

## ORIGINAL PAPER

# Immunophenotypical pleomorphism expression in sudden cardiac death

M. CEAUŞU<sup>1,2)</sup>, C. CURCĂ<sup>2)</sup>, CARMEN ARDELEANU<sup>1)</sup>,  
D. DERMENGIU<sup>2)</sup>

<sup>1)</sup>*Department of Pathology,  
"Victor Babeş" National Institute for Research and Development  
in Pathology and Biomedical Sciences, Bucharest*

<sup>2)</sup>*"Mina Minovici" National Institute of  
Forensic Medicine, Bucharest*

### Abstract

This study was undertaken to assess several histopathological and immunohistochemical markers regarding some lesional aspects of ischemically and hypoxically damaged myocardium in sudden cardiac death. Tissue samples of myocardium from 17 middle age and young patients with sudden cardiac death, following acute or chronic cardio(myo)pathies, were analyzed using standard HE stain and indirect tristodial ABC peroxidase immunohistochemical method, for a panel of 12 antibodies grouped in three categories: antibodies involved in programmed cell death (bcl-2, p53, Fas/CD95, Fas-L, bax, caspase 9), muscular markers (Myo-D1, myogenin, desmin, actin) and growth factor receptors (b-FGF, VEGF, NGF). Myogenin was more sensitive in identifying the ischemic perilesional myocardial fibers than Myo-D1, but less specific, while desmin had a greater sensitivity than myogenin and Myo-D1 taken separately, but with no specificity for myocardic fibers. Fas-L, caspase 9 and bax were expressed in more than 75% of cases in perilesional residual cardiomyocytes, correlating to each other ( $r = 0.45$ , respectively  $r = 0.6$ ,  $p < 0.05$ ). b-FGF, VEGF and NGF had a focally variable expression in subendocardial and subepicardial cardiomyocytes and were statistically independent. Even if there was a polymorphic expression of antibodies in the studied batch, our findings indicate that some parameters (Fas-L, b-FGF, Myo-D1) might be independent markers for predicting sudden cardiac death in patients with previously damaged myocardium.

**Keywords:** sudden death, predicting parameters, immunophenotype.

### Introduction

Sudden death in young adults is an increasing problem of modern society that has reached worrisome proportions. Despite this, our knowledge regarding individual causes and epidemiology, as well as morphologic and immunohistochemical investigations is limited. Despite their young age, these patients have extensive myocardial fibrosis, coronary atherosclerosis, anomalies of the conduction system and other cardiac lesions, all of which are uncommon in young adults. Therefore, in forensic practice there is a need for more sensitive methods for the postmortem diagnosis of myocardial injuries involved in sudden death.

In rabbit experimental models, it was determined that the inhibition of the pro-apoptotic protein p53 lowers the threshold of isoflurane-induced protection during early *in vivo* myocardial reperfusion [1], while other studies have shown that the immunohistochemical (IHC) expression of bcl-2 and bax (apoptotic regulator proteins in myocardial fibers) is significant during reperfusion after acute ischemia, and might be used as markers for sudden cardiac death in forensic pathology [2].

In cardiac failure secondary to myocarditis, the bcl-2 expression is high, but does not protect the myocytes from apoptosis, which can be detected as

small clusters of dying cells using the TUNEL technique and IHC for caspases [3].

The long-term intermittent hypoxia has negative effects on mitochondria and triggers the programmed cell death, via Fas/Fas-L dependent apoptotic pathways in rat hearts [4].

In addition, the HtrA2/Omi mitochondrial serine-protease released in cytosol promotes the activation of caspases (detectable by IHC), which inhibit the proteolysis of some apoptotic proteins; thus, the inhibition of this molecule ameliorates heart dysfunction following ischemia/reperfusion injury in rat heart *in vivo* [5].

