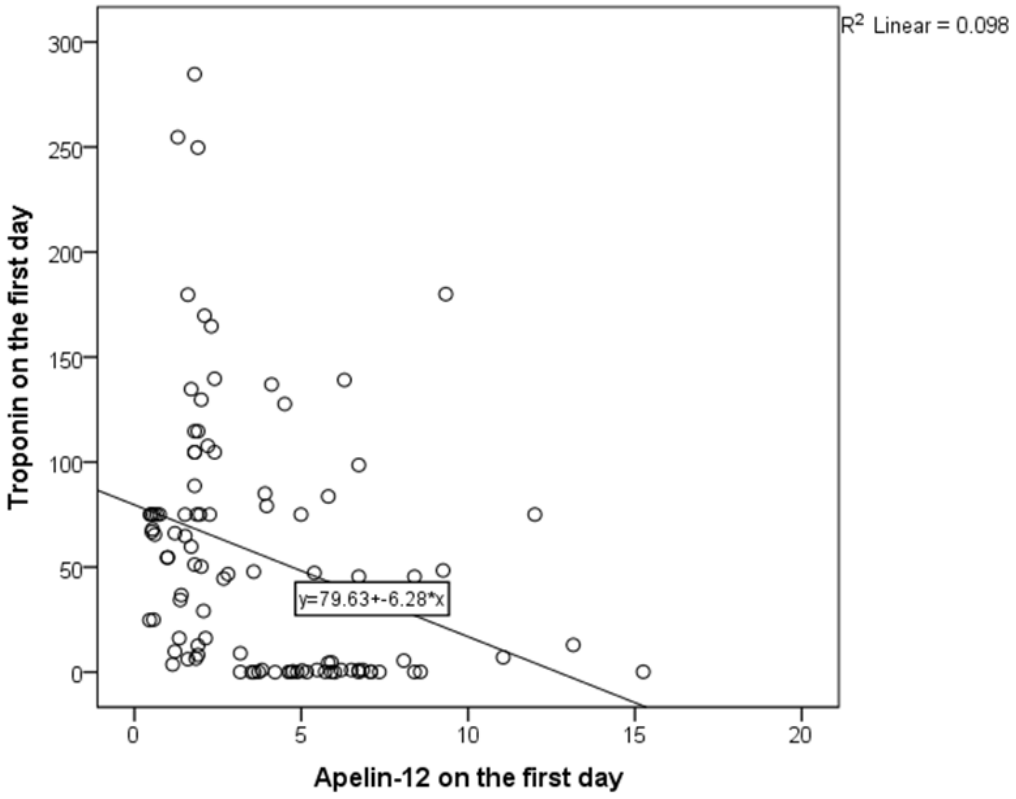


Figure 7. Inverse correlation between apelin-12 and troponin levels in patients with STEMI.

5.5. Apelin and major adverse cardiac events

There was variability in the apelin values on the seventh day (Kruskal-Wallis test) in relation to major adverse cardiac events (MACE) that was significantly different ($p<0.012$). Kaplan-Meier curves were used to show the number of MACE and the proportion of patients that survived at each event time point based on the cut-off value of apelin-12 on the seventh day (2.2 ng/mL) (Figure 8). The log-rank test for the difference in survival resulted in a p value of 0.002. A receiver operating characteristic (ROC) curve plots the true positive rate against the false positive rate at different cut-off points. Table

3 presents the area under the curve values for biomarkers, and Figure 9 presents the ROC curve for apelin-12 and MACE.

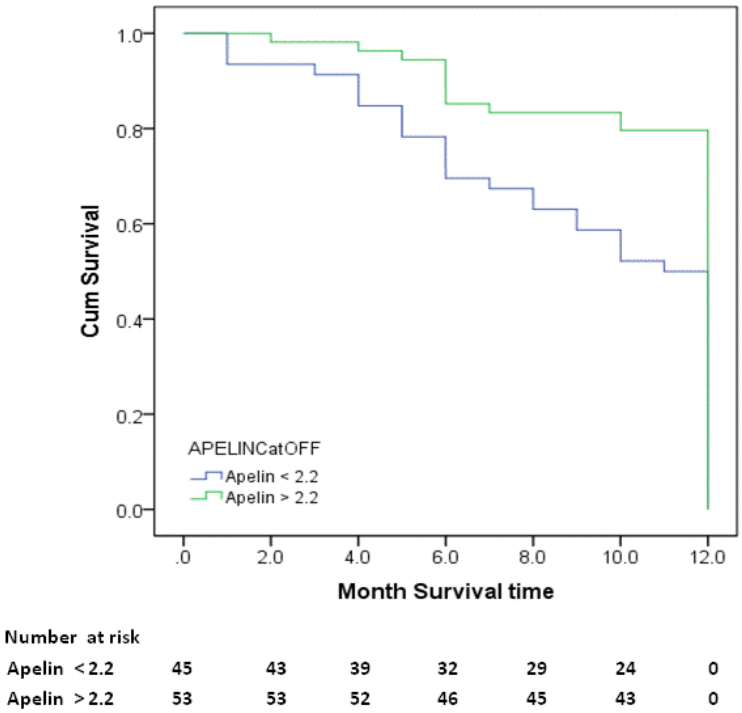


Figure 7. Kaplan-Meier estimates showing lower rates of MACE recurrence among patients with apelin levels >2.2 compared to lower apelin levels <2.2 (p=0.002).

