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UNIVERSITY OF MEDICINE AND PHARMACY,

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DOCTORAL SCHOOL

FIELD OF MEDICINE

Study of Immunohistochemical Markers in Post-Mortem Recognition of Myocardial Infarction.

SUMMARY OF THE DOCTORAL THESIS

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INTRODUCTION

Cardiovascular diseases represent the leading cause of mortality worldwide, with acute myocardial infarction and coronary atherosclerosis being among the most common causes of death. In cases of sudden death, which includes deaths of cardiac origin, autopsy plays an essential role in determining the cause of death, contributing to a deeper understanding of pathological mechanisms and improving preventive interventions.

The presented doctoral thesis includes three studies, each bringing valuable contributions to the field of histopathology and forensic medicine. The first study, a retrospective observational analysis, examines cases of sudden cardiac death autopsied at the Maramureș County Forensic Medicine Service. This study highlights the difficulty in identifying early-stage myocardial infarction, a major challenge in forensic medicine. It emphasizes the importance of histopathological diagnostic methods.

The second study presents a comprehensive review of the specialized literature, focusing on the use of immunohistochemical markers for diagnosing myocardial ischemia. Markers such as the C5b-9 complex, fibronectin, and cardiac troponins play a crucial role in detecting ischemia in its early stages, especially in cases where macroscopic and microscopic lesions are not evident. This study reiterates the importance of advanced immunohistochemistry techniques.

The third study, of an experimental nature, explores the immunohistochemical expression of ATP1A3 and PKP2 proteins as potential markers for diagnosing myocardial ischemia. The conclusions of this study suggest that ATP1A3 shows promising potential in diagnosing ischemia, while PKP2 does not demonstrate a significant correlation in this regard. In conclusion, this doctoral research emphasizes the importance of immunohistochemical markers in the early detection of myocardial ischemia and highlights the need for further studies. The development of more advanced and precise diagnostic methods has the potential to improve the accuracy of diagnosing acute myocardial infarction.

I. GENERAL PART

1. Sudden Cardiac Death

1.1 Introduction

Cardiovascular diseases are the leading cause of mortality worldwide. Among cardiovascular conditions, cardiac-related mortality ranks first. Examples of death causes associated with cardiovascular pathology include acute myocardial infarction, severe coronary atherosclerosis, ventricular fibrillation, dilated cardiomyopathy, hypertrophic cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, and myocarditis. Myocardial infarction and coronary atherosclerosis are the main causes of cardiovascular death of cardiac origin, accounting for 85% of all cardiac-related deaths. These two pathologies have considerable implications for social life and the healthcare system.

Cases of sudden cardiac death (SCD) are frequently classified as forensic cases, in accordance with the provisions of the Penal Code and the Code of Criminal Procedure, which refer to deaths suspected of being violent or with an unknown cause. The exact identification of the cause of SCD can be difficult even after post-mortem complementary investigations, as some conditions, such as coronary spasm, are hard to identify. The most common cause of SCD is atherosclerotic coronary disease, which can lead to myocardial ischemia, triggering ventricular arrhythmias or myocardial infarction (1). Myocardial ischemia is the precursor stage of any type of myocardial infarction. Therefore, the use of immunohistochemical markers is essential for the early detection of ischemia (2).

1.2 Epidemiology

Globally, SCD causes over 7,000,000 deaths annually. In the United States, SCD is responsible for approximately 300,000 deaths, while in the European Union, it accounts for about 250,000 deaths. Studies have shown that men are more susceptible to SCD due to a combination of factors that contribute to the differences in incidence between the two sexes. Additionally, the protective hormonal role in females is believed to contribute to the differences in SCD incidence (3). The most affected age group is between 50 and 60 years.

1.3 Pathophysiology of Sudden Cardiac Death

SCD is caused by interactions between structural, functional, and electrical gradient factors that affect the myocardium. Elevated levels of catecholamines, oxidative stress, and genetic mutations can trigger malignant ventricular arrhythmias (4). Endothelial damage associated with oxidative stress can lead to atherosclerosis and vasospasm, which result in myocardial ischemia and infarction.

1.4 Myocardial Ischemia and Acute Myocardial Infarction

Myocardial Ischemia

Myocardial ischemia represents an oxygen deficiency at the level of the cardiac muscle, which can be reversible, unlike acute myocardial infarction, which involves necrosis. Calcium channel blockers have been described as factors that may delay the irreversible damage to cardiomyocytes (5).

Myocardial Infarction

Myocardial infarction involves the necrosis of myocardial tissue and is clinically classified into five types, the most common being type one (atherosclerotic plaque rupture) and type two (imbalance between oxygen supply and demand). Type one is responsible for approximately 60% of cases (6), while type two is more common in women, the elderly, and those with multiple comorbidities. A histopathological classification guided by the clinical classification was carried out by the European Association of Cardiovascular Pathology (7).

1.4.1 Myocardial Infarction Caused by Acute Coronary Thrombosis

Acute coronary thrombosis is recognized as the leading cause of myocardial infarction, accounting for about 60% of acute myocardial infarction cases (6). Pathophysiologically, as atherosclerosis progresses, atherosclerotic streaks evolve into more advanced plaques, eventually leading to arterial blockage and reduced blood flow. Recent studies have demonstrated that, before platelet adhesion to the ruptured atheromatous plaque, plasma fibronectin and other proteins deposit on the plaque. This process has been recently identified as a mechanism that precedes platelet adhesion. After the rupture of the atherosclerotic plaque, the proteins in the lipid matrix, including collagen, von Willebrand factor (VWF), fibrinogen, and fibronectin, trigger a rapid platelet response. Subsequently, platelets adhere, activate, and aggregate. This can lead to partial or complete occlusion of the vessel, causing ischemic events and ultimately myocardial infarction (8).

1.4.2 Acute Myocardial Infarction Caused by Imbalance Between Oxygen Supply and Demand in Myocardial Tissue

Clinically classified as type II myocardial infarction, this type of infarction results from prolonged ischemia caused by an imbalance between oxygen supply and demand, other than that of thrombotic origin. This mechanism can be determined by various factors, including systemic, cardiac, or coronary pathology, which may compromise the balance between the oxygen supply and demand to the myocardial tissue (9). This mechanism includes both coronary atherosclerosis without acute thrombosis and relatively rare non-atherosclerotic coronary diseases, such as spasm, spontaneous dissection, or coronary embolism (10). The

causes that disrupt the balance between oxygen supply and demand can be classified based on increased myocardial oxygen demand or reduced supply to the myocardium (11-13).

1.4.2.1 Acute myocardial infarction due to reduced oxygen supply to the myocardial tissue

Atherosclerosis involves the accumulation of lipids and fibrous tissue on the arterial walls, leading to reduced blood flow and myocardial ischemia, which is a precursor to myocardial infarction (14). Coronary artery spasm represents an intense constriction of the coronary vessels, which can lead to partial or complete occlusion, resulting in acute ischemia. It usually occurs in the context of atherosclerosis but can also affect normal coronary vessels. Mechanisms include smooth muscle hyperreactivity, endothelial dysfunction, and magnesium deficiency. Physical and mental stress, as well as vasoconstrictive substances, are triggering factors (15-17). Spontaneous coronary artery dissection can lead to acute myocardial infarction by rupturing the vascular endothelium, affecting blood flow. Type A aortic dissection can extend into the coronary arteries. Coronary embolism, which can occur due to atrial fibrillation, cardiomyopathies, or endocarditis, obstructs coronary blood flow, causing ischemia and infarction. Shock, whether hypovolemic, cardiogenic, or septic, reduces coronary perfusion, leading to ischemia and infarction. Pulmonary embolism can cause myocardial ischemia by increasing right ventricular pressure, leading to infarction (18).

1.4.2.2 Acute myocardial infarction caused by increased oxygen demand

can have multiple causes, each contributing to myocardial overload. Arrhythmias, particularly tachyarrhythmias, increase oxygen demand in the heart and are a common cause of type II infarction (10). In cases of hypertrophic cardiomyopathy and aortic stenosis, left ventricular hypertrophy significantly increases oxygen demand, contributing to ischemia (19). Chronic kidney failure increases the risk of infarction through fluid accumulation, ventricular hypertrophy, and endothelial dysfunction (20). Takotsubo cardiomyopathy, often induced by physical or emotional stress, triggers a temporary increase in oxygen demand, causing ischemia. Thalassemias and sickle cell anemia can obstruct coronary microcirculation, causing vaso-occlusive crises and infarction. Additionally, rare cases of type II myocardial infarction are associated with embolism, pregnancy, or gastrointestinal conditions (21).

2. Immunohistochemical markers for identifying myocardial ischemia and necrosis

2.1 Immunohistochemical markers of the complement system

The C5b-9 complex, also known as the terminal complement complex or membrane attack complex, is recognized as a reference standard in evaluating ischemic lesions. This marker has high sensitivity, making C5b-9 detectable approximately 40 minutes after the onset of ischemia. Studies have demonstrated a correlation between C5b-9 expression and the severity of myocardial ischemia. Additionally, C1, C3, C8, and C9 components play important roles in inflammation associated with myocardial infarction. C1 and C3 are active in the early phases of ischemia, while C8 and C9 appear in the final stage of complement activation (22).

2.2 Immunohistochemical markers mediating inflammation

Inflammation plays a crucial role in the pathogenesis of myocardial infarction, significantly contributing to the worsening of cardiac lesions. Galectin-3 is a marker associated with inflammation and fibrosis, being active during ischemic periods. Studies indicate a correlation between the infiltration of Galectin-3-positive macrophages and the progression of fibrosis in myocardial infarction (23). Immunohistochemistry allows visualization of the localization and intensity of IL-1β, IL-6, and IL-15 expression in ischemic myocardial tissue (24). Markers such as CD15, CD18, and tryptase are present during ischemia and necrosis, being detectable in the early stages of cardiac lesions.

2.3 Immunohistochemical markers specific to cardiomyocyte proteins

Desmin, alpha-actinin, and vinculin are essential proteins for maintaining cardiomyocyte structure, and their degradation during ischemia indicates severe cellular structure damage. H-FABP (heart-type fatty acid-binding protein) is a sensitive marker for myocardial ischemia, considered superior to markers like creatine kinase and myoglobin. Cardiac troponins (I and T) are essential markers for diagnosing myocardial infarction, with their immunoreactive variations detectable within an hour of infarction. Zonula occludens-1 plays a role in maintaining cell junctions and the electrical activity of cardiomyocytes and is used as a marker for detecting myocardial ischemia. Connexin-4 forms gap junction channels, facilitating electrical activity between cardiomyocytes, and is considered a sensitive marker for myocardial ischemia. Natriuretic peptides, including B-type natriuretic peptide (BNP), are markers for cardiac stress and heart failure, being used to assess ischemia (2).

2.4 Immunohistochemical markers specific to plasma proteins and myocardial necrosis

Fibronectin is one of the first markers detectable in myocardial ischemia, playing an important role in assessing the extent of necrosis (25). S100A1, a sensitive marker, becomes visible shortly after the onset of ischemia and is particularly useful in forensic evaluations. Its presence indicates early myocardial damage, allowing for precise assessment of ischemic events (26).

2.5 Immunohistochemical markers specific to hypoxia

HIF1-alpha is a hypoxia marker detectable through immunohistochemistry in cases of myocardial infarction, being closely linked to the body's response to ischemia. Its expression increases under low oxygen conditions, contributing to cellular adaptation to ischemic stress (27). Galectin-1, also a hypoxia marker, shows rapid depletion after the onset of ischemia (28).

2.6 Immunohistochemical markers specific to atherosclerotic plaque destabilization

Matrix metalloproteinases, such as MMP-9, are involved in the degradation of the extracellular matrix and play an essential role in destabilizing atherosclerotic plaques, contributing to the progression of cardiovascular disease and the risk of infarction. Myeloperoxidase is an important inflammatory marker associated with both atherosclerotic plaque destabilization and post-infarction cardiac remodeling. Its presence indicates the activation of inflammatory processes and contributes to the progression of ischemia (29).

2.7 Immunohistochemical markers specific to apoptosis

Apoptosis is an essential process in myocardial necrosis. Markers such as BCL2 and caspase-3 are involved in detecting apoptosis in affected myocardial tissues (30, 31).