According to a study of 24 cases, which pointed out its presence both in the nuclei or cytoplasm of resting cardiomyocytes and on vascular endothelium surrounding the infarct area, b-FGF is another useful marker for the diagnosis of sudden cardiac death in early myocardial infarction [6].

The results of another study on 16 cases, regarding the expression of VEGF in myocardial infarction have shown that the intense positive reaction of VEGF in peri-infarction area, correlated with imagistic and statistic analysis could be an objective marker for the postmortem diagnosis of sudden coronary death [7].

Nuclear expression of Myo-D1 transcription factor in conditions of hypoxia was found in oncocytic

cardiomyopathy, a rare disease accompanied by rhythm disturbances involved in sudden cardiac death, where it was found [8]. Other muscle markers used in the diagnosis of myocardial damage involved in sudden cardiac death are desmin [9], myogenin and troponin [10].

The aim of this study was to correlate histopathological and immunohistochemical data using apoptotic markers, cellular growth factor receptors and muscle markers, in sudden cardiac death of young or middle age adults with previously damaged myocardium.

## ☒ Material and methods

### Tissue samples

We have retrieved randomly, from our database, in an interval between 2004–2006, 17 archived formalin-fixed paraffin-embedded samples of myocardial tissue (all from left ventricle) from young and middle-aged adults (sex ratio M:F = 2:1), ranging from 20 to 40 years, with sudden cardiac death, following acute or chronic cardio(myo)pathies or scarring myocardic fibrosis. Sections were cut at 5 microns and stained using the standard H&E stain, van Gieson and Weigert.

### Immunohistochemistry (IHC)

The indirect tristadial ABC peroxidase immunohistochemical method was used for a panel of 12 antibodies, grouped in three categories: antibodies involved in programmed cell death, muscle markers and growth factor receptors.

The immunohistochemistry (IHC) was performed on 3 µm thick sections from 10% formalin fixed paraffin embedded tissues, according to the indirect tristadial Avidin–Biotin–Complex method of Hsu SM *et al.* [11], modified by Bussolati G and Gugliotta P [12]. Briefly, the procedure was: deparaffinization in xylene and alcohol series, rehydration, washing in phosphate saline buffer (PBS), incubation with normal serum, for 20 minutes, incubation with primary antibody overnight, standard labeled streptavidine-antibody biotin (LSAB) kit (DAKO), washing in carbonate buffer and development in 3,3'-DAB hydrochloride/H<sub>2</sub>O<sub>2</sub>, microwave antigen retrieval in M-citrate buffer pH 6.0 was performed for certain antibodies.

All specimens were counterstained with Meyer's Hematoxylin, examined and photographed on a Nikon Eclipse 600 microscope. To ensure the reliability of the experimental study, internal quality control of immunohistochemical techniques was performed as a part of an implemented and certified quality assurance system (ISO 9001/2001). The antibodies used are shown in Table 1.

### Statistics

For correlation between parameters, statistical analysis has been done using the Student t-test, "paired two samples for mean" variant, one-group two tails, for uniform distributed data, from the *Analysis Tool Pack* of Microsoft Excel 2003, running under Windows XP Professional. A value of p<0.05 was considered significant.

**Table 1 – The antibodies used in the study**

Antibody	Producer	Clone	Dilution	Specificity
b-FGF	Santa Cruz	Poly	1:100	Fibroblastic growth factor
VEGF	Santa Cruz	C-1	1:100	Vascular endothelium growth factor
Myo-D1	Novocastra	5.8A	1:20/50	Nuclear transcription factor
NGF	Lab Vision	NGFR5	1:5/10	Nervous growth factor
Myogenin	DAKO	F5D	1:25/50	Striated muscle cells
Actin	Sigma	1A4	1:400	Microfilament muscle cells
Desmin	DAKO	D33	1:50/100	Intermediary filament striated cells
bcl-2	DAKO	124	1:40	Cytoplasmic protein of bcl-2 gene
p53	Neomarkers	DO7	1:50	Nuclear protein of p53 gene
CD95/Fas	Novocastra	GM30	1:40/80	Apoptotic receptor
Fas-L	Novocastra	5D1	1:50	Fas ligand
bax	Neomarkers	Poly	1:100	Apoptotic inducing protein
caspase 9	Novocastra	2C9B11	1:20/40	Apoptotic inducing protein