Table 3. Area under the curve values for biomarkers

Biomarker	AUC (95% CI)	P-value
Apelin 12 on the first day	0.52 (0.37-0.67)	0.71
Apelin 12 on the seventh day	0.71 (.58-0.84)	0.004
Troponin I on the first day	0.48 (0.33-0.63)	0.8

Troponin I on the seventh day	0.41 (0.27-0.55)	0.25
Creatine kinase	0.48 (0.33-0.63)	0.84
Creatine kinase-MB	0.58 (0.43-0.73)	0.27
NT-proBNP*	0.49 (0.33-0.64)	0.88
C-reactive protein	0.54 (0.39-0.69)	0.56

* NT-proBNB-*type B natriuretic peptide*

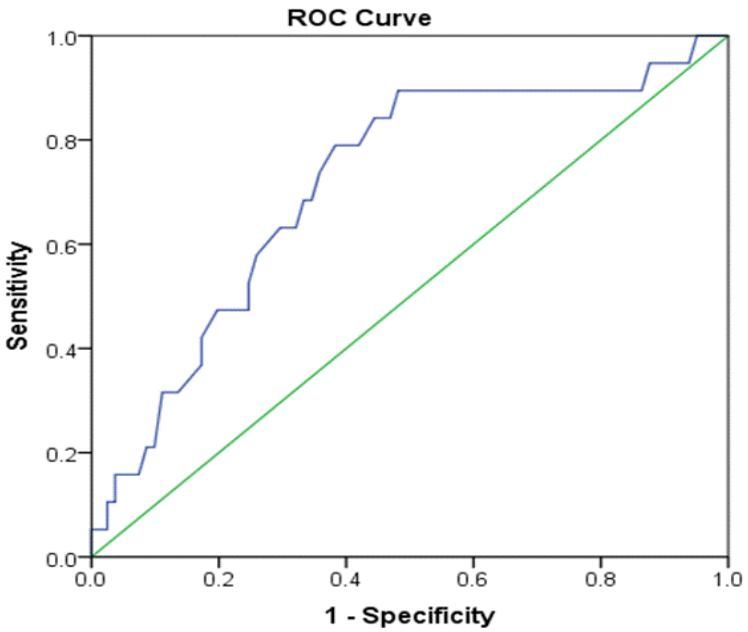


Figure 9. ROC curve analysis of the apelin values on the seventh day for the prediction of MACE. AUC=0.71 (95% CI, 0.58-0.84), p=0.004.

5.6. Association between apelin-12 and creatine kinase-MB depending reperfusion injury

In patients with TIMI flow ≤ 2 the median of apelin-12 level on the first day was 1.80 (0.46-9.25), and with TIMI flow 3, it was 3.93 (0.45-15.25). Variability was observed in the apelin values (Mann-Whitney test) based on TIMI flow grade (p<0.001), while it was not observed for creatine kinase-MB (p<0.54) (Table 4). The degree of association between apelin-12 and creatine kinase-MB was analyzed with Pearson's correlation resulting in patients without reperfusion injury (TIMI flow 3) p < 0.003, while in those with reperfusion injury (TIMI flow ≤ 2) p=0.23. Figure 10 shows the association of apelin-12 and creatine kinase-MB in patients with TIMI flow grade 3, while Figure 11 this association shows in patients with TIMI flow grade ≤ 2.

Table 4. Main variables and TIMI flow. *Kruskal-Wallis test, and #Mann-Whitney test

Variable	TIMI * flow ≤ 2	TIMI* flow 3	p
Apelin-12 on the first day (ng/mL) median (range)	1.8 (0.46-9.2)	3.93 (0.45-15.25)	<0.001
Apelin-12 on the seventh day (ng/mL) median (range)	1.94 (0.26-8.88)	2.42 (0.49-10.9)	<0.01
Creatine kinase-MB (IU/L) median (range)	145.0 (16.0-929.0)	148.5 (15.0-910.0)	0.54
Creatine kinase (IU/L) median (range)	1640.0 (57.0-4316.0)	1406.5 (42.0-7550.0)	0.95
Troponin I on the first day (ng/mL) mean±SD	78.27 ±68.94	47.39 ±56.75	<0.011
Troponin I on the seventh day (ng/mL) mean±SD	13.87±21.80	11.97±25.39	0.64
N-terminal pro-brain nariuretic peptide median (range)	1368.0 (350.0-9600.0)	835.3 (66.5-7383.0)	<0.031
C-reactive protein (mg/dL) median (range)	20.85 (1.8-255.6)	6.55 (0.5-72.4)	<0.001
Haemoglobin (g/dL) mean±SD	13.53±1.95	13.54±1.78	0.64
Creatinine (µmol/L) median (range)	91.5 (70.0-124.0)	93.5 (67.21-125.34)	0.67