II. PERSONAL CONTRIBUTIONS

3. Hypothesis/Objectives

The premises of this work stem from the observation that in cases of sudden cardiac death, the diagnosis of acute myocardial infarction may be missed during microscopic histopathological examination. Microscopic signs of ischemia may not be evident if the patient did not survive long enough from the onset of ischemia, which precedes acute myocardial infarction. The use of immunohistochemistry (IHC) can help detect ischemia even in the first minutes after onset, before changes are visible with H&E staining.

The goal of this thesis was to conduct a comprehensive analysis of the existing data in the literature to date concerning myocardial ischemia and acute myocardial infarction, with a special focus on pathoanatomical aspects and known IHC markers for detecting ischemia and myocardial infarction.

Objectives:

- Evaluate the existing data in the literature on myocardial ischemia and acute myocardial infarction, focused on pathoanatomical aspects.
- Assess the existing data in the literature regarding immunohistochemical markers known for detecting ischemia and acute myocardial infarction.
- Perform a retrospective statistical analysis of the importance of sudden cardiac death cases encountered in forensic pathology labs, with the goal of establishing the final diagnoses reported in forensic investigations of sudden cardiac death.
- Create a classification of immunohistochemical markers for detecting myocardial ischemia and acute myocardial infarction based on the mechanism involved and/or the location of the markers in cardiomyocytes.
- Investigate potential new markers of myocardial ischemia and acute myocardial infarction, such as the ATP1A3 and PKP2 proteins, located intracellularly and at the intercalated discs of cardiomyocytes, which exhibit high specificity and sensitivity.

4. General Research Methodology

Study Design and Data Collection

Study 1 was a retrospective observational study conducted over a period of 3 years (2021-2023). Study 2 was a systematic review. Study 3 was a retrospective observational study conducted over 4 years (2020-2023).

The data used for the studies were collected from:

Study 1 and 3: based on medico-legal data and cases from the Maramureș County Forensic Medicine Service.

Study 2: conducted using databases such as PubMed, Scopus, Science Direct, American Chemical Society, Springer, ProQuest, and Wiley. The present research adheres to the Helsinki Declaration.

Location and Research Infrastructure

The database was studied at the headquarters of the Maramureș County Forensic Medicine Service. H&E-stained slides from the cases included in Study 3 were reviewed by a pathologist at the Maramureș County Forensic Medicine Service. Immunohistochemical staining was performed at the MedLife Humanitas Hospital laboratory in Cluj-Napoca.

Immunohistochemical Markers Used

Three antibodies were purchased:

Anti-ATP1A3 antibody [XVIF9-G10] ab2826, quantity 100µg, concentration 0.1 mg/mL (ABCAM);

Anti-Plakophilin 2/PKP2 antibody ab189323, quantity 100µg, concentration 0.1 mg/mL (ABCAM);

Anti-C5b-9 antibody ab55811, quantity 100µg, concentration 0.5 mg/mL (ABCAM).

The immunohistochemical staining procedure followed the manufacturer's recommendations.

Statistical Analysis

Descriptive statistical analysis was performed using Microsoft Office 2010 and SPSS 17 statistical software packages. Various variables were used for descriptive analysis, including the Chi-square test, and Pearson correlation and ROC tests were used for immunohistochemical correlations.

5. STUDY I: Statistical Analysis of Causes of Death and Histopathological Diagnosis in Forensic Investigations of Sudden Cardiac Deaths

5.1 Hypothesis and Specific Objectives

Sudden cardiac deaths (SCD) are increasing globally and are associated with multiple causes. Annually, approximately 350,000 people in Europe and between 300,000 and 400,000 people in the United States die from SCD-related pathologies. The main causes include advanced coronary atherosclerosis, acute myocardial infarction, arrhythmias, cardiomyopathies, coronary artery spasms, and electrolyte imbalances.

The premise of this study is based on the observation that many cases of acute myocardial infarction remain undiagnosed, although this diagnosis should be commonly established. This situation may be explained by the fact that early myocardial infarction can be missed as a diagnosis if the patient did not survive long enough for microscopic and, later, macroscopic changes to become evident.

The purpose of this study is to investigate the pathological background of patients who died from sudden cardiac death and to perform a descriptive statistical analysis of the encountered cases.

Objectives:

- Perform a statistical analysis of the causes of death recorded between 2021-2023 using the database of the Maramureș County Forensic Medicine Service in the context of SCD cases.
- Conduct a statistical analysis of microscopic diagnoses in SCD cases to identify patterns and their frequency.

5.2 Materials and Methods

Data Collection and Inclusion Criteria

This was a descriptive observational study that investigated the cardiac pathological causes of death and the histopathological diagnosis associated with SCD between 2021-2023. The cases analyzed came from various regions of Maramureș County, Romania, and included all cases of sudden cardiac death reported during this period.

Exclusion criteria focused on cases where toxicological examinations revealed the presence of substances of abuse or acute intoxications, as well as cases where another cause of death was identified through histopathological examination, initially attributed to SCD. These cases were excluded from the study, ensuring the inclusion of only true SCD cases, which allowed for a precise evaluation of the cardiac pathological background associated with SCD.

Sample Size

A total of 1,186 autopsies were conducted between 2021-2023 at the Maramureș County Forensic Medicine Service. The number of autopsies per year was as follows: 417 in 2021, 394 in 2022, and 375 in 2023. Of these, 126 cases in 2021, 89 cases in 2022, and 61 cases in 2023 were identified as sudden cardiac deaths. Thus, the study sample consisted of 276 cases of sudden cardiac death, which formed the database for this study.

Histopathological Examination

Each case included in the study underwent detailed microscopic histopathological examination of myocardial tissue, with some cases including multiple tissue fragments collected from different areas of the myocardium. Most slides examined were from the left ventricle.

Microscopic histopathological diagnoses were classified into five distinct groups to facilitate statistical analysis:

1. Myocardial lesions: Includes cases of acute coronary thrombosis (indirect myocardial lesion), acute myocardial infarction, organizing myocardial infarction (connective tissue formation), and old myocardial infarction (scarring).
2. Advanced myocardial fibrosis (AMF): Includes all cases where advanced myocardial fibrosis was observed.
3. Lipomatosis: Characterized by the abnormal accumulation of adipose cells within the myocardial tissue.
4. Coronary atherosclerosis: Includes cases with advanced coronary atherosclerosis.
5. Cardiomyopathy: Includes cases of dilated and hypertrophic cardiomyopathy.

The final diagnoses of the forensic reports (2021-2023) in cases of sudden cardiac death encountered in the forensic laboratory fell into one of six categories. These diagnoses were supported by microscopic histopathological examinations and are among the most commonly encountered in cases of SCD:

1. Acute myocardial infarction (AMI): Histological changes appear between 6 and 12 hours after the event, with the first change being coagulative necrosis. After necrosis, a neutrophil influx is observed between 12-24 hours. Karyolysis occurs between days 1

and 3, phagocytosis by macrophages between days 3 and 7, and granulation tissue formation at the edges occurs thereafter (12).

2. Advanced myocardial fibrosis (AMF): This diagnosis is often one of exclusion, used when no other cardiac pathology can explain the cause of death. Myocardial fibrosis occurs in a multitude of cardiac pathologies of ischemic or non-ischemic origin, such as hypertension, dilated cardiomyopathy, coronary atherosclerosis, myocarditis, etc. (32). Pathophysiologically, advanced fibrosis can lead to ventricular arrhythmia, which can result in death (33, 34). AMF is characterized by increased myofibroblast activity with collagen deposition in the extracellular matrix (35).
3. Old myocardial infarction (old MI): Complete formation of the myocardial scar takes about 2 months. Most often, this cardiac pathology leads to death through the occurrence of ventricular arrhythmia. Old myocardial infarction is also associated with post-myocardial infarction dilated cardiomyopathy (12).
4. Dilated cardiomyopathy (DCM): This includes dilation of the atria and ventricles, along with other pathological changes associated with this condition (36).
5. Cardiac hypertrophy (CH): Macroscopically, left ventricular wall thickening is observed in most cases. The causes of left ventricular hypertrophy are multiple: hypertension, valvular pathologies, amyloidosis, etc. Microscopically, enlarged nuclei with disorganization of hypertrophic myocyte architecture, which appear branched and may be accompanied by a variable amount of interstitial fibrosis, are observed (37).
6. Coronary atherosclerosis (CAD): This category includes narrowing of the coronary vessels due to atherosclerotic plaques, which can lead to reduced blood flow and, ultimately, sudden cardiac death (38).

Statistical Analysis

The data was entered into Excel tables and analyzed using descriptive statistics and other analytical techniques. The Chi-square test was used to evaluate statistical differences between various groups, which was particularly useful in analyzing categorical data, such as differences in death diagnoses by gender (39). Additionally, the one-way ANOVA test was used to examine age differences between various death diagnoses, helping determine whether age variations significantly impacted the prevalence of different cardiac pathologies (40).

Data Interpretation

The results were presented through tables and graphical representations, allowing for a clear interpretation of death diagnoses in SCD and associated microscopic diagnoses. This approach provided a deeper understanding of the pathological background and trends associated with SCD cases in the studied population.

5.3 Results

During the years 2021, 2022, and 2023, the Maramureș County Forensic Medicine Service performed a total of 1,186 autopsies, including both traumatic and non-traumatic deaths. Of these, 276 cases were attributed to sudden cardiac death. Approximately 23% of all autopsies, including both traumatic and non-traumatic deaths, were due to SCD (Figure 5.1). A second graphical representation illustrates the proportions between the total number of autopsies performed and those attributed to SCD in the years 2021, 2022, and 2023.

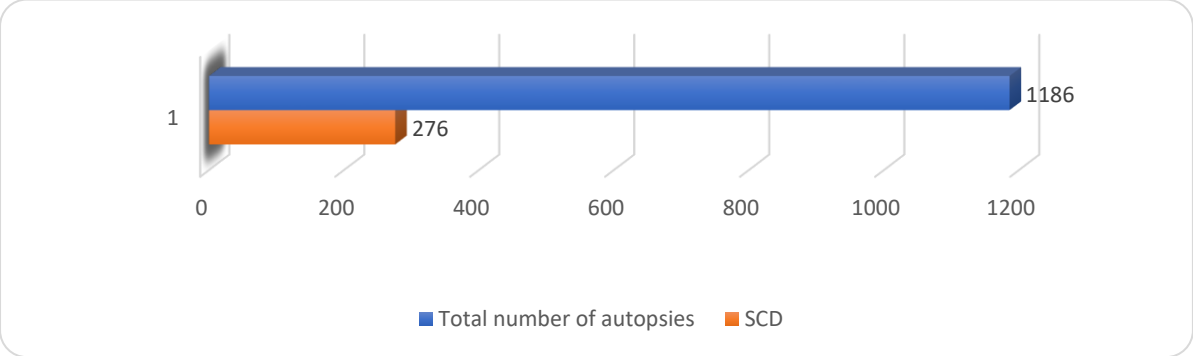

Figure 5.1: showing the total number of autopsies (blue) and sudden cardiac deaths (SCD) (red) during the study period from 2021 to 2023. Let me know if you need any adjustments or further details!

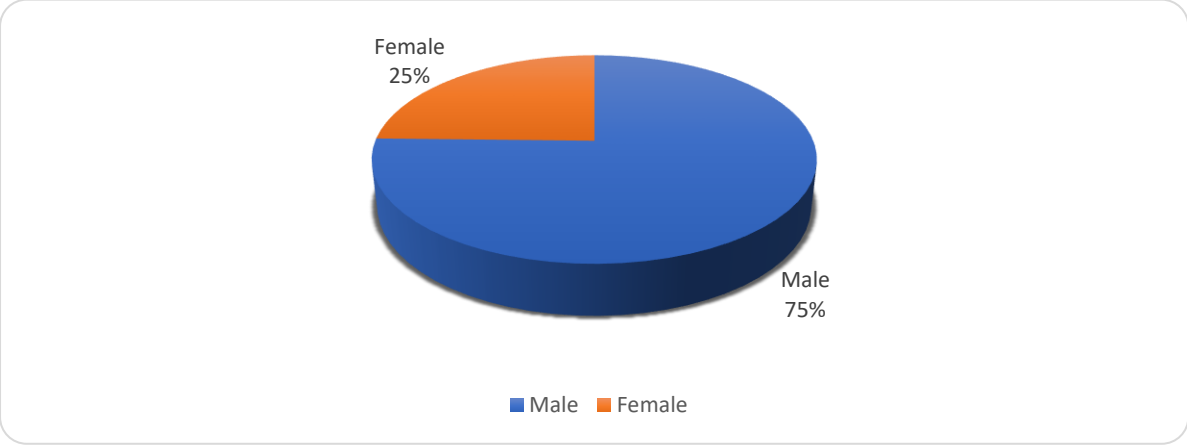

Figure 5.2: showing the gender distribution in sudden cardiac death (SCD) cases over the study period from 2021 to 2023. The distribution remains consistent, with approximately 75% of cases being male (blue) and 25% female (red) each year.