## ☒ Results

The classic histopathology investigation of the study batch has shown various degrees of fibrosis in eight of 17 (47%) cases. The myocardial fibrosis was evaluated semi-quantitatively as negative/absent (grade 0), mild (grade 1), moderate (grade 2) or diffuse (grade 3), with various types of anatomic localization: subendocardial, interstitial or substitution fibrosis. Other microscopic

data recorded were granular or vacuolar changes of myocardic fibers, following hypoxic status in the neighboring microenvironment in six (35.3%) of cases, a moderate interstitial inflammatory infiltrate in six (35.3%) of cases, coagulation necrosis in five (~30%) of cases, and other changes, such as: focal lymphomatosis, hyperemia, fragmented myocardic fibers and coronary atherosclerosis, sometime with calcification and mild inflammation (Figures 1 and 2).

For the apoptotic markers, the highest IHC expression was recorded in perilesional residual myocardic fibers for Fas-L in 15 (88%) of cases, caspase 9 in 13 (76.5%) of cases (Figure 3) and for bax mitochondrial protein in 14 (82.35%) of cases (Figure 4).

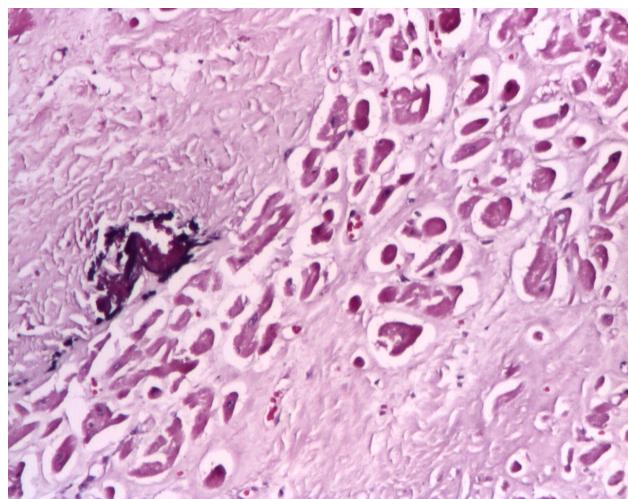
Fas-L had a linear membranar stain, while caspase 9 and bax were positive in the cytoplasm. Even if the wild type of p53 has a short half-life, the mutant isoform of this antibody was expressed in seven (41%) of cases (Figure 5), with a stain intensity ranging between 5–33%, in the nuclei of residual cardiomyocytes (Figure 6). Bcl-2 expression was absent or inconclusive in almost studied cases.

A direct correlation, statistically significant was recorded between the grade of interstitial fibrosis and Fas-L expression in perifibrotic cardiomyocytes ( $r = 0.4$ ,  $p < 0.001$ , Figure 7). Statistically significant positive correlations, were also noted between Fas-L and caspase 9 ( $r = 0.45$ ,  $p = 0.0002$ ) and between caspase 9 and bax ( $r = 0.6$ ,  $p = 0.05$ ); there was no correlation between other apoptotic markers in the

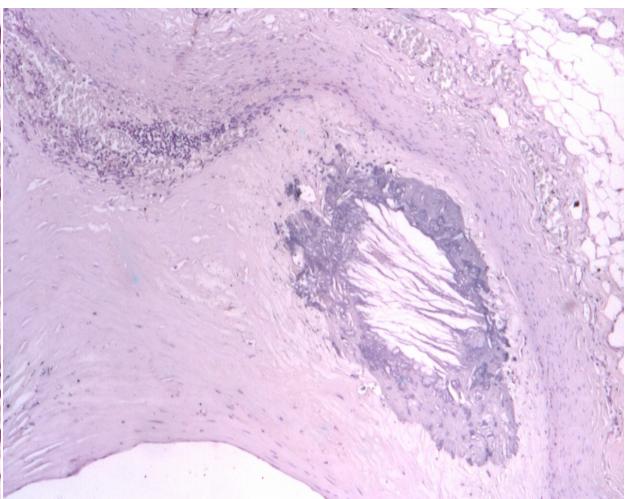
study, so they were considered as independent parameters.