*TIMI: *thrombolysis in myocardial infarction.*

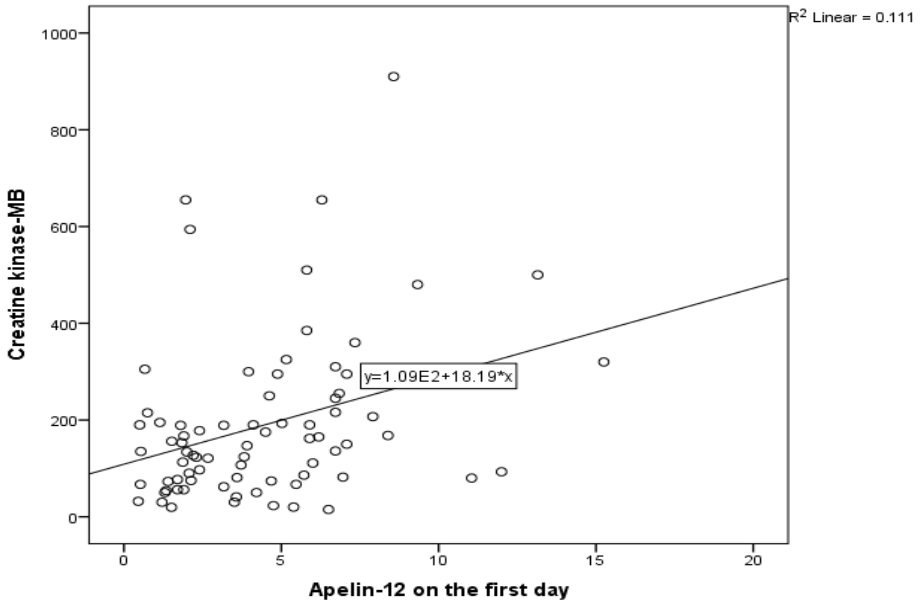


Figure 10. Correlation between apelin-12 on the first day and creatine kinase-MB in patients with TIMI flow 3.

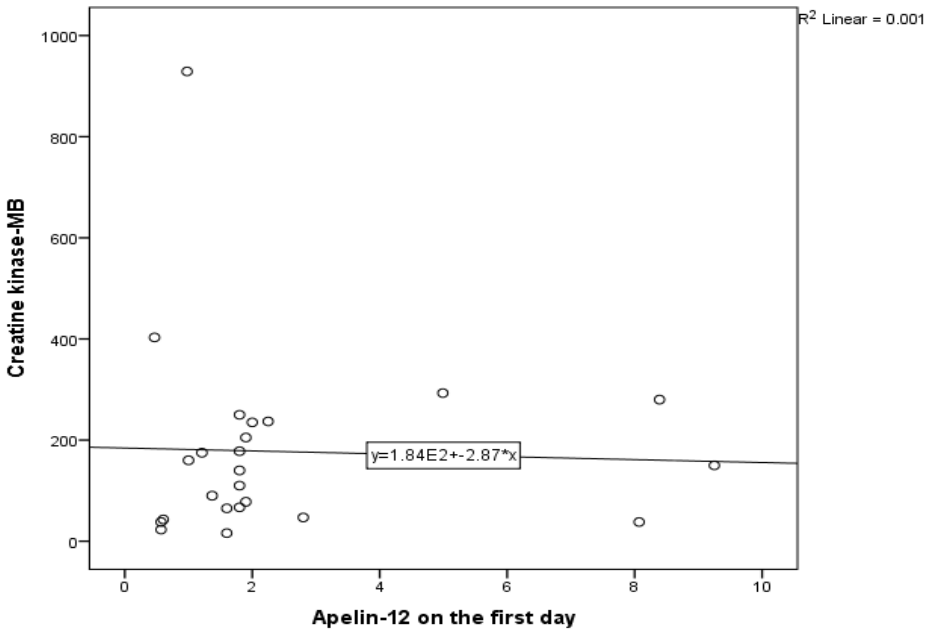


Figure 11. Correlation between apelin-12 on the first day and creatine kinase-MB in patients with TIMI flow ≤2.