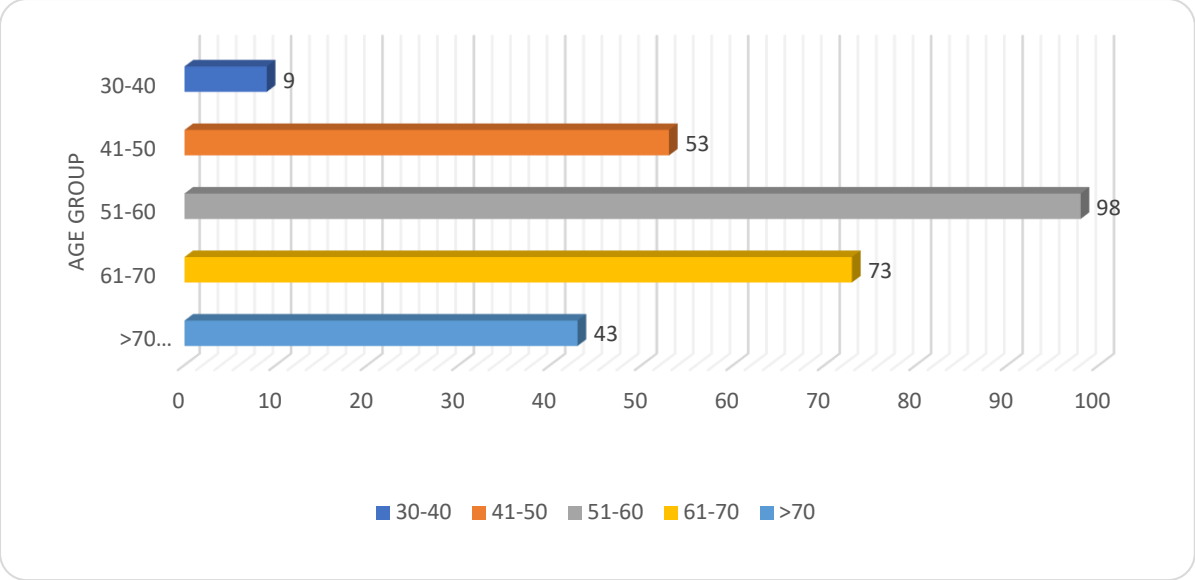

Figure 5.3: showing the number of sudden cardiac death (SCD) cases for each age group.

Table 5.1: Frequencies of different SCD causes per each age range.

Age ranges	AMF	AMI	CAD	CH	DCM	OLD MI	Total
>70	22 (51%)	5 (11.65%)	6 (14%)	5 (11.65%)	3 (7%)	2 (4.7%)	43
30-40	2 (22.3%)	4 (44.4%)	1 (11.1%)	1 (11.11%)	0 (0%)	1 (11.1%)	9
41-50	21 (39.6%)	8 (15.1%)	9 (17%)	5 (9.4%)	10 (18.9%)	0 (0%)	53
51-60	38 (39%)	19 (19.2%)	20 (20.3%)	7 (7.2%)	12 (12.2%)	2 (2.1%)	98
61-70	32 (43.8%)	12 (16.4%)	14 (19.2%)	4 (5.5%)	10 (13.7%)	1 (1.4%)	73
Total	**115**	**48**	**50**	**22**	**35**	**6**	**276**

The % represents the percentage of each diagnosis in each age group.

Table.5.1 provides a breakdown of the frequencies of different causes of sudden cardiac death (SCD) across various age ranges. In the dataset, the most common cause of SCD for the >70 age group is AMF (22 cases), which significantly contributes to the total of 43 cases in that age range For individuals aged 51-60, AMF also predominates with 38 cases. Notably, the incidence of AMI and CAD shows a rising trend with increasing age, while DCM becomes

more significant in the older age ranges. The data indicates a shift in predominant SCD causes as age increases, with AMF being more prevalent among the older age groups. The total counts show that AMF is the most frequent cause overall, with 115 cases out of 276. The analysis highlights the importance of considering age-specific risk factors and prevention strategies for AMF, particularly in older populations where its impact is most pronounced. Figure.5 shows the frequencies of different SCD causes per each year.

Table 5.2: Comparative Analysis of Sudden Cardiac Death Causes by Gender and Age

		Gender			
Diagnosis	*Values*	Males	Females	Total	P value
AMF	Frequency per gender (%)	38.46%	51.47%	41.67 %	0.13
	Age (M±SD)	58.4±10.4	64±11	60±10.9	0.4**
AMI	Frequency per gender (%)	20.19%	8.82%	17.39 %	0.045***
	Age (M±SD)	56±10	59.8±10.8	56.6±10	
CAD	Frequency per gender (%)	15.87%	25.00%	18.12 %	0.09
	Age (M±SD)	57.2±9.6	62.2±11	58.9±11.3	
CH	Frequency per gender (%)	9.62%	2.94%	7.97 %	0.12
	Age (M±SD)	58.75±11.7	67±18.4	59.5±12	
DCM	Frequency per gender (%)	13.46%	10.29%	12.68 %	0.53
	Age (M±SD)	55.35±8.9	63±14.3	56.9±10.5	

OLD MI	Frequency per gender (%)	2.40%	1.47%	2.17 %	0.39
	Age (M±SD)	55.6±13.6	72	58.3± 13.9	

** P value from one way ANOVA showing no significant differences in the mean ages across the six groups (AMF, AMI, CAD, CH, DCM, and OLD MI)

*** Indicates significant difference between males and females regarding the frequency of AMI in favor of males.

Table 5.2 presents a comparative analysis of sudden cardiac death based on sex and age. The data show that men experience a higher frequency of acute myocardial infarction (AMI) compared to women, with a significant difference observed (20.19% in men vs. 8.82% in women, $p = 0.045$). Other causes of SCD (cardiac hypertrophy, dilated cardiomyopathy, and scarring myocardial infarction) showed higher differences in men compared to women, but without statistical significance. Conversely, women show a higher frequency of coronary atherosclerosis (CAS) (25.00%) compared to men (15.87%) and a higher frequency of advanced myocardial fibrosis (AMF) (51.47%) compared to men (38.46%), although this difference is not statistically significant ($p = 0.09$).

The average age at which each condition occurs varies, with no significant differences found in the average age between groups ($p = 0.4$), as determined by the one-way ANOVA test. Despite these variations, the general trend indicates that both sexes are affected by different causes of sudden cardiac death at different ages, with AMI being more common in men and CAS more frequent in women.

5.4 Discussion

Sudden cardiac death (SCD) is defined as death presumed to be of cardiac origin that occurs within 1 hour of the onset of cardiac symptoms or within 24 hours from the last time the person was seen in apparent good health (41). Therefore, establishing universally accepted definitions would help guide a more accurate classification and facilitate more precise studies on SCD. A national protocol would allow for more accurate coding of SCD cases where medico-legal or pathoanatomical autopsies have been performed.

As observed in this study, sudden cardiac deaths (SCD) represent approximately 23% of all medico-legal autopsies performed in the region between 2021 and 2023, with a predominant

occurrence in males (75%). The studied population closely reflects the global trend of a higher male predisposition to developing pathologies leading to sudden cardiac death (42).

Advanced myocardial fibrosis (AMF) emerged as the most frequent cause of SCD, especially in older age groups, accounting for 41.6% of cases. Other significant causes include acute myocardial infarction (AMI) and coronary atherosclerosis (CAS), with AMI being much more frequent in males (20.19%) compared to females (8.82%), a difference that reached statistical significance ($p = 0.045$). The 51-60 age group showed the highest prevalence of all SCD causes. This is consistent with other studies that have reported similar findings (43-45). The second age group with the highest number of patients is the 61-70 age group.

Patients in these age groups should be evaluated for lifestyle changes, daily routine adjustments, and other factors that can be modified to reduce susceptibility to conditions that could lead to SCD. AMF was the most widespread diagnosis in all age groups, except for the 31-40 age group, where AMI had the highest prevalence (44.4%). These findings highlight the importance of considering both gender and age in understanding and preventing SCD.

Many risk factors have been identified in the context of SCD, including all risk factors for atherosclerotic cardiovascular diseases (ASCVD), left ventricular hypertrophy, and genetic pathologies. The risk of SCD has been reported to be relatively low in populations with a lower incidence of ASCVD and structural heart diseases.

According to the literature, coronary artery disease, including coronary atherosclerosis, is considered responsible for the majority of SCD cases (46). In this study, it was found that myocardial fibrosis is the leading cause of death in SCD. Myocardial fibrosis can occur independently of coronary atherosclerotic disease and represents a lesion found in many other cardiac pathologies. In this study, CAS accounted for only 18% of SCD cases. This situation can also be explained by the frequent use of myocardial fibrosis as a diagnosis of exclusion, as well as the omission of some acute myocardial infarction cases where microscopic lesions are not evident. This underdiagnosis may lead to incomplete statistics on the causes of SCD.

This histopathological controversy can be explained by several factors. If death occurs immediately after the onset of ischemia, it may not be detectable through microscopy using standard H&E staining. In addition, microinfarctions may be missed during routine autopsy, especially if they occur in scarred areas or in areas with advanced myocardial fibrosis.

SCD can also result from non-ischemic triggers, such as arrhythmias from certain genetic channelopathies. As reported in our study, the most common cause of death and microscopic diagnosis observed in autopsies of sudden cardiac death was AMF. Cardiac fibrosis is a pathological remodeling process of the extracellular matrix, which affects

myocardial function. Cardiac muscle fibrosis most commonly occurs after myocardial infarction; however, various other conditions can promote cardiac fibrosis, such as hypertensive heart disease, myocarditis, aortic stenosis, pulmonary hypertension, atrial fibrillation, diabetic hypertrophic cardiomyopathy, and idiopathic dilated cardiomyopathy (34, 47).

Ischemic heart disease involves reduced blood supply due to atherosclerosis, leading to fibrosis; chronic hypertension can strain the myocardium and lead to structural changes; cardiomyopathies are various conditions of the myocardium that affect function and lead to fibrosis; myocarditis is inflammation caused by infections or autoimmune conditions, resulting in fibrosis as the tissue heals; diabetic hypertrophic cardiomyopathy; aortic stenosis; pulmonary hypertension; atrial fibrillation; exposure to cardiotoxic agents; aging naturally leads to the accumulation of fibrous tissue; genetic disorders predispose individuals to fibrosis due to mutations affecting cardiac muscle proteins. This diagnosis of advanced myocardial fibrosis (myocardial sclerosis) is frequently used in cases of SCD in Romania.

It is well established that atherosclerosis is the leading cause of death in cardiovascular diseases and that myocardial infarction most commonly occurs as a complication of coronary atherosclerosis (48, 49). However, our statistical analysis showed that the most common cause of death in cardiac and microscopic diagnoses is diffuse advanced myocardial fibrosis. It is important to emphasize that this diagnosis is one of exclusion, used only when no other cardiac pathology can explain the cause of death.

Immunohistochemistry is a method by which a more accurate diagnosis can be made in cases where myocardial ischemia, the precursor to myocardial infarction, is suspected. In cases with significant legal implications, immunohistochemistry is recommended to determine the presence and extent of ischemia in the myocardium.

Macroscopic and microscopic examinations performed in SCD cases are essential for a better understanding of the underlying cardiac pathology. Sudden cardiac death represents a major threat to anyone, due to its unpredictable nature and the difficulty of being effectively detected and prevented. These investigations significantly contribute to clarifying hidden causes and developing appropriate preventive strategies.

A multidisciplinary approach with evidence-based research is necessary to further improve diagnostic capabilities in SCD cases. It is also widely accepted that genetic factors play a key role in the incidence of SCD. Therefore, evaluating the genetic basis of sudden cardiac deaths with the help of genetics laboratories becomes a necessity (50-52).

