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HIF-1α expression by immunohistochemistry and mRNA-210 levels by real time polymerase chain reaction in post-mortem cardiac tissues: A pilot study

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ABSTRACT

Introduction: The postmortem diagnosis of acute myocardial ischemia (AMI) represents a challenging issue in forensic practice. Immunohistochemical studies and gene expression studies are becoming a promising field of research in forensic pathology. The present study aims to evaluate HIF-1α expression through immunohistochemistry (IHC), and mRNA-210 level using real-time polymerase chain reaction (RT-PCR), in order to define if HIF-1α and mRNA-210 in post-mortem myocardium could be adopted in the diagnosis of AMI.
Materials and Methods: Thirty-five deceased individuals, who underwent forensic autopsy at the Legal Medicine Service of the University of Parma, between 2010 and 2018, were investigated. The cohort was divided into two groups according to the cause of death (sudden deaths caused by AMI vs control cases). Cardiac specimens were collected during autopsy, then samples were processed for morphological evaluation using haematoxylin–eosin staining, for IHC, and for RT-PCR. HIF-1α expression and mRNA-210 levels were investigated.
Results: Statistical evaluation demonstrated statistically significant differences in terms of number of IHC positive vessels, leukocytes, and cardiomyocytes between the two groups. Moreover, in the majority of cases, immunostaining positivity was observed only in myocardial and subendocardial samples. With reference to mRNA-210, the difference between the two groups proved to be statistically significant.
Conclusions: The present study indicates that HIF-1α and mRNA-210 in post-mortem cardiac specimens could represent appropriate biomarkers in the diagnosis of AMI. The current study was primarily limited by the scarcity of the cohort, so further research is required to confirm these preliminary observations.

1. Introduction

Sudden cardiac death (SCD) is defined as an unexpected sudden death attributed to a cardiac cause, that occurs within one hour of symptoms onset [1,2]. SCD is recognized as a leading cause of death in western countries and is responsible for the majority of deaths from cardiovascular disease [3].

The spectrum of SCD causes varies with age. In the young, there is a predominance of channelopathies, cardiomyopathies and myocarditis. In older populations, most frequent causes are represented by chronic degenerative diseases like coronary artery disease, valvular heart diseases, and heart failure. Approximately 80 % of SCD cases are attributed to atherosclerotic coronary artery disease. As most SCD are caused by coronary heart disease, they have similar risk factors such as hypertension, diabetes mellitus, obesity, smoking, and psychosocial factors [4,5].

According to the recommendations of the 2022 ESC Guidelines for the management of patients with ventricular arrhythmias and the prevention of sudden cardiac death, a comprehensive autopsy should be ideally performed in all cases of unexpected sudden death, and always in

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those younger than 50 years of age [6]. Thus, autopsy is crucial to diagnose the underlying pathology in SCD and to define screening and investigation of relatives of victims [7].

Many forensic studies have attempted to identify acute myocardial ischemia (AMI) as accurately as possible. In the earliest stage of cell death (first 4 h), light microscopic changes consist of a regional wavy pattern of myocytes as a result of stretching of dead non-contractile myocytes by adjacent functional myocardium during the cardiac cycle; interstitial oedema also appears. Pathological contraction bands, oedema, homogeneous eosinophilia, and neutrophils infiltration can be found from the 4th to the 12th hour. Nonetheless, these changes may have poor reliability and are related to over-interpretation. Even though transmission electron microscopy reveals very early ischemic changes such as mitochondrial swelling in cardiomyocytes, this method can only be used in experimental conditions because of the similarities between early ischemic changes and autolysis [8–10]. As a result, nowadays there is no specific and sensitive gold standard for the post-mortem diagnosis of early myocardial ischemia [11].

Post-mortem gene expression studies are becoming a promising field of research in forensic pathology to investigate the cause of death at the molecular level. González-Herrera et al. (2019) examined mRNA expression patterns in myocardial tissue, pericardial fluid and blood in samples from cadavers. They found increased MYL3, VEGFA and MMP9 mRNA expression in the anterior wall of the right ventricle in cases of acute myocardial infarction (AMI), so they suggested that the duration of myocardial ischemia might be determined on the basis of mRNA expression profiles [12]. Gao et al. (2023) investigated CircRNA, unique type of RNA with a closed loop structure. They found that circSMARCC1 and circLRBA may be potential biomarkers for postmortem diagnosis of AMI [13].