5.7. Association between apelin-12 and N-terminal pro-brain natriuretic peptide depending reperfusion injury

In patients with TIMI flow ≤ 2 the median of apelin-12 level on the seventh day was 1.94 (0.26-8.88), and with TIMI flow 3, it was 2.42 (0.49-10.90). Variability was observed in the apelin values (Mann-Whitney test) based on TIMI flow grade (p<0.010), and in the the N-terminal pro-brain natriuretic peptide (p<0.031) (Table 4). The degree of association between apelin-12 and NT-proBNP was analyzed with Pearson's correlation resulting in patients without reperfusion injury (TIMI flow 3) p=-0.042, while in those with reperfusion injury (TIMI flow ≤ 2) p=-0.25. Figure 12 shows the association of apelin-12 and NT-proBNP in patients with TIMI flow grade 3, while Figure 13 this association shows in patients with TIMI flow grade ≤2.

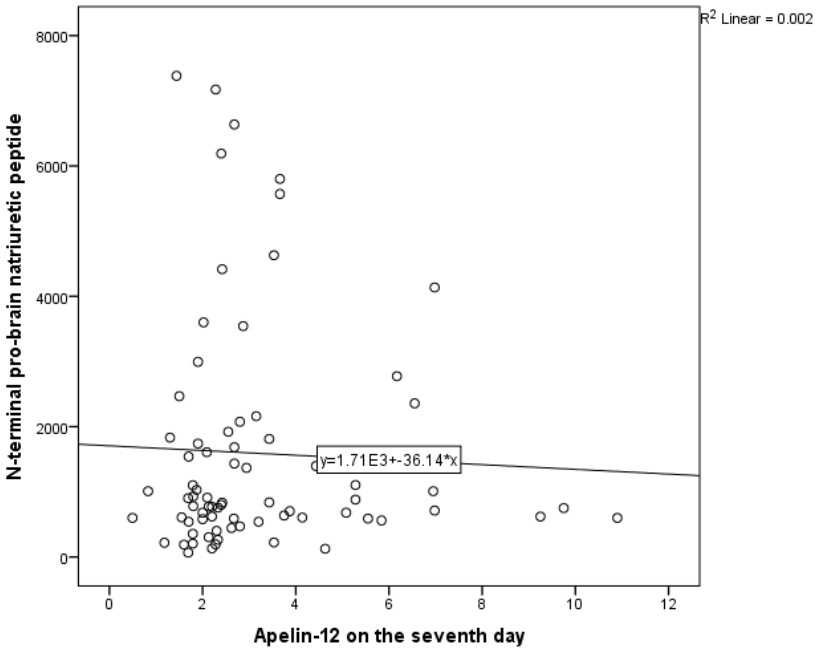


Figure 12. Correlation between apelin-12 on the seventh day and NT-proBNP in patients with TIMI flow 3.

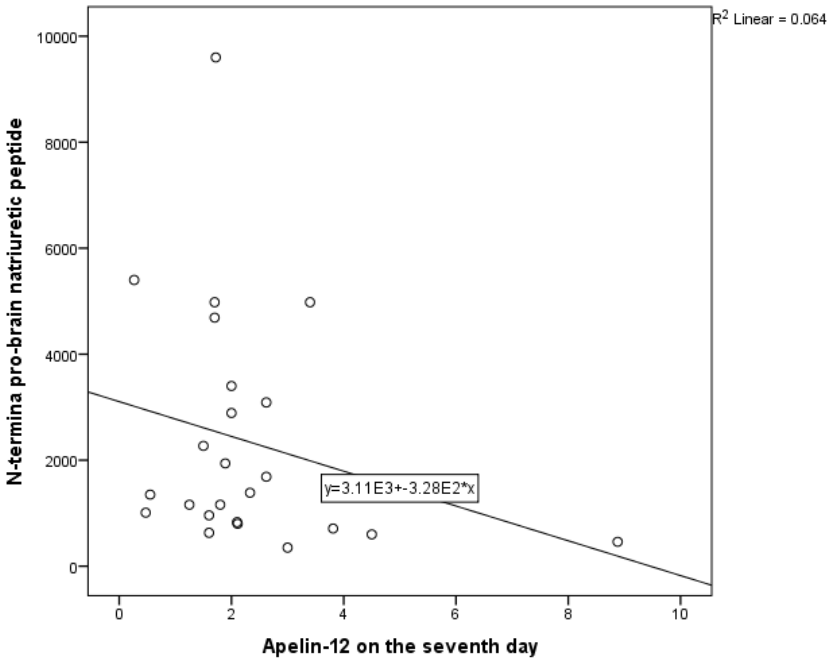


Figure 13. Correlation between apelin-12 on the seventh day and NT-proBNP in patients with TIMI flow ≤2.