Although this study provides valuable insights into the epidemiology and pathology of SCD in Maramureș County, Romania, the study has limitations. The histopathology laboratory at the Maramureș County Forensic Medicine Service lacks the infrastructure to perform immunohistochemistry techniques. This limitation may lead to the underdiagnosis of myocardial infarctions. Implementing immunohistochemistry techniques would significantly improve diagnostic accuracy in such cases. Additionally, the study does not take into account the presence of comorbidities or other factors that may contribute to SCD. Including these data would allow for a more detailed and conclusive analysis, offering a more comprehensive perspective on the multiple causes that may influence SCD. The sample size, although significant, is limited to a specific geographic area and time frame, which may affect the generalizability of the results to other regions. Moreover, human factors may play an important role in diagnosing SCD, with professional experience influencing the accuracy of the final diagnosis. Therefore, standardizing procedures at the national level would minimize the impact of human factors in diagnosing SCD.

5.5 Conclusion

As observed in the statistical analysis conducted in the region, there is a trend consistent with other studies indicating that the male population is more affected by SCD, particularly in the 50-60 age group. A new perspective brought by this study relates to the frequent use of AMF as the primary cause of death, which may result from the limitations of microscopic histopathological examination using standard H&E staining. This highlights the need for improved diagnostic protocols, including the integration of specific immunohistochemical markers for ischemia, with the aim of increasing the accuracy of postmortem investigations.
Given the complex nature of SCD, it is vital to develop standardized protocols that involve both immunohistochemical and genetic techniques. Such an approach could provide more accurate diagnoses and a better understanding of the underlying mechanisms of SCD.

The ultimate goal of this research is not only to improve the accuracy of SCD diagnoses but also to pave the way for better preventive strategies. This research represents a call for the integration of immunohistochemistry into microscopic histopathological diagnostics for a more precise evaluation of SCD pathology.

6. STUDY II: New Perspectives on the Expression of Immunohistochemical Markers in Post-Mortem Recognition of Myocardial Infarction

6.1 Working Hypothesis and Specific Objectives

Immunohistochemistry plays a crucial role in detecting myocardial infarction through the use of specific markers in the histological analysis of cardiomyocytes. These markers allow the identification of molecular and structural changes that occur in ischemic cardiomyocytes or following the onset of necrosis. By highlighting specific proteins associated with necrosis and cellular inflammation, immunohistochemistry offers an accurate tool for early diagnosis and evaluation of the extent of myocardial lesions, thus contributing to diagnostic accuracy.

The premise of this study was established due to the difficulty encountered in identifying the specificities of immunohistochemical (IHC) markers in the specialized literature. An updated synthesis of markers, based on their sensitivity and specificity, is essential for selecting the appropriate marker according to the specific characteristics of each case. This issue is amplified by the fragmented nature of available information in the literature, complicating the process of diagnosing and assessing myocardial ischemia in various pathological contexts. The lack of a coherent synthesis of data regarding specific markers for post-mortem myocardial ischemia makes it difficult to apply clear protocols in forensic and pathoanatomical practice. This highlights the need for more extensive and detailed studies to clarify the role of immunohistochemical markers. A better understanding of these variables would not only allow for a more accurate evaluation of acute myocardial infarction (AMI) but also help develop more efficient diagnostic methodologies tailored to each case in post-mortem diagnosis.

Ongoing advances in the use of immunohistochemical markers for diagnosing ischemia in cardiomyocytes led us to conduct a systematic review to provide a detailed overview of the markers used to detect myocardial ischemia.

The goal of this study is not only to present the most relevant immunohistochemical markers but also to classify them based on the mechanism involved and/or their location in cardiomyocytes. The data has been synthesized into an accessible table that provides a clear picture of the expression of markers based on their location, the time they become detectable after the onset of ischemia, and the main characteristics of each marker, including specificity. The table aims to assist pathologists and forensic professionals in selecting the appropriate markers for diagnosing myocardial ischemia, the precursor stage to acute myocardial

infarction. Therefore, this study aims to provide a comprehensive synthesis, offering a unified perspective on myocardial ischemia markers and how to apply them in post-mortem diagnosis.

Objectives:

- Identify the direct markers used in immunohistochemistry, as well as secondary markers involved in detecting ischemia and acute myocardial infarction.
- Develop a classification of markers based on their mechanism of action.
- Create a table that includes the specific characteristics of markers used to detect ischemia and AMI.

6.2 Materials and Methods

We conducted a systematic review of published studies on immunohistochemistry, focusing on myocardial ischemia and acute myocardial infarction.

Search Strategy

We searched databases such as PubMed, Scopus, Science Direct, American Chemical Society, Springer, and Wiley using keywords such as "Acute Myocardial Infarction," "Ischemia," "Hypoxia," "Forensic Medicine," "WHO," "Immunohistochemical Detection," "Forensic Pathology," "Markers," "C5b-9," "Troponin," "CK-MB," "GBPP," and "HIF." We included studies from the last 10-15 years.

The full text of relevant studies was evaluated, and we identified more than 25 immunohistochemical markers used to detect myocardial ischemia and acute myocardial infarction.

6.3 Results

Following a systematic review, several markers associated with myocardial ischemia were identified. These markers include proteins and other molecules directly involved in the cellular response mechanisms to ischemic and necrotic events.

In Table 6.1, the markers were classified according to their mechanism of action, providing a clear understanding of the specific role they play in the pathological cascade associated with myocardial ischemia. An important aspect highlighted was the reaction time of each marker from the onset of ischemia, as well as how they manifest positive expression or depletion at the cytoplasmic, membranous, or nuclear levels. The specific characteristics of each marker are also relevant from a morphopathological perspective.

This information allows for a better understanding of the temporal and spatial dynamics of markers in the context of myocardial ischemia, providing a solid foundation for assessing their diagnostic potential.

Table 6.1: Classification and Characteristics of Immunohistochemical Markers Used for Post-Mortem Recognition of Myocardial Ischemia and Acute Myocardial Infarction (2).

MARKER	EARLY DETECTION OBSERVED IN STUDIES	MARKER EXPRESSION	CHARACTERISTICS
Complement and Inflammation Markers			
C5b-9 Complex	40 minutes	Positive IHC staining	- High sensitivity in detecting ischemic/necrotic cardiomyocytes among viable ones - Resuscitation does not affect C5b-9 complex expression - Resistant to post-mortem decomposition (53)
C1, C3, C8, and C9	2 hours (animal study)	Positive IHC staining	- Earliest detection of complement C3 in experimental animal studies (54)
Inflammatory Mediator Markers			
Galectin-3	20 minutes	Positive IHC staining	- Cardiomyocytes and endothelial cells express GAL-3 during ischemic events - After 24 hours post-myocardial infarction, neutrophils express GAL-3
IL-1β, IL-6, IL-15, IL-8, ST-2 Family	4 hours	Positive IHC staining	- 0-6 hours: mild positivity for IL-1β, IL-6, IL-8, and intense IL-15 reaction in the infarction area (55)
TNF-alpha	4 hours	Positive IHC staining	- Not specific to myocardial infarction; TNF-α production can be promoted by ischemia, reperfusion, cardiac surgery, and chronic heart failure (56)

CD15, CD18, and Tryptase	4 hours	Positive IHC staining	- 0-6 hours: mild positivity for CD15, CD18, and tryptase - 6-8 hours: strong immune response visible in the infarct area (55)
Cardiac Cellular Proteins			
Heart-type Fatty Acid-Binding Protein (H-FABP)	15 minutes	Loss of IHC staining	- H-FABP expression loss reaches a peak after four hours of myocardial ischemia onset - No consensus on whether H-FABP is affected by autolysis; studies show varying conclusions (57, 58)
Desmin, alpha-actinin, and Vinculin	1 hour	Loss of IHC staining	- Suitable markers, but not resistant to autolysis/putrefaction; useful up to 1-2 days post-mortem (54, 59)
Cardiac Troponins (CTns)	1 hour	Loss of IHC staining	- Very specific to myocardial injury, released from cardiomyocytes only during irreversible cell death (60-63)
Creatine Kinase MM, BB, and MB	-	Loss of IHC staining	- CK-MB is widely used as an ischemia marker - CK-MB loss is more evident at the periphery than in the center of the infarct (63, 64)
Cx43	-	Loss of IHC staining	- Quickly loses expression after ischemia onset - mRNA levels of these proteins can be markers for ventricular arrhythmias (65)
ZO-1	-	Positive IHC staining	- Marker of myocardial ischemia (65)

Natriuretic Peptides	-	Positive IHC staining	- Not specific, elevated in other myopathies - BNP and NT-proBNP are resistant to post-mortem decomposition and may be objective biomarkers of cardiac function (66, 67)
Myoglobin	3 hours	Loss of IHC staining	- One of the most reliable biomarkers for diagnosing myocardial infarction - IHC expression can be used to detect infarction up to 2-3 days post-mortem (59, 63)
Plasma Proteins and Myocardial Necrosis Markers			
Fibronectin	2 hours	Positive IHC staining	- Not specific, can also be seen in myocarditis - Advanced putrefaction does not affect fibronectin staining (25)
S100A1	15 minutes (animal study)	Loss of IHC staining	- Ischemic cardiomyocytes show depletion of S100A1 (26)
Hypoxia Markers			
HIF and HIF1-alpha	2 hours	Positive IHC staining	- Expressed during ischemia due to oxygen deficiency (68)
Galectin-1	20 minutes (animal study)	Loss of IHC staining	- Significant role as a diagnostic marker in early myocardial infarction - Cells surviving in the ischemic area, located at the infarct zone's edge, show increased GAL-1 expression (28)
Plaque Destabilization Markers			

Matrix Metalloproteinases	-	Positive IHC staining	- Immunopositivity for MMP-9 mainly observed in polymorphonuclear endovascular cells; indirect marker of early myocardial ischemia (69-71)
Myeloperoxidase	-	Positive IHC staining	- MPO is an enzyme found in leukocytes and is released during myocardial infarction - Low specificity, MPO release is not exclusive to myocardial infarction (72)
Pregnancy-Associated Plasma Protein A (PAPP-A)	-	Positive IHC staining	- Detectable in blood and cardiac tissue for days after myocardial infarction - Not specific, may be elevated in other myopathies (73, 74)
Other Markers			
Apoptosis Markers	-	Positive IHC staining	- Proteins regulating apoptosis, such as Bcl-2 and Caspase-3, have been used in recent myocardial infarction, but they are not specific (30, 31, 75)

6.4 Discussions

In this study, over 25 immunohistochemical markers were identified and analyzed, each being evaluated for their specificity and sensitivity in detecting myocardial ischemia and acute myocardial infarction. Each marker was individually assessed and subsequently classified based on the pathological mechanisms involved and/or their specific location in cardiomyocytes. This classification aims to simplify the selection of appropriate markers in the context of myocardial ischemia diagnosis, ensuring their optimal use depending on the specificities of each case. For clear and concise presentation, the study results were synthesized and organized into a table. This table provides an overview of each marker, highlighting its contribution to myocardial ischemia evaluation.

6.5 Conclusions

We discussed the main types of diagnostic markers that can be used in the form of antibodies for immunohistochemical staining of myocardial tissue sections embedded in paraffin blocks. Given the challenges of post-mortem diagnosis of acute myocardial infarction (AMI), especially in the absence of necrosis and visible cellular lesions, it is essential to identify relevant markers that can be detected in the early stages of ischemic events before necrosis occurs. Among the essential markers specific to acute myocardial infarction are cardiac troponins, which are a sensitive and specific indicator of myocardial injury, as well as complement components, especially the C5b-9 complex, which reflects immune cascade activation. Additionally, natriuretic peptides play a significant role in evaluating cardiac dysfunction and are frequently used to assess hemodynamic stress. Alongside these, markers associated with ischemia provide valuable information regarding the presence and severity of ischemic events.

However, some of these markers have notable limitations. For instance, in cases where autolysis is advanced, the accuracy of their detection and interpretation can be significantly reduced, limiting their diagnostic utility post-mortem. To enhance diagnostic accuracy and reduce the risk of errors, it is recommended to use these markers in a combination of at least three or four. This approach can provide a more comprehensive evaluation of cardiac pathologies, with a special focus on early myocardial infarction detection.

7. STUDY III: Immunohistochemical Study of ATP1A3 and Plakophilin 2 as Potential New Markers in the Diagnosis of Myocardial Ischemia

7.1 Working Hypothesis and Specific Objectives

In cases where an individual dies within the first 6 hours of the onset of myocardial ischemia, the precursor to acute myocardial infarction, histological changes are not visible microscopically using standard hematoxylin-eosin staining.