Aside from the approaches aforementioned, research interest has been recently concentrated on post-mortem immunohistochemistry (IHC) [14]. Indeed, Sabatasso et al. (2018) found that dephosphorylated connexin 43, JunB, and TUNEL assays were early, sensitive markers for AMI, even though they were not specific [15]. According to Kondo et al. (2021), immunostaining of thrombomodulin may be useful for supplementing conventional techniques in the diagnosis of ischemic heart disease [16]. Furthermore, Kuninaka et al. (2021) suggested that intracardiac Heme Oxygenase-1 expression would be considered a valuable marker to diagnose acute ischemic heart diseases [17]. Kunisek et al (2021) hypothesized that the expression of calpain 2 in cardiomyocytes should be specific and sensitive diagnostic forensic marker for SCD caused by early myocardial ischemia and an indicator of the duration of myocardial agonal period, that could even confirm the cause of death [18].

Kondo et al. (2022) found that von Willebrand factor, vimentin and CD31 immunostaining may be used in order to diagnose ischemic heart disease, and may allow to analyse the timing of myocardial remodelling [19–21]. Hiyamizu et al. (2024) recently argued that the immunohistochemical examination of nuclear factor erythroid 2-related factor 2 combined with fibronectin and/or terminal complement complex (C5b-9) has the potential to identify early-stage myocardial ischemic lesions in SCD cases [22].

Although various immunohistochemical markers have been investigated for improving postmortem detection of early myocardial ischemia, no single marker appears to be both sufficiently specific as well as sensitive [23,24].

In particular, the so-called “adaptive markers” are related to molecular changes following the ischemia-induced damage. Recent adaptive markers include hypoxia-inducible factor 1-alpha (HIF-1α), mitochondrial B cell lymphoma 2, and vascular endothelial growth factor. HIF-1α, a pro-angiogenic transcription factor, appears during the initial 2 h of myocardial ischemia. The post-mortem stability of this marker in the myocardium remains elusive, as well as its specificity [23,25]. Tayae et al (2023) suggested that HIF-1α antisense RNA 2, a LncRNA (Long non-coding RNA), may be an early diagnostic biomarker of AMI in vivo with high sensitivity [26]. Furthermore, Bavelloni et al. (2017) reported that HIF-1α-binding sites are carried in mRNA-210 promoter, so hypoxic conditions result in a significant up-regulation of mRNA-210 [27].

In the light of these considerations, the role of HIF-1α and mRNA-210 as possible markers of AMI remain questionable and further research is needed to test and validate these markers for application in routine diagnostics in clinical and forensic pathology. The present study aims to compare HIF-1α expression through IHC staining, and mRNA-210 levels through real-time polymerase chain reaction (RT-PCR), in samples of post-mortem myocardium, in order to define if HIF-1α and mRNA-210 could be adopted in the diagnosis of AMI.

2. Materials and methods

2.1. Cases

A cohort of thirty-five deceased individuals, who underwent forensic autopsy at the Legal Medicine Service of the University of Parma, a Northern Italian city, between 2010 and 2018, was investigated. The post-mortem interval ranged between 1 and 3 days. The cause of death was identified on the basis of medical records, scene of death, autopsy, toxicological and histological findings. Out of 35 total cases, two groups were identified; group 1: sudden deaths caused by acute myocardial infarction (n = 19, 14 males and 5 females; age: 33–84 years); group 2: control cases (n = 16, 14 males and 2 females; age: 18–45 years): deaths caused by traumatic shock related to road accident (n = 12); deaths caused by homicidal or suicidal gunshot wounds to the head (n = 4). Data are reported in Table 1.

2.2. Exclusion criteria

Exclusion criteria applied in the study comprised subjects whose specimens tested positive for drugs, cases of traumatic cardiac and pericardial injuries, cases of asphyxial deaths, and cases in which resuscitation occurred. The presence of chronic cardiac pathologies was a further exclusion criterion for cases belonging to group 2 (control cases).

2.3. H&E And IHC staining

The dissection of the heart at autopsy was performed following a standard procedure [28]. According to our protocol, five cardiac specimens were sampled in each case (left ventricle, right ventricle, septum, and apex). In the present study, two samples, corresponding to left ventricle and septum, were evaluated for each case. Cardiac samples from each case were fixed in 4 % PBS–formaldehyde solution, dehydrated through alcohol and paraffin-embedded [29]. 5-μm-thick paraffin-embedded sections were cut from each lung specimen and processed for morphological evaluation using haematoxylin-eosin

Table 1
Sample characteristics.