In line with all these, analyzing the CRP level and its association with Apelin 12 at the site of reperfusion injury (TIMI flow grade), there were different values of this biomarker. The level of CRP at the TIMI flow ≤2 was 20.85 compared with the level for TIMI flow 3 of 6.55, and when tested it showed a significant difference $p<0.001$. In opposite of this, the difference in levels of haemoglobin and creatinine showed no significance (Table 4).

6. DISCUSSION

Our prospective observational study is conducted on one hundred consecutive patients in two centers with the aim to evaluate apelin-12 levels and its correlation to the rate of MACE in STEMI.

To the best of our knowledge this is the first study investigating the apelin-12 levels in STEMI in humans. The main findings of this study are the following.
During acute myocardial infarction the apelin showed protective effect from reperfusion injury. Variability in the apelin values based on the different numbers of coronary lesions and stenoses exhibited a statistically significant difference. The apelin influenced troponin levels and the rate of major adverse cardiac events (MACE). There was a positive correlation between increased levels of apelin and increased activity of creatine kinase–MB, but an inverse correlation between the apelin-12 and N-terminal pro-brain natriuretic peptide levels.

Our study was conducted to investigate the level of different markers and their association and possible effects and impacts during acute myocardial infarction in humans.
Based on our data during the acute phase of STEMI high level of apelin-12 resulted in better TIMI flow and showing effect also on cardiac contractility by influencing troponin I level. But during the non-acute phase of myocardial infarction high rate complications was inversely associated with low levels of this marker.

According to our study, a difference in the median apelin-12 level measured in patients on the first day after myocardial infarction and the level measured on the seventh day is observed. The median level recorded at the first measurement is higher and the difference between these two separate measures is statistically significant. Acute myocardial infarction (AMI) remains one of the main causes of cardiovascular mortality and morbidity worldwide, particularly in patients with ST-segment elevation myocardial infarction (STEMI) (3, 4). Under these conditions, the levels of apelin and angiotensin receptor like-1 (APJ), which are widely expressed throughout the cardiovascular system (17), are increased. The apelin peptide exerts its biological effects by inducing

angiogenesis, limiting the infarct size, and improving myocardial function (39, 121-123); meanwhile, in the non-acute phase, HIF-1a is rapidly degraded as the oxygen levels increase, thereby decreasing the level of apelin.

Our data show variability in apelin values that are consistent with segmental wall motion abnormalities, which rely on the protective effect of apelin during the acute phase of myocardial infarction, and a statistically significant association between the apelin-12 level and ejection fraction (EF). Echocardiography remains the method of choice for detecting segmental wall motion abnormalities and is used not only to assess the extent of myocardial ischemia during the acute phase but also to predict the final infarct size (124).

In our study population, the correlations between low levels of apelin measured on the seventh day and the different numbers of stenotic coronary arteries were statistically significant. Apelin/APJ expression patterns were inversely correlated with human aortic and coronary atherosclerosis in previous studies (125, 126). Apelin expression promotes cholesterol efflux and reduces foam cell formation in subjects with a low grade of atherosclerosis. A low serum apelin level is associated with plaque vulnerability. Plasma apelin levels correlate with the severity of coronary artery stenosis and the stability of atherosclerotic plaques in humans, representing a useful indicator of the severity of coronary artery stenosis and the stability of coronary atherosclerotic plaques (125).

In our study, myocardial ischemia-reperfusion injury after reperfusion therapy was present in approximately one-fourth of our patients, according to the angiographic criteria. In the acute phase of myocardial infarction, the levels of apelin in two groups of patients stratified according TIMI flow were statistically significant different. Apelin-12 levels correlated with TIMI flow independent of other risk factors (age, gender, hypertension, dyslipidaemia and diabetes mellitus). In the study by Zhang et al., apelin/APJ was reported to serve as a potential target for the prevention of hypoxic/ischemic injury in the cardiovascular system (127). Other authors suggested that a structural analogue of apelin-12, chemically modified apelin-12 (MA), reduces irreversible cardiomyocyte damage, improves cardiac dysfunction, and enhances metabolic restoration and

membrane integrity in experimental models of myocardial I/R injury (128, 129). Based on the findings reported in the study by Wang et al., a lack of apelin increases the potential for ischemia reperfusion (IR) injury by exacerbating adverse remodeling induced by myocardial infarction. Therefore, the loss of apelin affects remodeling, angiogenesis and functional recovery after a myocardial infarction, and it exacerbates myocardial ischemic reperfusion injury (130).