Some of the most reliable and frequently used ischemia markers include the C5b-9 complex, fibronectin, and cardiac troponins (2).

Gap junction proteins, ion channel proteins, transporter proteins, as well as those involved in signaling and regulation, play a crucial role in maintaining the integrity and efficient functioning of cardiomyocytes. These studies emphasize the complexity of the mechanisms involved at the level of intercalated discs.

PKP2 is a plakophilin protein class with cell adhesion properties. In addition to their structural role in desmosomes, plakophilins are involved in various intracellular signaling and regulatory processes (24, 25).

ATP1A3 has been identified in the intercalated discs and intracellularly, and animal models have confirmed its presence in the sarcolemma of cardiomyocytes. ATP1A3 is a subfamily of the Na+/K+-ATPase and is an integral membrane protein responsible for establishing and maintaining the electrochemical gradients of sodium (Na) and potassium (K) ions across the plasma membrane. It is primarily expressed in the brain and cardiomyocytes. Recent studies have highlighted its complex role in cardiac and neural pathologies (26-30). ATP1A3 plays a role in the process of neuronal autophagy, "autosis," induced by hypoxia (76).

The premise of this study is based on the observation that the available immunohistochemical markers for detecting ischemia and myocardial infarction do not provide a sufficient level of sensitivity and specificity. Detecting myocardial ischemia in cardiomyocytes as early as possible after its onset remains a challenge in forensic medicine.

In this context, identifying and validating new immunohistochemical markers for detecting ischemia, the precursor to AMI, is a priority in forensic medicine.

The aim of this study was to discover new immunohistochemical markers for myocardial ischemia with increased specificity and sensitivity that would be of real use in pathoanatomical and forensic practice. For this purpose, two proteins localized at the intercalated discs, namely PKP2 and ATP1A3, were chosen.

The objectives of this study were to identify the possibility that the expression and distribution of the PKP2 and ATP1A3 markers might be influenced in the context of myocardial ischemia, the precursor to acute myocardial infarction. The distribution and expression levels of PKP2 and ATP1A3 were compared with those of the C5b-9 complex to evaluate the potential of these markers to provide results consistent with a known ischemia marker.

7.2 Materials and Methods

For this retrospective study, we had access to death certificates and histopathological examinations of 1,184 autopsied cases in the forensic medicine department, from 2020 to 2023. From this sample, 85 cases were selected, and paraffin blocks and histopathological sections stained with H&E of myocardial tissue and coronary arteries were extracted from the histological archive. Tissue samples were predominantly taken from the left ventricle, and the number of H&E sections examined varied between cases.

Inclusion criteria allowed for the selection of cases regardless of age, sex, comorbidities, or whether resuscitation was performed. Exclusion criteria targeted cases where autopsies were performed more than 72 hours after death and cases showing signs of autolysis in the histopathological sections stained with H&E, although they fell within the 72-hour interval. Additionally, cases where resuscitation was initially successful, followed by a variable survival period, were excluded.

Thus, the 85 cases were divided into four groups based on the post-mortem cause of death determined from the forensic reports. In determining the cause of death, investigation data, negative toxicological findings, macroscopic and/or microscopic examinations performed on hematoxylin and eosin sections were considered.

In the first group, 10 cases were selected where the cause of death was attributed to AMI. In each of these 10 cases, histopathological examination in H&E staining confirmed acute myocardial infarction. Myocardial sections stained with H&E revealed areas of coagulative necrosis, band necrosis, karyolysis, along with neutrophil polymorphonuclear infiltration.

The second group included 25 cases of sudden cardiac death attributed to acute coronary thrombosis, CAS, old myocardial infarction, DCM, CH, and AMF. For all these cases, microscopic histopathological examination did not show signs of myocardial ischemia in the H&E-stained sections of myocardial tissue.

The third group consisted of 25 cases where the cause of death was attributed to mechanical asphyxia by hanging. In this group, histopathological macroscopic and/or microscopic examination revealed one of the following conditions: coronary atherosclerosis, old myocardial infarction, cardiac hypertrophy, dilated cardiomyopathy, and advanced myocardial fibrosis. Microscopic histopathological examination did not reveal signs of myocardial ischemia in H&E staining.

The fourth group included 25 cases involving myocardial tissue from individuals who died from acute mechanical asphyxia. These cases showed no histological pathology at the macroscopic and/or microscopic level, with no signs of ischemia visible microscopically.

All H&E-stained histopathological sections from the 85 cases were re-examined by an experienced pathologist. The primary focus was on identifying signs of myocardial ischemia.

Immunohistochemistry

The ATP1A3, Plakophilin 2, and C5b-9 sections were stained using an automated system (DAKO Autostainer Plus). The process included deparaffinization, rehydration, and epitope extraction using the PT-link module (10 minutes at 97°C).

The same pathologist carefully examined the immunohistochemically stained slides using the ATP1A3, PKP2, and C5b-9 markers from the 85 cases. The examination was carried out at different magnifications (10x, 20x, 40x, and 100x with immersion). Marker expression and distribution were carefully examined, including histopathological changes at the intercalated discs, nucleus, and cytoplasm.

Table 7.1: Investigated Antibodies and Grading Scale

Marker	Grade 0	Grade 1	Grade 2
ATP1A3	A uniform positive expression was observed, with minimal exceptions in the subendocardial region and papillary muscles.	A slight loss of marker expression.	Loss of marker expression in cell groups, presenting a mosaic-like appearance.
PKP2	Positive marker expression at the level of intercalated discs.	Displays a slight loss in marker expression intensity at the	Very weak or absent marker expression compared to an internal standard of normal myocardium.

		level of intercalated discs.	
C5b-9 Complex	Negative, no marker expression.	Reduced positivity, indicating minimal marker presence.	Intense and/or diffuse positivity over a larger area, demonstrating widespread or strong marker expression in the cytoplasm of cardiomyocytes.

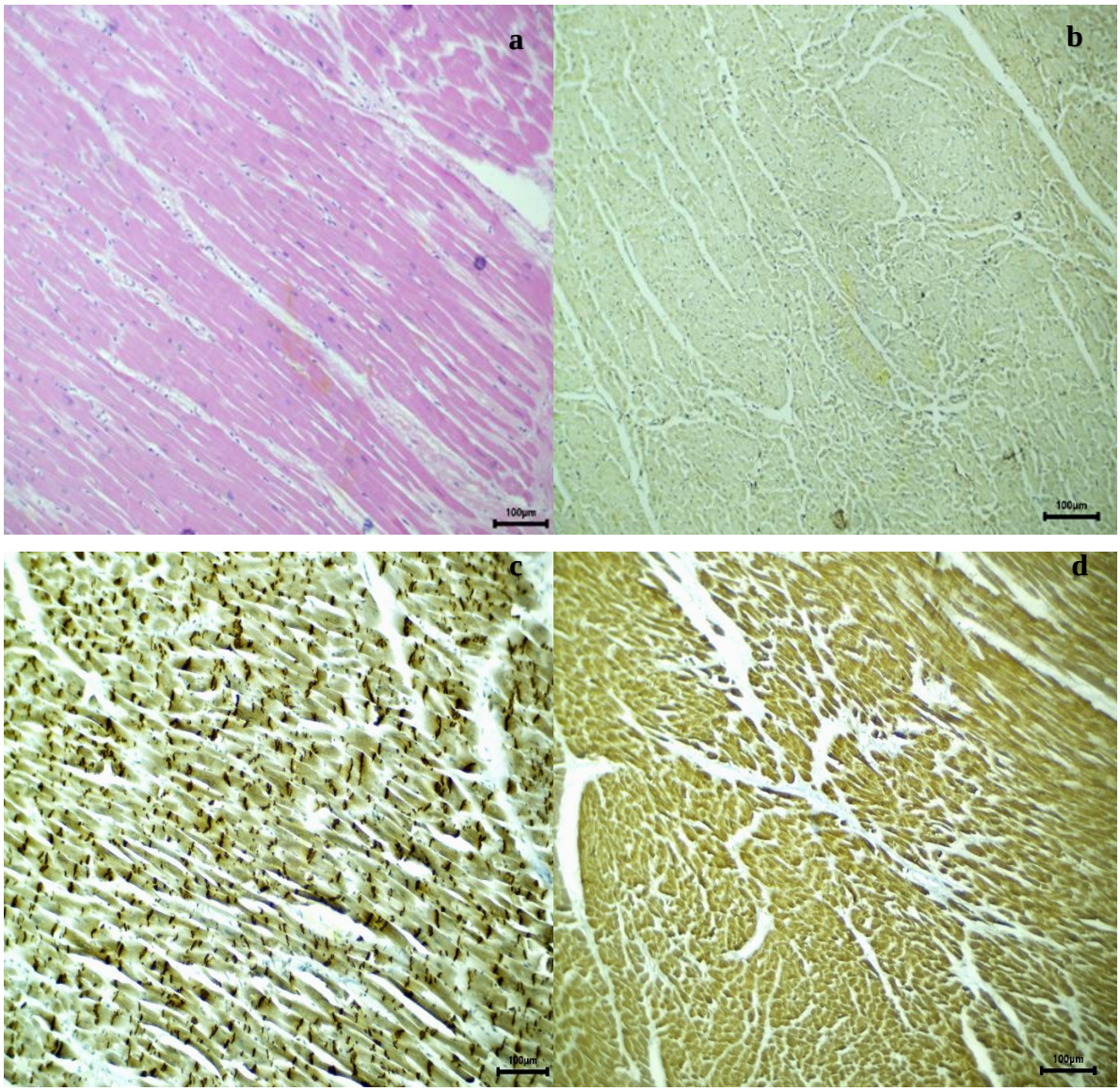

Figure 1: Grade 0 semiquantitative grading scale same histological slid representation for H&E and IHC, (20x), scale bar 100 µm: (a) H&E staining cardiomyocytes in a longitudinal section with no signs of ischemia; (b) C5b-9 complex negative staining; (c) PKP2 highly

expressed at the intercalated discs; (d) ATP1A3 uniform pattern of staining with no depletion of the marker.