	Group 1	Group 2 (control cases)
Nr. Cases	19	16
Cause of death	sudden deaths caused by acute myocardial infarction	n = 12: deaths caused by traumatic shock related to road accident (75 %) n = 4: deaths caused by homicidal or suicidal gunshot wounds to the head (25 %)
Gender	14 (≈ 74 %)	14 (87,5%)
Male	5 (≈ 26 %)	2 (12,5%)
Female		
Age (years)	58 (33–84)	34 (18–45)
Postmortem interval (hours)	48 (24–72)	60 (24–72)

(H&E) staining and for IHC.

IHC analysis was performed using the human anti-hypoxia-induced factor 1-α (HIF-α) mouse monoclonal antibody (1:100 clone H1alpha67 by Novus Biologicals). For the IHC, samples were de-waxed, hydrated, and subjected to a 10-minutes incubation in a 3 % hydrogen peroxide solution and an antigen retrieval procedure using EDTA buffer pH 9.0 in a thermostatic bath at 98 °C. Incubation was performed (60′) with the primary antibody at RT; (15′) with a biotinylated secondary antibody; (15′) with avidin–biotin-peroxidase-complex (ABC of UltreTek HRP Anti-Polyvalent kit, ScyTek Laboratories, USA); (1′) with DAB substrate (Roche, Italy); then slides were washed and nuclear counter-staining (30″) with hematoxylin was performed. Optimal dilution of the antibody was set up and established using serial sections from neoplastic brain tissue, as positive control tissue; negative controls were carried out omitting the primary antibody. Overnight incubation was performed with the primary antibody at room temperature; reactions were detected thanks to Ultraview Detection DAB; ROCHE. DAB (3 3′-diaminobenzidine) revealed antibody-antigen binding. Nine random microscopic fields at 40x original magnification for each immunostained tissue section were assessed, classified as follows: three sub-endocardial sections, three myocardial sections, three sub-pericardial sections. Vessels, cardiomyocytes, and leucocytes were investigated for each field.

2.4. RT-PCR

RNA extraction was performed using the RecoverAll™ Total Nucleic Acid Isolation Kit – Invitrogen™, according to 2016 Thermo Fisher Scientific protocol (for technical details: https://www.thermofisher.com). RNA reverse transcription was performed through the TaqManTM Advanced miRNA cDNA Synthesis Kit – Applied BiosystemsTM. mRNA-210 levels were evaluated (assay name = hsa-miR-210-3p; miRBase Accession Number = MIMAT0000267; target sequence = CUGUGCGUGUGACAGCGGCUGA); 2016 Thermo Fisher Scientific protocol was carried out. Finally, RT- PCR was performed thanks to QuantStudio™ 7 Flex (Applied Bisystems), TaqMan™ Advanced MicroRNA Assays (Applied Biosystems), and TaqMan™ Fast Advanced Master Mix (Applied Biosystems), which contains AmpliTaq Fast DNA Polymerase, uracil-N-glycosylase (UNG), dNTPs with dUTP, ROX dye (passive reference), and optimized buffer components.

2.5. Statistical analysis

All the statistical analysis were performed using RStudio (RStudio 2022.12.0 + 353). Descriptive statistics were presented and then the Shapiro-Wilk test was performed. If the normal distribution was satisfied, the ANOVA test was performed; otherwise, the Kruskal-Wallis H test was chosen. A probability value of less than 0.05 was deemed to be statistically significant.

3. Results

3.1. H&E Staining

In group 1 (sudden deaths caused by acute myocardial infarction, for a total of 19 cases), light microscope observation of H&E-stained samples documented, in 6 cases, the presence of contraction bands, nuclear pyknosis, oedema, myocytolysis, and wavy, fragmented fibrocells (Fig. 1a). In the remaining 13 cases, contraction bands, interstitial oedema, myocytolysis, and wavy, fragmented fibrocells, severe coronary artery atherosclerosis and arteriolosclerosis, increased amount of pericardial fat, and fatty infiltration of the myocardium were found. In 2 out of 19 cases, microscopic observation revealed initial leukocyte margination. In 9 out of 19 cases, myocardial fibrosis was found (Fig. 1b). In 7 out of 19 cases, focal microhemorrhages were observed.

In group 2 (control cases), light microscope observation of H&E-stained samples documented mild coronary artery atherosclerosis in 4 out of 16 cases. In 3 out of 16 cases, interstitial oedema was found.