In our study, the (strong) correlation between apelin-12 and troponin I was statistically significant. Additionally, the regression analysis revealed that those two parameters predicted a change in one variable based on the level of other. In the acute phase of myocardial infarction, the atheromatous plaque in the lumen suffers from complete or incomplete acute blockade, resulting in ischaemia in the myocardium. During hypoxia, apelin gene expression and secretion are increased through the activation of hypoxia-inducible factor (HIF) (39, 121, 123). Hypoxia requires a functional mitochondrial electron transport chain to inhibit prolyl hydroxylases and stabilize HIF (109). Under anoxic conditions, HIF is stabilized due to the lack of a functioning mitochondrial respiratory chain, and thus, the apelin gene is neither expressed nor is its expression increased (110). Apelin increases the intracellular Ca^{2+} concentration through protein kinase C initially by increasing the activity of the sarcolemmal Na^{+}/H^{+} exchanger (NHE), leading to an increase in pH, which indirectly increases the intracellular Ca^{2+} concentration through the reverse Na^{+}/Ca^{2+} exchanger (NCX). Second, apelin increases the intracellular Ca^{2+} concentration through the activation of the calcium release channels associated with ryanodine receptors (RyRs) and the activation of protein kinase C, which decreases the phosphorylation of phospholamban (PLB), reducing the function of the SR Ca^{2+} ATPase (SERCA) (94, 95). Intracellular alkalinzation resulting from sarcolemmal NHE activation increases myofilament sensitivity to Ca^{2+} and potentiates the positive inotropic effect of apelin. Through protein kinase C, apelin activates its sites on troponin I, thereby regulating Ca^{2+} sensitivity and ATPase activity in the myocardium. The phosphorylation of one or more of these sites on cTnI plays a central role in suppressing myofilament ATPase activity and increasing the Ca^{2+} sensitivity of myofilaments. The increased Ca^{2+} sensitivity may also be associated with a slowing of Ca^{2+} dissociation from troponin C and

prolongation of attached crossbridges during the force-generating or work-performing cycle (96, 97). During ischaemia/infarction, the levels of phosphorylated TnI, Ca^{2+} sensitivity and ATPase activity are decreased in the myocardium, and the increased Ca^{2+} levels subsequently activate protease I (calpain I), leading to the proteolytic degradation of troponins and ventricular dysfunction (103-106).

In our study, low levels of apelin in the non-acute phase of STEMI were associated with high rates of MACE after STEMI, with significantly variability in the apelin values on the seventh day observed in patients stratified according to major adverse cardiac events (MACE). A cut-off value for apelin-12 levels on the seventh day was established for the survival analysis examining the number of MACE and proportion of surviving patients. Differences in the survival were statistically significant, and the analyses of sensitivity to specificity for different cut off values were also significantly different. After acute myocardial infarction, the left ventricle undergoes a series of changes in shape, size, and thickness, which is referred to as ventricular remodeling; this process precedes the development of clinically evident MACE by months to years. Consistent with these changes, the apelin-APJ axis may be upregulated in response to good left ventricular remodeling or downregulated in response to cardiac troponin degradation and the release of cardiac natriuretic hormones that inhibit the pathophysiological mechanisms responsible for ventricular remodeling (17-19), which is a strong predictor of heart failure and mortality. The involvement of the left coronary artery, large territory or transmurality of myocardial infarction, and echocardiographic evidence of diastolic dysfunction are associated with progressive LV dilation. The findings of other studies have confirmed the efficiency of the effects of apelin on myocardial protection by limiting myocardial infarction and its action as a regulatory peptide to increase cardiac contractility (131-132).

During acute myocardial infarction, a positive correlation was observed between increased levels of apelin and increased activity of creatine kinase–MB in the subgroup of patients with no reperfusion injury. According to a previous study, creatine kinase (CK) structurally associates with sarcoplasmic reticulum (SR) membranes and is capable of linking energy production and utilization using phosphocreatine (PCr) to rephosphorylate all of the ADP produced by the ATP-ases (108). Therefore, one of the enzymes that is

activated after the binding of apelin to its receptor is protein kinase C, through which apelin activates its sites on troponin I, thereby regulating Ca^{2+} sensitivity and ATPase activity in the myocardium (96, 97).

In our study, the subgroup with reperfusion injury exhibited a decreased level of apelin that did not correlate with the level of CK-MB. Presumably, the apelin/APJ axis may serve as a potential target for the prevention of myocardial reperfusion injury in patients with ST-segment elevation myocardial infarction. Reperfusion therapy may induce pathological events in patients with acute myocardial infarction, leading to myocardial tissue injury. Increased generation of highly reactive oxygen species in the heart within minutes of reperfusion has been postulated to affect glycolysis, erythropoiesis, angiogenesis, and apoptosis by decreasing stability of hypoxia-inducible factor 1 alpha (HIF-1α) (133, 134).

The inverse correlation between apelin-12 and NT-proBNP levels identified in our study depends on the TIMI flow grade that determines ventricular dysfunction. The inverse correlation was only observed for patients with a TIMI flow of 3. This finding shows the variability in apelin-12 and NT-pro-BNP levels on the seventh day, depending on the TIMI flow grade. Injury to the heart during myocardial reperfusion causes cardiac dysfunction. The recovery of the myocardium from this reversible form of injury is delayed for several days or weeks (135, 136). Natriuretic peptides are expressed at higher levels in advanced atherosclerotic lesions and are sensitive markers of ischaemia-dependent LV dysfunction (137, 138). After the acute phase of myocardial infarction, the release of natriuretic peptides results from systolic and diastolic dysfunction and increased stress of the left ventricle wall (139, 140).