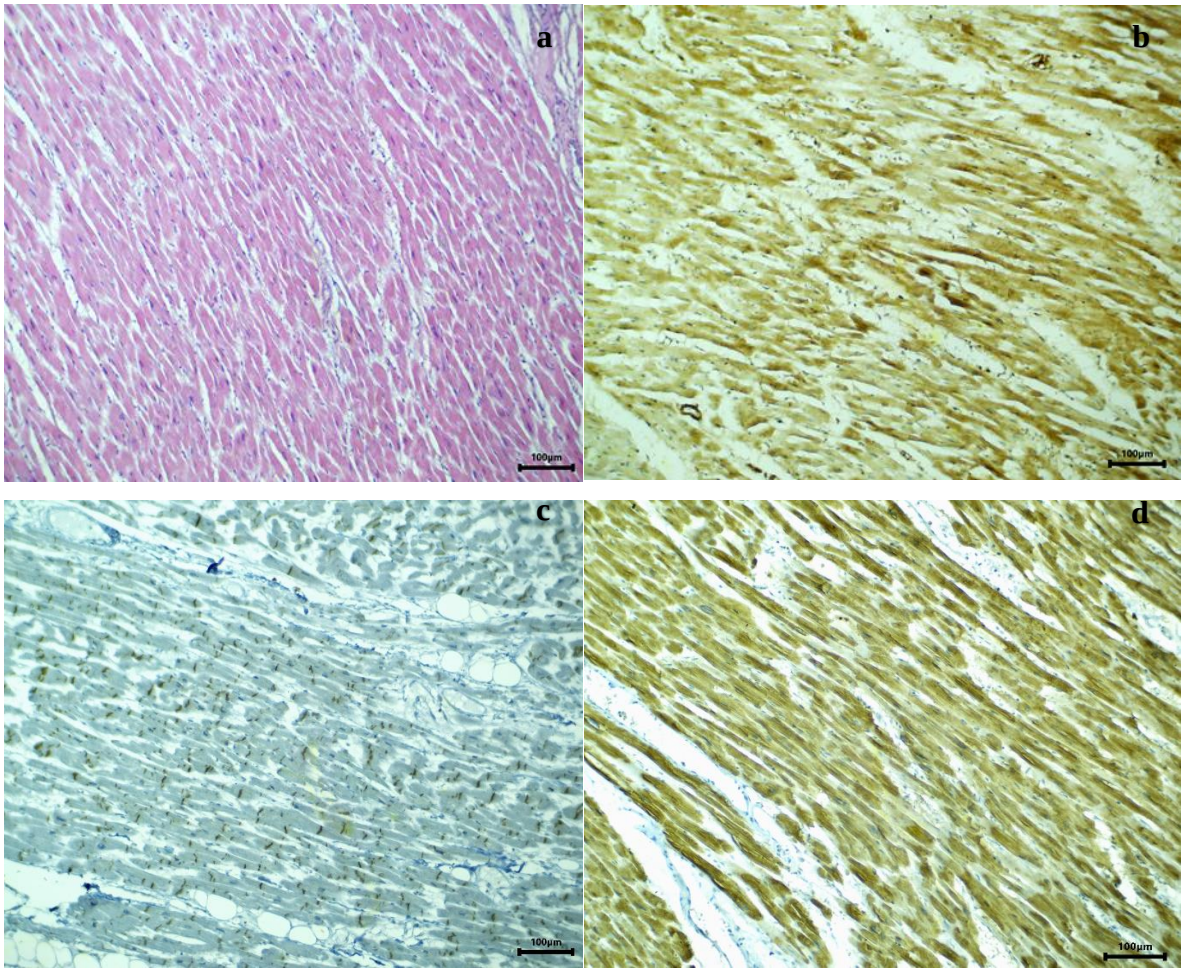

Figure 2: Grade 1 semiquantitative grading scale same histological slid representation for H&E and IHC, (20x), scale bar 100 µm: (a) H&E staining cardiomyocytes in a longitudinal section with no signs of ischemia; (b) C5b-9 complex slight positivity, reflecting the presence of the marker; (c) PKP2 weak staining at the intercalated discs in the ischemic region; (d) ATP1A3 a slight loss of staining intracellularly and the intercalated discs observed in the group of cells.

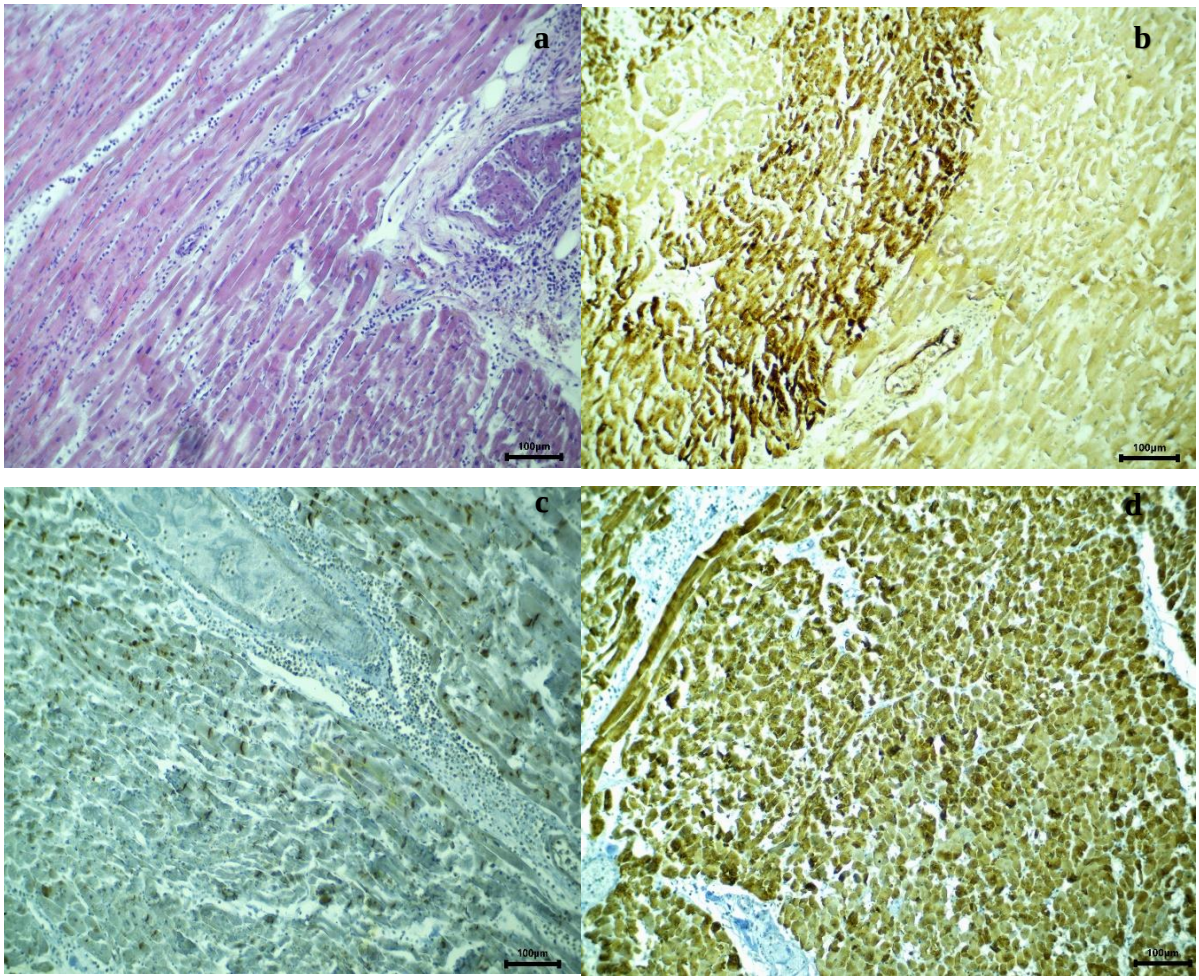

Figure 3: Grade 2 semiquantitative grading scale with same histological slid representation for H&E and IHC, (20x), scale bar 100 µm: (a) H&E coagulative necrosis of the myocytes is observed, characterized by absent nuclei, along with interstitial neutrophilic infiltrate. Additionally, a blood vessel shows evidence of thrombosis; (b) C5b-9 complex positive staining shows an abrupt border between vital tissue and necrotic areas; (c) PKP2 shows very weak and absent staining of the intercalated discs in the infarcted area; (d) ATP1A3 mosaic-like staining in group cells.

Table 7.2: Frequencies of staining grades (0, 1, and 2) using the three markers in each group.

Marker		**Group 1 (n = 10)**	**Group 2 (n = 25)**	**Group 3 (n = 25)**	**Group 4 (n = 25)**
	Grade 0	0%	12%	76%	88%
	Grade 1	10%	36%	16%	8%

ATP1A3 (%)	**Grade 2**	90%	52%	8%	4%
PKP2 (%)	**Grade 0**	30%	64%	96%	96%
	Grade 1	50%	16%	4%	4%
	Grade 2	20%	20%	0%	0%
C5b-9 (%)	**Grade 0**	0%	44%	92%	96%
	Grade 1	10%	32%	8%	4%
	Grade 2	90%	24%	0%	0%

Table 7.3: Results of the Pearson Correlation Test

Group	Marker 1	Marker 2	Pearson Correlation Coefficient	p-value	Relationship	Statistical Significance
Group 1 (n = 10)	PKP2	C5b-9	-0.063	0.8627	Very weak negative correlation, no relationship between markers	Not statistically significant
Group 1 (n = 10)	ATP1A3	C5b-9	1.0	6.65e-64	Strong positive correlation, markers provide information about myocardial infarction	Statistically significant
Group 2 (n = 25)	PKP2	C5b-9	0.184	0.3784	Weak positive correlation, no significant relationship between markers	Not statistically significant
Group 2 (n = 25)	ATP1A3	C5b-9	0.674	0.00022	Strong positive correlation, markers provide relevant information	Statistically significant

Group 3 (n = 25)	PKP2	C5b-9	-0.060	0.7750	Very weak negative correlation, no relationship between markers	Not statistically significant
Group 3 (n = 25)	ATP1A3	C5b-9	0.548	0.0046	Moderate positive correlation, markers suggest a significant relationship	Statistically significant
Group 4 (n = 25)	PKP2	C5b-9	-0.042	0.8432	Very weak negative correlation, no relationship between markers	Not statistically significant
Group 4 (n = 25)	ATP1A3	C5b-9	0.602	0.0015	Strong positive correlation, markers provide relevant information	Statistically significant

7.4 Discussions

The aim of this study was to evaluate the potential of ATP1A3 and PKP2 proteins as specific markers for myocardial ischemia. To achieve this, the cases were divided into four distinct groups.

The first group included 10 cases of acute myocardial infarction (AMI), confirmed as the cause of death. This group was selected because it is known that IHC marker expression may vary depending on the time elapsed since the onset of ischemia. Certain markers show positive expression in the early stages or undergo rapid depletion, while others become detectable only in the later stages of ischemia or after necrosis has set in (54). This group was used to evaluate changes in the expression or distribution of the two new markers in the early stages of myocardial infarction. If no significant changes were observed, it could be concluded that these markers lack the sensitivity necessary to detect ischemia or the initial stages of myocardial infarction.

In all 10 cases, no collagen deposition was observed, as the collagen deposition process usually begins around one week after the onset of infarction (12, 77). It is known that on the same histopathological section examined with H&E staining, some areas may show coagulation necrosis, while other areas may present a wavy appearance of the myocytes.

For PKP2, marker expression was observed at the intercalated discs. The areas with histopathological signs of acute myocardial infarction showed weak expression (Grade 1) and very weak or absent expression (Grade 2), especially in regions with increased polymorphonuclear neutrophil infiltration and coagulation necrosis. The grading of PKP2 expression at the intercalated discs was easier to assess in longitudinal sections of the cardiomyocytes. In areas of myocardial fibrosis, evaluating the distribution and intensity of PKP2 expression becomes more difficult. H&E staining identified areas of AMI with neutrophil infiltration and cardiomyocytes showing hyper-eosinophilia, being less affected by coagulation necrosis. The same region was then analyzed on the histopathological slide stained with PKP2. The marker showed intense positive expression, classified as Grade 0, in the area of myocardial infarction. The control marker, C5b-9 complex, tested positive for ischemia and necrosis in the same areas. This pattern was consistently observed in multiple cases, leading to the conclusion that PKP2 might lose its expression later in the course of AMI.

For ATP1A3, marker expression was observed both at the intercalated discs and intracellularly (78). In the first group, depletion of the marker was observed in all cases, with most being classified as Grade 2. In this grade, transverse sections of cardiomyocytes in the area of myocardial infarction presented a mosaic pattern in cell groups, with some cardiomyocytes showing total depletion of the intracellular marker, others showing partial loss, and some appearing normal compared to an internal standard. At higher magnifications (40x and 100x with immersion), variable intracellular depletion could be seen, with histopathological changes interpreted as myofibrillar edema and rhexis. In longitudinal sections, cardiomyocytes revealed intercalated discs with variable depletion of staining and histopathological changes, such as distortions and ruptures of the intercalated discs.

The second group was chosen to assess whether the markers alter their expression in the early stage of ischemia. For this purpose, cases with a high probability of identifying myocardial ischemia using IHC but without evident histopathological changes in H&E staining were included. This group also included cases of acute coronary thrombosis and advanced coronary atherosclerosis with old myocardial infarcts, but without histopathological signs of ischemia. In some cases, acute coronary thrombosis observed during autopsy does not present signs of ischemia or infarction when examined with H&E staining. Due to the short interval between the onset of ischemia and death, histopathological signs of ischemia and necrosis, which are usually observed in H&E staining, do not have sufficient time to develop. The purpose of this study was to determine whether these markers can specifically detect early-stage ischemia rather than necrosis. This group was not included in the ROC analysis because

it was not possible to definitively classify subjects as having or not having a myocardial infarction (MI) due to the use of a single control marker, C5b-9.

The third group included cases of mechanical asphyxia (hanging) associated with myocardial pathology. The cardiac pathology associated with these cases was documented either through macroscopic examination or microscopic examination. This group was used as a control to determine whether the myocardial pathology in these cases could influence the distribution or expression of APT1A3 and PKP2 markers, and to evaluate whether the changes observed in the first and second groups are present when death occurs within minutes due to mechanical asphyxia. In cases of mechanical asphyxia, cardiac arrest usually occurs within minutes, either through asystole or pulseless electrical activity, both being non-shockable rhythms.