The IHC expression of growth factors and the receptors for cytokines showed that b-FGF was focally positive in 10 cases (58.82%), in subendocardial and subepicardial residual cardiomyocytes (Figure 8), VEGF was focally positive in just two cases (11.76%) and NGF was variable positive in 10 cases (58.82%) in subepicardial nerve fascicles, rarely in interstitial fibers. All markers had a linear membrane stain.

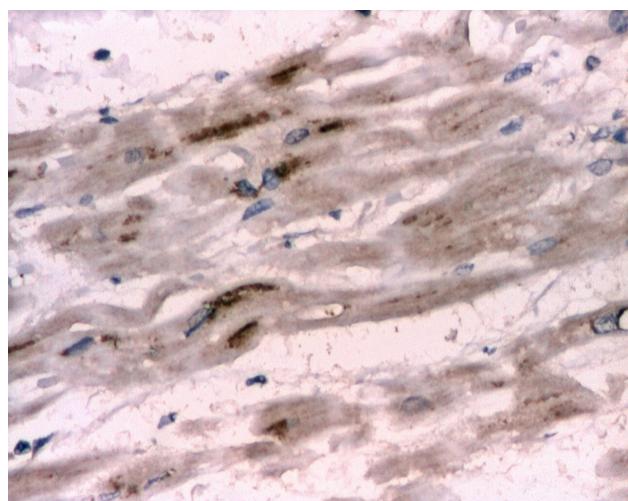
The muscle markers used in the study have shown that Myo-D1 was variably positive in the nuclei of residual myocardic fibers in nine cases (53%), especially in subendocardial and subepicardial regions (Figure 9), myogenin was positive in the cytoplasm of dispersed hypoxic myocardic fibers (Figure 10) in 12 cases (70.58%), actin was negative in all cases, but was positive in the interstitial vessels of the myocardium in 11 cases and desmin was frequently positive (Figure 11) in five cases (29.41%) in subendocardial or subepicardial residual myocytes (Figure 12).



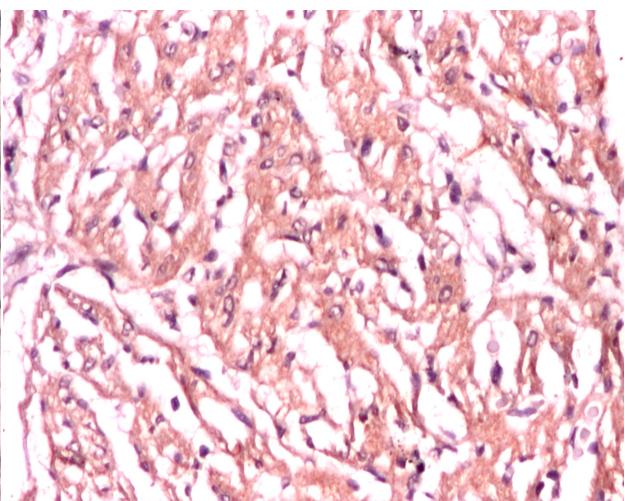
**Figure 1 – Diffuse interstitial fibrosis, after myocardial infarct, with calcification focus, in condition of long-standing intermittent hypoxia (HE, ob. 4×)**