6.1. Study limitations

The results of the present study should be interpreted in (the light of) several limitations. Firstly, this was dual-center experience study. However, increased number of centers and study group would increase power of the study. Secondly, relatively limited number of patients related to the limited number of biochemical laboratory tests. Thirdly, the study

included only STEMI patients, apelin levels and correlation with troponin in non-STEMI acute chest pain differential admissions to the emergency department would be interesting as control group. Fourthly, assessment of the infarct size using other sophisticated techniques would be of a benefit, but would claim for field experienced staff and would raise the expencies of the study.

7. CONCLUSIONS

Based on our results, the following conclusions were drawn:

1. Apelin-12 levels were significantly higher in the acute phase in comparison to the non-acute phase of STEMI.

2. Variability of apelin-12 values in the acute phase based on segmental wall motion abnormalities and in the non-acute phase based on the different numbers of coronary lesions and stenoses was significant.

3. The difference between apelin-12 levels and final TIMI flow was significant.

4. Apelin-12 levels correlated with TIMI flow independent of other risk factors (age, gender, hypertension, dyslipidemia and diabetes mellitus).

5. There was a significant correlation between apelin-12 and troponin I on the first day of acute phase of STEMI.

6. Variability of apelin levels was significant in the non-acute phase in relation to major adverse cardiac events (MACE).

7. The cut-off value for the apelin-12 levels measured on the seventh day (2.2 ng/mL) identified the number of MACE and the proportion of patients who survived at each event time point.

8. Apelin-12 levels correlated with creatine kinase-MB levels in STEMI patients without reperfusion injury.

9. Apelin-12 levels inversely correlated with N-terminal pro-brain natriuretic peptide levels depending reperfusion injury.

8. ABSTRACT IN CROATIAN

Uvod: Razina fosforiliranog troponina I, osjetljivost Ca^{2+} i aktivnost ATP-aze snižena je u miokardu tijekom akutnog infarkta miokarda. Porast razine Ca^{2+} aktivira proteazu I (kalpain I) koja proteolizom razgrađuje troponine. Istovremeno, hipoksija potiče ekspresiju hipoksijom induciranog faktora 1 alfa (HIF-1a) koji potiče porast razine apelina. Apelin ograničava veličinu infarkta i poboljšava funkciju miokarda.

Metode: U ovu prospektivnu opservacijsku studiju bilo je uključeno 100 uzastopnih bolesnika koji su ispunjavali sljedeće kriterije: neprekinuta bol u prsima u trajanju > 30 min, elevacija ST-spojnice u elektrokardiogramu (EKG) (mjerena od J-točke) ≥ 2.5 mm u muškaraca < 40 godina, ≥ 2 mm u muškaraca ≥ 40 godina ili ≥ 1.5 mm u žena u odvodima V2-V3 i/ili ≥ 1mm u drugim odvodima (uz odsutnost hipertrofije lijeve klijetke ili bloka lijeve grane), porast specifičnih biomarkera poput troponina I, MB frakcije kreatin kinaze (CKMB), i bolesnici koji su bili podvrgnuti reperfuzijskoj terapiji. Mjerena je razina apelina-12, kreatin kinaze (CK), MB frakcije kreatin kinaze (CKMB), troponina I, NT-proBNP, CRP, triglicerida, LDL i HDL kolesterola te rutinskih laboratorijskih parametara. Posebna pozornost data je na mjerenje razine apelina-12 i troponina I prvi i sedmi dan nakon reperfuzijske terapije kod svih bolesnika.

Rezultati: Pronađena je varijabilnost u razini apelina prvog dana (Kruskal-Walisov test) relativna u odnosu na segmentalne abnormalnosti gibanja stijenke (SWMAs) ($p=0.046$) i sedmog dana relativna u odnosu na različiti broj koronarnih lezija i stenoza ($p<0.001$). Prema Mann-Whitneyevom testu, povezanost uzmeđu apelina-12 i konačnog stupnja TIMI protoka u akutnoj fazi infarkta miokarda bila je statistički značajna ($p=0.001$). Razina apelina-12 bila je obrnuto proporcionalna razini troponina (Spearmanova korelacija =-0.40) sa p-vrijednosti <0.001. Postojala je varijabilnost u razini apelina sedmog dana (Kruskal-Waslisov test) temeljena na pojavi velikih neželjenih kardijalnih događaja (MACE) ($p=0.012$). Koristeći ROC krivulju, granična vrijednost testa vrijednosti od > 2.2 bila je određena za povezanost apelina s MACE, a površina ispod krivulje (AUC) bila je 0.71 (95% interval podudarnosti CI, 0.58-0.84). Analiza preživljenja (koristeći Kaplan-Meierovu metodu) pokazala je nižu stopu MACE u bolesnika s razinom apelina > 2.2 ($p=0.002$), a ROC krivulja je pokazala statistički značajnu razliku u površini ispod krivulje