There were also a few cases where differences in grades for APT1A3 and the C5b-9 complex were observed. This group included only cases of mechanical asphyxia, where the individuals were found deceased at the scene. Grades 1 and 2 for APT1A3 and the C5b-9 complex in cases of asphyxia may be explained by a prolonged period of agony. It is known that different types of hanging can lead to a prolonged period of agony, which may influence certain biomarkers.

The fourth group was used as a baseline control to evaluate the markers in myocardial tissue without pathological changes observed macroscopically and microscopically in cases of mechanical asphyxia. This group served as a negative reference, contributing to the assessment of the negative predictive value of the new markers.

Changes in the expression of different immunohistochemical markers in myocardial ischemia or necrosis can be explained by various factors. The specific location and functions of each marker are determining factors. The expression of the C5b-9 complex depends primarily on complement-mediated lesions or an inflammatory response, ATP1A3 depletion can be associated with energy depletion and ischemic stress, and PKP2 loss is influenced by molecular changes at the intercalated discs. Time is a crucial factor, as the molecular changes triggered by ischemia occur in a sequence of events. Other factors, such as selective protein vulnerability, variations in cellular stress responses, post-mortem intervals, and variability in histological sampling, may also contribute to these variations in marker expression.

Study Limitations

The study had several limitations. Its retrospective nature, combined with the small size of the histopathological sections analyzed, is a factor. Additionally, there is the possibility of false-positive results with ATP1A3 in cases where death was attributed to causes other than

myocardial ischemia, which cannot be explained in this study. Furthermore, resuscitation efforts were not considered in the statistical analysis due to the small number of cases in the sample, although it is known that resuscitation can influence distribution patterns and marker expression. Another limitation is the exclusion of cases where autopsies were performed more than 72 hours after death, meaning we could not assess whether the markers are affected by autolysis.

7.5 Conclusion

The majority of non-violent deaths examined in medico-legal autopsies are attributed to sudden cardiac death. Sometimes, establishing myocardial infarction as the cause of death represents a significant challenge. In such situations, immunostaining for early ischemia detection preceding myocardial infarction has become an essential tool. Finding new immunohistochemical markers that offer higher specificity and sensitivity is of great importance. In this study, we investigated for the first time the potential of the immunohistochemical markers APT1A3 and PKP2 in detecting ischemia in myocardial tissue. In the statistical analysis of the four groups, APT1A3 and C5b-9 markers demonstrated strong, statistically significant positive correlations, suggesting a reliable relationship in which both markers provide correlated information about myocardial infarction. PKP2 and C5b-9 did not show any correlation in the first two groups, indicating the absence of a solid or reliable relationship between these markers in the analyzed datasets. For APT1A3, there were cases where Grades 1 or 2 were observed, while the C5b-9 complex showed Grades 0 or 1, suggesting that APT1A3 might detect early ischemia more effectively than C5b-9. We can conclude that the APT1A3 marker could be used in combination with other markers to detect ischemia in myocardial tissue. Future research on this immunohistochemical marker could be beneficial to further establish and improve its sensitivity and specificity.

8. Conclusions and Personal Contributions

The three studies analyzed within this doctoral thesis address essential and current topics in cardiac pathology and forensic medicine. They focus particularly on myocardial ischemia, the precursor to myocardial infarction, a subject of great relevance in the context of forensic medicine, and on the use of immunohistochemical markers for its diagnosis. These studies contribute significantly to advancing knowledge in cardiac pathology and forensic medicine, offering new methods for diagnosing and assessing myocardial infarction in histopathological examinations. This work is the first conducted in Romania to focus on the use of immunohistochemical markers in the context of ischemia and acute myocardial infarction. Additionally, it is unique in the specialized literature, as no previous studies have been identified that investigate the use of ATP1A3 and PKP2 markers for the early identification of myocardial ischemia, the stage preceding acute myocardial infarction.

The results of the three studies contribute to a better understanding of the following essential aspects:

- The increased prevalence of sudden cardiac death (SCD) in men (75%), with the highest incidence in the 50-60 age group (Chapter 5.3).
- Advanced myocardial fibrosis (AMF) was identified as the most frequent cause of SCD, representing 41.6% of all cases. This finding raises concerns related to diagnosing myocardial ischemia, as advanced myocardial fibrosis is often a diagnosis of exclusion (Chapters 5.3, 5.4, and 5.5).
- The need for developing national protocols to standardize the diagnosis and reporting of SCD cases in anatomical pathology and forensic investigations (Chapters 5.3, 5.4, and 5.5).
- H&E microscopy has proven insufficient in certain cases for diagnosing acute myocardial infarction, highlighting the need for using immunohistochemical markers in histopathological diagnosis.
- Rapid identification of appropriate markers for each case contributes to diagnostic accuracy (Chapter 6.3).
- The classification of markers based on their mechanism of action (Chapter 6.3).
- ATP1A3 has high potential as an immunohistochemical marker for myocardial ischemia, particularly due to its sensitivity (Chapter 7.3).

- Another important aspect highlighted is that ATP1A3 can be used in combination with other known markers, such as the C5b-9 complex, to provide a complete picture of ischemia (Chapter 7.5).
- PKP2 did not show a clear correlation in all cases studied, suggesting that this marker is not optimal for diagnosing cardiac ischemia or myocardial infarction (Chapter 7.5).

Recommendations and Future Perspectives

The three studies conducted within this thesis provide valuable conclusions and clear directions for future research in myocardial ischemia and myocardial infarction. One of the main recommendations is the need to integrate immunohistochemistry techniques into the standard diagnosis of sudden cardiac death and acute myocardial infarction, especially in cases where conventional histopathological examinations do not provide conclusive results.

Standardization of Protocols: It is recommended to develop standardized protocols that include the use of immunohistochemical markers in post-mortem examinations to improve diagnostic accuracy and prevent the underdiagnosis of acute myocardial infarction.

Combined Use of Markers: To increase the specificity and sensitivity of the diagnosis, it is recommended to use a combination of immunohistochemical markers. For example, the combination of ATP1A3, C5b-9, and fibronectin markers can provide a comprehensive view of ischemia and significantly improve the diagnosis of acute myocardial infarction.

In conclusion, these studies have made significant contributions to the diagnosis of ischemia, the precursor to acute myocardial infarction, and have highlighted the need for more precise diagnostic methods in cases of sudden cardiac death and myocardial infarction. A multidisciplinary approach and the integration of advanced immunohistochemistry and genetic techniques can lead to significant improvements in diagnosis and, subsequently, in the prevention of myocardial infarction.

Original Articles Published in National and International Peer-Reviewed Journals Indexed as First Author:

1. **Moldovan R,** Ichim VA, Beliș V. Recent perspectives on the early expression immunohistochemical markers in post-mortem recognition of myocardial infarction. Legal Medicine. 2023;64:102293.
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2. **Moldovan R,** Ichim VA, Pufu G.A, Beliș V. Comprehensive Statistical Analysis of Causes of Death and Histopathological Diagnosis in Medico-Legal Investigations of Sudden Cardiac Deaths RM.J Vol 71 No 3. Year 2024; DOI: 10.37897/RMJ
   Indexat: Scopus.
   Chapter from which the article derived: Chapter 5

Selective Bibliography

1. Milroy CM. Coronary artery disease, sudden death and implications for forensic pathology practice. Diagnostic Histopathology. 2017;23(11):479-85.
2. Moldovan R, Ichim VA, Beliș V. Recent perspectives on the early expression immunohistochemical markers in post-mortem recognition of myocardial infarction. Legal Medicine. 2023;64:102293.
3. Iorga A, Cunningham CM, Moazeni S, Ruffenach G, Umar S, Eghbali M. The protective role of estrogen and estrogen receptors in cardiovascular disease and the controversial use of estrogen therapy. Biology of Sex Differences. 2017;8(1).
4. Goyal V, Jassal DS, Dhalla NS. Pathophysiology and prevention of sudden cardiac death. Canadian Journal of Physiology and Pharmacology. 2016;94(3):237-44.
5. Monticello TM, Sargent CA, McGill JR, Barton DS, Grover GJ. Amelioration of ischemia/reperfusion injury in isolated rat hearts by the ATP-sensitive potassium channel opener BMS-180448. Cardiovascular Research. 1996;31(1):93-101.
6. Thygesen K, Alpert JS, Jaffe AS, Chaitman BR, Bax JJ, Morrow DA, White HD. Fourth Universal Definition of Myocardial Infarction (2018). Circulation. 2018;138(20).
7. Michaud K, Basso C, D'Amati G, Giordano C, Kholová I, Preston SD, et al. Diagnosis of myocardial infarction at autopsy: AECVP reappraisal in the light of the current clinical classification. Virchows Archiv. 2020;476(2):179-94.
8. Xu XR, Carrim N, Neves MAD, McKeown T, Stratton TW, Coelho RMP, et al. Platelets and platelet adhesion molecules: novel mechanisms of thrombosis and anti-thrombotic therapies. Thrombosis Journal. 2016;14(S1).
9. Collinson P, Lindahl B. Type 2 myocardial infarction: the chimaera of cardiology? Heart. 2015;101(21):1697-703.
10. Coscia T, Nestelberger T, Boeddinghaus J, Lopez-Ayala P, Koechlin L, Miró Ò, et al. Characteristics and Outcomes of Type 2 Myocardial Infarction. JAMA Cardiology. 2022;7(4):427.
11. Kumar V AA, Aster JC. Robbins and Cotran Pathologic Basis of Disease. 10th ed. Philadelphi: Elsevier; 2020.
12. Ghafoor M, Kamal M, Nadeem U, Husain AN. Educational Case: Myocardial Infarction: Histopathology and Timing of Changes. Academic Pathology. 2020;7:237428952097663.
13. Wang G, Zhao N, Zhong S, Li J. A systematic review on the triggers and clinical features of type 2 myocardial infarction. Clinical Cardiology. 2019;42(10):1019-27.
14. Libby P. The changing landscape of atherosclerosis. Nature. 2021;592(7855):524-33.
15. Macalpin RN. Some observations on and controversies about coronary arterial spasm. International Journal of Cardiology. 2015;181:389-98.
16. Lanza GA, Careri G, Crea F. Mechanisms of Coronary Artery Spasm. Circulation. 2011;124(16):1774-82.
17. Hung M-J, Hu P, Hung M-Y. Coronary Artery Spasm: Review and Update. International Journal of Medical Sciences. 2014;11(11):1161-71.
18. Freund Y, Cohen-Aubart F, Bloom B. Acute Pulmonary Embolism. JAMA. 2022;328(13):1336.
19. Braşoveanu AM, Mogoantă L, Mălăescu GD, Predescu OI, Cotoi BV, Ifrim Chen F. Hypertensive cardiomyopathy - histopathological and immunohistochemical aspects. Rom J Morphol Embryol. 2019;60(2):487-94.