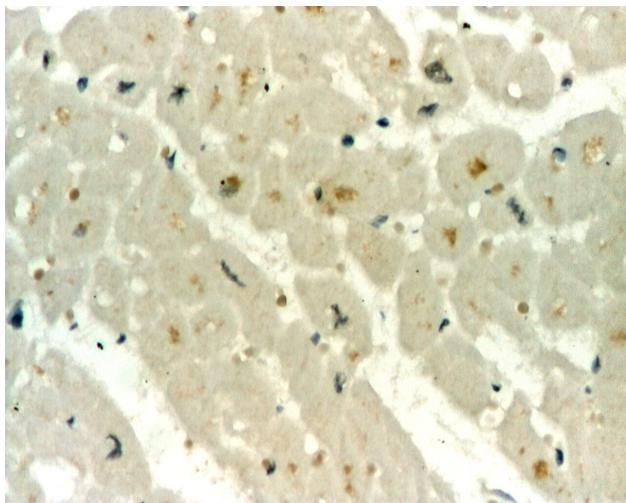
**Figure 2 – Subepicardial coronary vessel with severe atherosclerosis and mild chronic inflammatory infiltrate (HE, ob. 4×)**



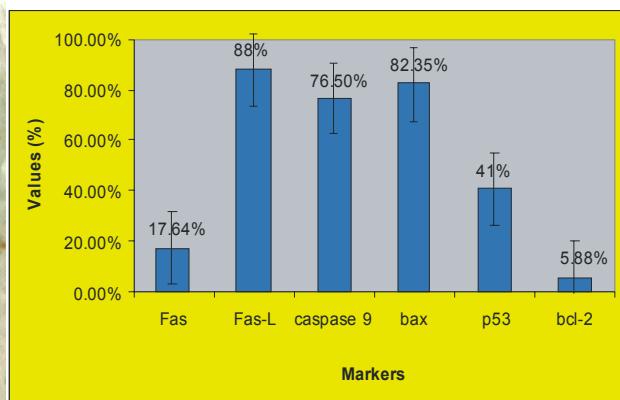
**Figure 3 – Immunohistochemical staining for caspase 9 in several cardiomyocytes (ob. 20×)**



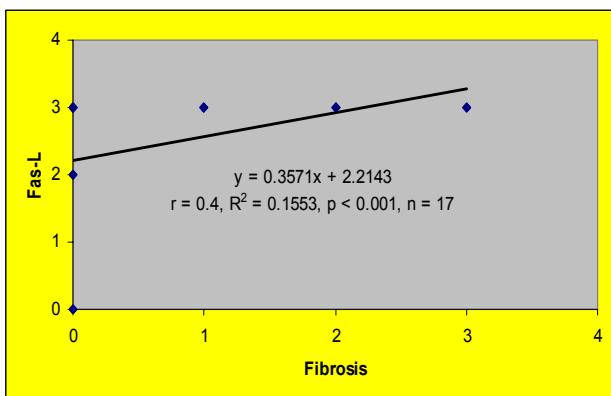
**Figure 4 – Positive immunohistochemical reaction for bax (ob. 20×)**



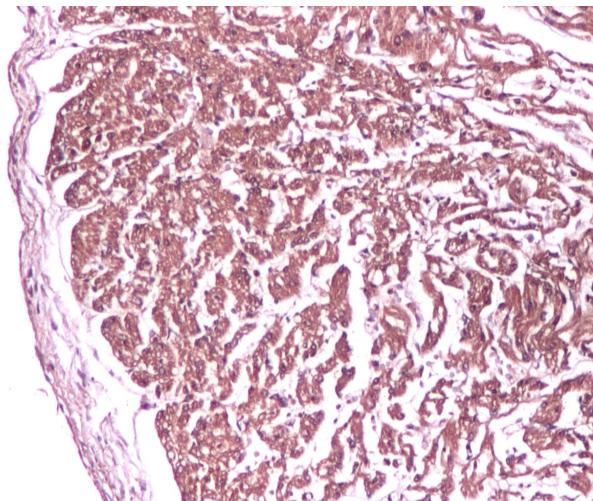
**Figure 5 – Positive IHC reaction for p53 in the nuclei of resting cardiomyocytes (ob. 20×)**