(p=0.004). Pearsonova korelacija između apelina-12 i kreatin kinaze-MB prvog dana u bolesnika bez reperfuzijske ozljede bila je okarakterizirana sa p-vrijednosti <0.003, dok je p-vrijednost korelacije između apelina 12 i NT-proBNP-a sedmog dana u bolesnika bez reperfuzijske ozljede bila -0.042.

Zaključak: Porast razine apelina tijekon akutne faze infarkta miokarda ukazuje na protektivni učinak na reperfuzijsku ozljedu dok je niska razina apelina tijekom neakutne faze infarkta miokarda u obrnuto proporcionalnoj vezi s brojem koronarnih stenoza. Apelin-12 utječe na razinu troponina I u akutnoj fazi STEMI-ja, dok su tijekom neakutne faze, niske razine apelina povezane s većom pojavnosti MACE-a u razdoblju od 12 mjeseci praćenja. U bolesnika sa STEMI-jem koji su podvrgnuti reperfuzijskoj terapiji, apelin-12 je povezan s kreatin kinazom-MB ovisno o reperfuzijskoj ozljedi koja određuje ulogu apelina u sustavu kreatin kinaze.

9. ABSTRACT IN ENGLISH

Background: During acute myocardial infarction, phosphorylated TnI levels, Ca^{2+} sensitivity and ATPase activity are decreased in the myocardium, and the elevation in Ca^{2+} levels activates protease I (calpain I), leading to the proteolytic degradation of troponins. Concurrently, hypoxia enhanced the expression of hypoxia inducible factor 1 alpha (HIF-1a) increasing the level of apelin, which limits infarct size, and improves myocardial function.

Methods: In this prospective observational study, 100 consecutive patients meeting the following criteria were included: continuous chest pain lasting > 30 min, an electrocardiogram (ECG) with ST-segment elevation (measured at the J-point) ≥ 2.5 mm in men < 40 years, ≥ 2 mm in men ≥ 40 years, or ≥ 1.5mm in women in leads V2-V3 and/or ≥ 1mm in other leads [in the absence of left ventricular (LV) hypertrophy or left bundle branch block (LBBB)], rise of specific biomarkers such as troponin I and the MB fraction of creatine kinase (CKMB), and patients who underwent reperfusion therapy. The levels of apelin-12, creatine kinase (CK), the MB fraction of creatine kinase (CKMB), troponin I, NT-proBNP, CRP, triglycerides, LDL and HDL cholesterol, and other routine laboratory parameters are measured. In particular, we evaluated the levels of apelin-12 and troponin I on the first and seventh days after reperfusion therapy in all patients.

Results: There was variability in apelin values on the first day (Kruskal-Wallis test) relative to segmental wall motion abnormalities (SWMAs) ($p=0.046$) and on the seventh day relative to different numbers of coronary lesions and stenoses ($p<0.001$). Based on the Mann-Whitney test, the relationship between apelin-12 and the final TIMI grade flow in the acute phase of myocardial infarction was statistically significant ($p=0.001$). Apelin-12 was inversely correlated with troponin I levels (Spearman's correlation = −0.40) with a p value <0.001. There was variability in the apelin values on the seventh day (Kruskal-Wallis test) based on major adverse cardiac events (MACE) ($p = 0.012$). Using ROC curve analyses, a cut-off value of >2.2 for the association of apelin with MACE was determined, and the AUC was 0.71 (95% CI, 0.58–0.84). Survival analysis using the

Kaplan-Meier method showed a lower rate of MACE among patients with apelin levels >2.2 (p = 0.002), and the ROC curve analysis showed a statistically significant difference in the area under the curve (p = 0.004). Pearson's correlation between apelin-12 and creatine kinase-MB on the first day in patients without reperfusion injury was characterized with a p value <0.003, while between apelin-12 and NT-proBNP on the seventh day in patients without reperfusion injury p value was -0.042.

Conclusion*:* The increase level of apelin during acute phase of myocardial infarction indicate a protective effect from reperfusion injury while low level of apelin during non-acute phase of myocardial infarction is found to be inversely associated with number of coronary stenoses. Apelin-12 influences troponin I levels in the acute phase of STEMI, whereas during the non-acute phase, low apelin levels were associated with high rate of MACE. In STEMI patients undergoing reperfusion therapy, apelin-12 was associated with creatine kinase-MB depending reperfusion injury determining role of apelin in creatine kinase system.

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