20. Tanaka Y, Joki N, Hase H. Ischemic Heart Disease in Patients with End-Stage Kidney Disease. Blood Purification. 2015;40(4):332-6.
21. Shi Y, Zong Y. Type 2 Myocardial Infarction Resulted from the Left Thoracic Stomach. Brazilian Journal of Cardiovascular Surgery. 2020;35(5).
22. Bally I, Rossi V, Lunardi T, Thielens NM, Gaboriaud C, Arlaud GJ. Identification of the C1q-binding Sites of Human C1r and C1s. Journal of Biological Chemistry. 2009;284(29):19340-8.
23. Tsai T-H, Sung P-H, Chang L-T, Sun C-K, Yeh K-H, Chung S-Y, et al. Value and level of galectin-3 in acute myocardial infarction patients undergoing primary percutaneous coronary intervention. Journal of atherosclerosis and thrombosis. 2012;19(12):1073-82.
24. Katkenov N, Mukhatayev Z, Kozhakhmetov S, Sailybayeva A, Bekbossynova M, Kushugulova A. Systematic Review on the Role of IL-6 and IL-1β in Cardiovascular Diseases. Journal of Cardiovascular Development and Disease. 2024;11(7):206.
25. Sepideh Saleki1 FA-A, Hamidreza Eftekhari2, Hayedeh Haeri2,Sahra Emamzadehfard2,3. Fibronectin as an Immunohistochemical Marker for Postmortem Diagnosis of Myocardial Infarction. M J Immu 1(1): 001. 2016;Volume 1(Issue 1 - 2017).
26. Bi H, Yang Y, Huang J, Li Y, Ma C, Cong B. Immunohistochemical detection of S100A1 in the postmortem diagnosis of acute myocardial infarction. Diagnostic Pathology. 2013;8(1):84.
27. Cecchi R, Camatti J, Bonasoni MP, Maria CG, Simona N, Nicoletta C, Paola M. HIF-1α expression by immunohistochemistry and mRNA-210 levels by real time polymerase chain reaction in post-mortem cardiac tissues: A pilot study. Legal Medicine. 2024:102508.
28. Al-Salam S, Hashmi S. Galectin-1 in Early Acute Myocardial Infarction. PLoS ONE. 2014;9(1):e86994.
29. Mondello C, Ventura Spagnolo E, Bartoloni G, Alibrandi A, Cardia L, Sapienza D, et al. Dystrophin and metalloproteinase 9 in myocardial ischemia: A post-mortem immunohistochemical study. Legal Medicine. 2021;53:101948.
30. Korshunova A, Blagonravov M, Neborak E, Syatkin S, Sklifasovskaya A, Semyatov S, Agostinelli E. BCL2-regulated apoptotic process in myocardial ischemia-reperfusion injury (Review). International Journal of Molecular Medicine. 2020;47(1):23-36.
31. Zidar N, Dolenc-Stražar Z, Jeruc J, Štajer D. Immunohistochemical expression of activated caspase-3 in human myocardial infarction. Virchows Archiv. 2006;448(1):75-9.
32. Hinderer S, Schenke-Layland K. Cardiac fibrosis – A short review of causes and therapeutic strategies. Advanced Drug Delivery Reviews. 2019;146:77-82.
33. Corrado D, Zorzi A, Cipriani A, Bauce B, Bariani R, Brunetti G, et al. Scarring/arrhythmogenic cardiomyopathy. European Heart Journal Supplements. 2023;25(Supplement_C):C144-C54.
34. Disertori M, Masè M, Ravelli F. Myocardial fibrosis predicts ventricular tachyarrhythmias. Trends in Cardiovascular Medicine. 2017;27(5):363-72.
35. Galati G, Leone O, Pasquale F, Olivotto I, Biagini E, Grigioni F, et al. Histological and Histometric Characterization of Myocardial Fibrosis in End-Stage Hypertrophic Cardiomyopathy. Circulation: Heart Failure. 2016;9(9):e003090.
36. Mitrut R, Stepan AE, Pirici D. Histopathological Aspects of the Myocardium in Dilated Cardiomyopathy. Curr Health Sci J. 2018;44(3):243-9.
37. Tejado BSM, Jou C. Histopathology in HCM. Global Cardiology Science and Practice. 2018;2018(3).
38. Abedinzadeh N, Pedram B, Sadeghian Y, Nodushan SMHT, Gilasgar M, Darvish M, Mokarizadeh A. RETRACTED ARTICLE: A histopathological analysis of the epidemiology of coronary atherosclerosis: an autopsy study. Diagnostic Pathology. 2015;10(1).
39. McHugh ML. The Chi-square test of independence. Biochemia Medica. 2013:143-9.

40. Ross A, Willson VL. Basic and Advanced Statistical Tests. 2017.
41. Zipes DP, Wellens HJJ. Sudden Cardiac Death. Circulation. 1998;98(21):2334-51.
42. Skjelbred T, Rajan D, Svane J, Lynge TH, Tfelt-Hansen J. Sex differences in sudden cardiac death in a nationwide study of 54 028 deaths. Heart. 2022;108(13):1012-8.
43. Mahapatra R, Gouda K, Das R, Mohanty P, Prusty G. Histopathological Spectrum of Cardiac Lesions in Sudden Cardiac DeathAn Autopsy Study. JOURNAL OF CLINICAL AND DIAGNOSTIC RESEARCH. 2023;17.
44. Zeppenfeld K, Tfelt-Hansen J, De Riva M, Winkel BG, Behr ER, Blom NA, et al. 2022 ESC Guidelines for the management of patients with ventricular arrhythmias and the prevention of sudden cardiac death. European Heart Journal. 2022;43(40):3997-4126.
45. Stecker EC, Reinier K, Marijon E, Narayanan K, Teodorescu C, Uy-Evanado A, et al. Public Health Burden of Sudden Cardiac Death in the United States. Circulation: Arrhythmia and Electrophysiology. 2014;7(2):212-7.
46. Fox CS, Evans JC, Larson MG, Kannel WB, Levy D. Temporal Trends in Coronary Heart Disease Mortality and Sudden Cardiac Death From 1950 to 1999. Circulation. 2004;110(5):522-7.
47. Jellis C, Martin J, Narula J, Marwick TH. Assessment of Nonischemic Myocardial Fibrosis. Journal of the American College of Cardiology. 2010;56(2):89-97.
48. Salari N, Morddarvanjoghi F, Abdolmaleki A, Rasoulpoor S, Khaleghi AA, Hezarkhani LA, et al. The global prevalence of myocardial infarction: a systematic review and meta-analysis. BMC Cardiovascular Disorders. 2023;23(1).
49. Huikuri HV, Castellanos A, Myerburg RJ. Sudden Death Due to Cardiac Arrhythmias. New England Journal of Medicine. 2001;345(20):1473-82.
50. Hayashi M, Shimizu W, Albert CM. The Spectrum of Epidemiology Underlying Sudden Cardiac Death. Circulation Research. 2015;116(12):1887-906.
51. Deyell MW, Krahn AD, Goldberger JJ. Sudden Cardiac Death Risk Stratification. Circulation Research. 2015;116(12):1907-18.
52. Bezzina CR, Lahrouchi N, Priori SG. Genetics of Sudden Cardiac Death. Circulation Research. 2015;116(12):1919-36.
53. Jasra SK, Badian C, Macri I, Ra P. Recognition of Early Myocardial Infarction by Immunohistochemical Staining with Cardiac Troponin-I and Complement C9. Journal of forensic sciences. 2012;57(6):1595-600.
54. Mondello C, Cardia L, Ventura-Spagnolo E. Immunohistochemical detection of early myocardial infarction: a systematic review. International journal of legal medicine. 2017;131:411-21.
55. Turillazzi E, Di Paolo M, Neri M, Riezzo I, Fineschi V. A theoretical timeline for myocardial infarction: immunohistochemical evaluation and western blot quantification for Interleukin-15 and Monocyte chemotactic protein-1 as very early markers. Journal of Translational Medicine. 2014;12(1):188.
56. Tian M, Yuan YC, Li JY, Gionfriddo MR, Huang RC. Tumor necrosis factor-α and its role as a mediator in myocardial infarction: A brief review. Chronic Diseases and Translational Medicine. 2015;1(1):18-26.
57. Pyati DAK. Heart-Type Fatty Acid Binding Protein: A Better Cardiac Biomarker than CK-MB and Myoglobin in the Early Diagnosis of Acute Myocardial Infarction. JOURNAL OF CLINICAL AND DIAGNOSTIC RESEARCH. 2015.
58. Zhu ZL, Wang P, You JB, Yue Q, Wang PF, Wang XL, et al. [Application of IMA and H-FABP in Forensic Diagnosis of Sudden Cardiac Death]. Fa Yi Xue Za Zhi. 2017;33(4):393-6.

59. Bing-Jie Hu1, *, Yu-Chuan Chen2, J-ZZ. IMMUNOHISTOCHEMICAL STUDY ON THE DEPLETION OF DESMIN, ACTIN AND MYOGLOBIN IN AUTOLYZED AND PUTREFIED NORMAL HUMAN MYOCARDIA. Rom J Leg Med. 2020; 121-126 [2020].
60. Jia J-z, Shen Y-w, Xue A-m, Zhao Z-q. Immunohistochemical analysis of cardiac troponin inhibitor in an experimental model of acute myocardial infarction experimental model and in human tissues. Pathology-Research and Practice. 2015;211(6):456-61.
61. Park KC, Gaze DC, Collinson PO, Marber MS. Cardiac troponins: from myocardial infarction to chronic disease. Cardiovascular research. 2017;113(14):1708-18.
62. Jaffe AS, Wu AHB. Troponin Release—Reversible or Irreversible Injury? Should We Care? Clinical Chemistry. 2012;58(1):148-50.
63. Wang Q, Michiue T, Ishikawa T, Zhu B-L, Maeda H. Combined analyses of creatine kinase MB, cardiac troponin I and myoglobin in pericardial and cerebrospinal fluids to investigate myocardial and skeletal muscle injury in medicolegal autopsy cases. Legal medicine. 2011;13(5):226-32.
64. Barranco R, Ventura F. Immunohistochemistry in the Detection of Early Myocardial Infarction: Systematic Review and Analysis of Limitations Because of Autolysis and Putrefaction. Applied Immunohistochemistry & Molecular Morphology. 2018;28(2):95-102.
65. Xue Y, Zhao R, Du S-H, Zhao D, Li D-R, Xu J-T, et al. Decreased mRNA levels of cardiac Cx43 and ZO1 in sudden cardiac death related to coronary atherosclerosis: a pilot study. International Journal of Legal Medicine. 2016;130(4):915-22.
66. Schwuchow-Thonke S, Göbel S, Emrich T, Schmitt VH, Fueting F, Klank C, et al. Increased C reactive protein, cardiac troponin I and GLS are associated with myocardial inflammation in patients with non-ischemic heart failure. Scientific Reports. 2021;11(1).
67. Cao Z, Jia Y, Zhu B. BNP and NT-proBNP as Diagnostic Biomarkers for Cardiac Dysfunction in Both Clinical and Forensic Medicine. International Journal of Molecular Sciences. 2019;20(8):1820.
68. Hashimoto T, Shibasaki F. Hypoxia-Inducible Factor as an Angiogenic Master Switch. Frontiers in Pediatrics. 2015;3.
69. Bass-Zubek AE, Godsel LM, Delmar M, Green KJ. Plakophilins: multifunctional scaffolds for adhesion and signaling. Current Opinion in Cell Biology. 2009;21(5):708-16.
70. Mondello C, Ventura Spagnolo E, Cardia L, Sapienza D, Scurria S, Gualniera P, Asmundo A. Membrane Attack Complex in Myocardial Ischemia/Reperfusion Injury: A Systematic Review for Post Mortem Applications. Diagnostics. 2020;10(11):898.
71. Guo X, Wang J, Elimban V, Dhalla NS. Both enalapril and losartan attenuate sarcolemmal Na+-K+-ATPase remodeling in failing rat heart due to myocardial infarction. Canadian Journal of Physiology and Pharmacology. 2008;86(4):139-47.
72. Omran MM, Zahran FM, Kadry M, Belal AAM, Emran TM. Role of myeloperoxidase in early diagnosis of acute myocardial infarction in patients admitted with chest pain. Journal of Immunoassay and Immunochemistry. 2018;39(3):337-47.
73. Bayes-Genis A, Conover CA, Overgaard MT, Bailey KR, Christiansen M, Holmes DR, et al. Pregnancy-Associated Plasma Protein A as a Marker of Acute Coronary Syndromes. New England Journal of Medicine. 2001;345(14):1022-9.
74. Nilsson E, Kastrup J, Sajadieh A, Boje Jensen G, Kjøller E, Kolmos HJ, et al. Pregnancy Associated Plasma Protein-A as a Cardiovascular Risk Marker in Patients with Stable Coronary Heart Disease During 10 Years Follow-Up—A CLARICOR Trial Sub-Study. Journal of Clinical Medicine. 2020;9(1):265.
75. Misao J, Hayakawa Y, Ohno M, Kato S, Fujiwara T, Fujiwara H. Expression of bcl-2 Protein, an Inhibitor of Apoptosis, and Bax, an Accelerator of Apoptosis, in Ventricular Myocytes of Human Hearts With Myocardial Infarction. Circulation. 1996;94(7):1506-12.

76. Depierre P, Ginet V, Truttmann AC, Puyal J. Neuronal autosis is Na(+)/K(+)-ATPase alpha 3-dependent and involved in hypoxic-ischemic neuronal death. Cell Death Dis. 2024;15(5):363.
77. Duncanson ER, Mackey-Bojack SM. Histologic Examination of the Heart in the Forensic Autopsy. Academic Forensic Pathology. 2018;8(3):565-615.
78. Uhlén M, Fagerberg L, Hallström BM, Lindskog C, Oksvold P, Mardinoglu A, et al. Tissue-based map of the human proteome. Science. 2015;347(6220):1260419-.