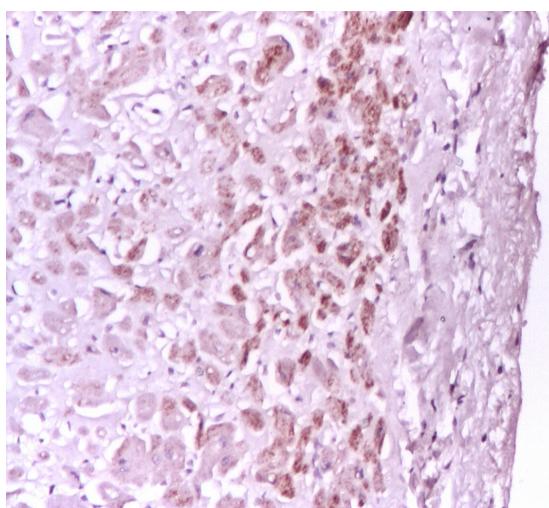
**Figure 6 – Apoptotic markers expression in studied cases**



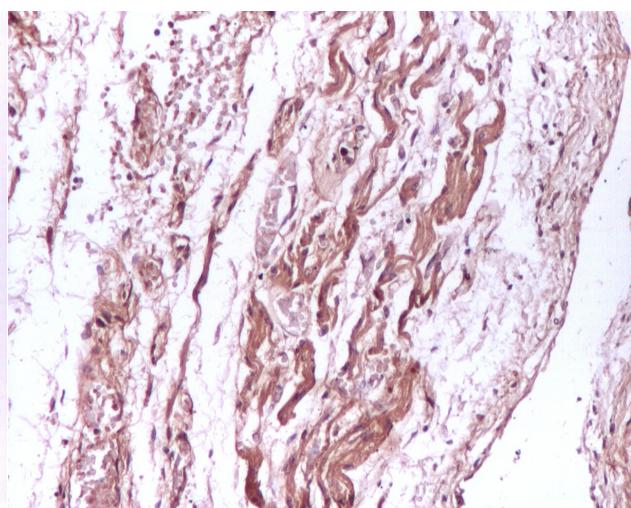
**Figure 7 – Correlation between myocardial fibrosis and Fas-L expression**



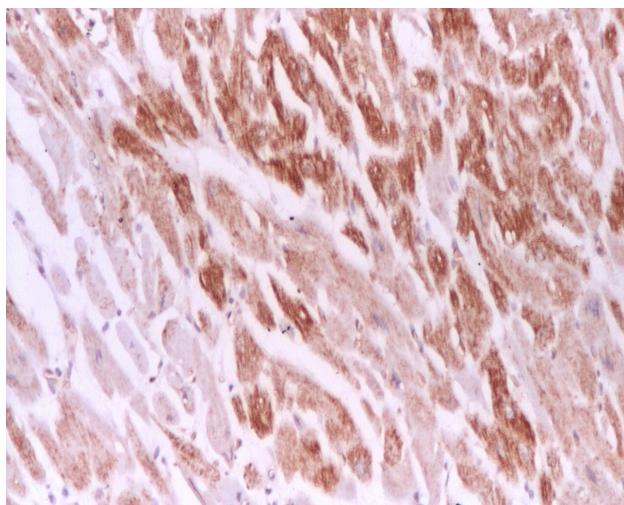
**Figure 8 – Diffuse positive IHC reaction for b-FGF in subendocardial cardiomyocytes (ob. 4×)**



**Figure 9 – Subendocardial cardiomyocytes focally positive for Myo-D1 (ob. 10×)**



**Figure 10 – Hypoxic subendocardial cardiomyocytes staining for myogenin (IHC, ob. 10×)**

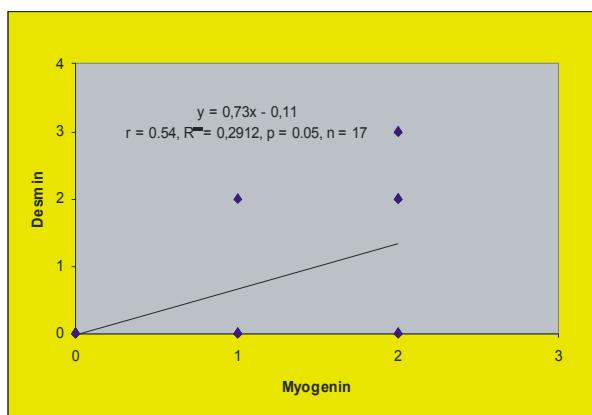


**Figure 11 – Desmin focally positive in myocardial fibers (ob. 10×)**



**Figure 12 – Muscular markers and growth factors expression in studied cases**

A statistically significant direct correlation between myogenin and desmin in perilesional myocardic fibres was recorded ( $r = 0.54$ ,  $p = 0.05$ , Figure 13); other correlations were also noted, between b-FGF and desmin ( $r = 0.34$ ,  $p = 0.1$ ) and between Myo-D1 and desmin ( $r = 0.31$ ,  $p = 0.5$ ), but were not statistically significant. The other parameters were statistically independent.



**Figure 13 – Correlation between myogenin and desmin in studied cases**

## Discussion

In some patients, sudden cardiac death is due to a global remodeling of ventricular interstitium, after myocardial infarct or because of an idiopathic myocardial fibrosis. Thus, according to a study, idiopathic myocardial fibrosis involves diffuse and heterogeneous remodeling of the ventricular interstitium, with a predilection for the inferior wall of left ventricle, TGF-beta 1 being a potential mediator of interstitial remodeling [13].

The direct proportion relation between Fas-L, caspase 9 and bax expression, supports the idea, that in conditions of a longstanding chronic intermittent myocardic ischemia, the activation of caspases and bax expression take place in cytosol; following this, an apoptotic signal transduction, mediated by

Fas/Fas-L pathway, triggers the programmed cell death mechanism in perilesional residual cardiomyocytes. Some data from the literature reveal that the apoptosis contributes to the development of cardiomyocyte injury in ischemia and apoptotic death of cardiomyocytes may lead to electric instability of the myocardium in early ischemia and cause sudden cardiac death [14]. In addition, another study has shown that cardiomyocytes apoptosis can be detected using IHC and TUNEL assay to prove myocardial ischemia in early myocardial damage [15].

Regarding the muscular markers, myogenin was more sensitive in identifying the ischemic perilesional myocardial fibers than Myo-D1, but less specific, while desmin had a greater sensitivity than myogenin and Myo-D1 taken separately, but with no specificity for myocardial fibers.

The nuclear transcription factor Myo-D1, which had a focal variable expression, in approximately 50% of cases, in either subendocardial or subepicardial localization, together with myogenin expression might increase the specificity of predicting more accurately possible post-infarct lethal rhythm disturbances. In addition, if desmin has the greatest sensibility in identifying the perilesional ischemic myocardic fibers, its expression must be correlated with that of myogenin, which is more sensitive for finding the hypoxic lesions.

## Conclusions

It should be noted that even if there was a polymorphic expression of antibodies in the studied batch, our findings indicate that some parameters might be independent markers for predicting sudden cardiac death in patients with previously damaged myocardium, which require further studies.

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## Corresponding author

Mihai Ceaușu, Assistant Professor, MD, PhD, "Victor Babeș" National Institute for Research and Development in Pathology and Biomedical Sciences, 99–101 Independenței Avenue, sector 5, 050 096, Bucharest, Romania; Phone/Fax +4021–319 27 34, E-mail: ceausu\_mihai@yahoo.com

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