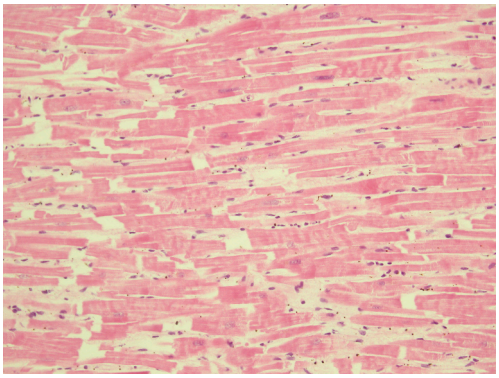


Fig. 1a. Left ventricle showing oedema and contraction bands (h&e) – group 1.

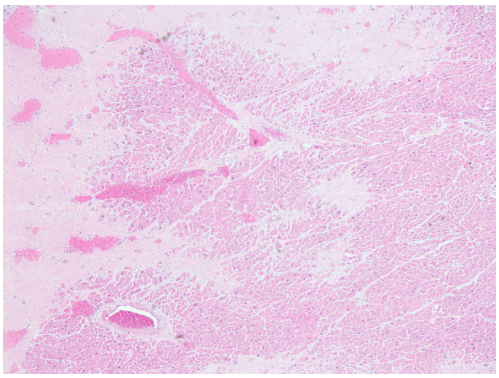


Fig. 1b. Septum showing areas of myocardial fibrosis (h&e) – group 1.

3.2. IHC staining (HIF-1α marker)

IHC positive vessels, cardiomyocytes, and leucocytes were investigated in nine random microscopic fields at 40x original magnification, classified as follows: three sub-endocardial sections, three myocardial sections, three sub-pericardial sections. As is well known, detection of HIF-1α by immunohistochemistry is not ideal, because positivity tends to be tenuous. in the present study, HIF-1 α nuclear positivity is documented by a tenuous brown staining that distinguishes positive from negative nuclei, which appear stained with H&E (haematoxylin & eosin).

In group 1 (n = 19), a mean value of positively stained vessels of 9.7 per case, a mean value of positively stained leukocytes of 19.7 per case and a mean value of positively stained cardiomyocytes of 9.4 per case

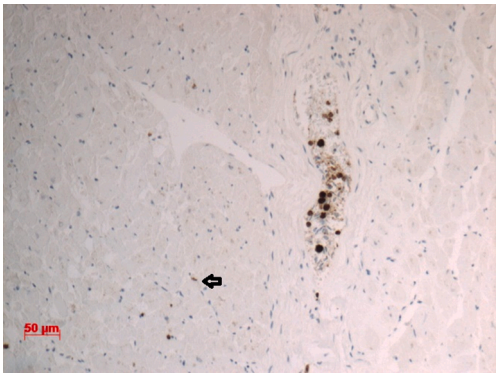


Fig. 2a. Anti-hif-1α positive capillaryindicated by arrow (IHC) – Group 1.

were found (Fig. 2a, Fig. 2b).

Moreover, immunostaining positivity was observed only in myocardium and sub-endocardium samples in 10 out of 19 cases, while, only in a single case, immunohistochemical staining positivity was detected exclusively in the myocardium.

Comparative observation on H&E-stained samples demonstrated that, in cases with absence of histological aspects of coronary artery disease (n = 6), the majority of IHC positive leukocytes were represented by tissue-resident macrophages. In cases characterized by coronary artery disease diagnosed through histological observation (n = 13), the majority of positively stained leukocytes showed intravascular location, and comparative observation on H&E-stained samples demonstrated that the majority of IHC positive leukocytes were associated to microhemorrhages. In 9 out of 19 cases, no positive cardiomyocytes were found. In 3 out of 19 cases, wavy cardiomyocytes showed immunohistochemical staining positivity.

The mean value of IHC positive cardiomyocytes in cases characterized by coronary artery disease diagnosed through histological observation was about five times as high as in cases characterized by the absence of histological aspects of coronary artery disease (12.7 vs 2.4).

In group 2 (n = 16), positively stained vessels and cells (leukocytes and cardiomyocytes) were found only in 3 out of 16 cases. (in 2 cases of them, a relevant number of IHC positive cells was found only in myocardium and in sub-endocardium). In group 2, a mean value of positively stained vessels of 3.4 per case, a mean value of positively stained leukocytes of 3 per case and a mean value of positively stained cardiomyocytes of 0.8 per case were found.

Descriptive statistics are resumed in Table 2.

First, Shapiro-Wilk test was performed, as reported in Table 3.

Since the normal distribution was not satisfied for all data sets obtained (not in all cases the p-value was less than 0.05), a nonparametric test was adopted for statistical analysis of the data. Thus, the Kruskal-Wallis H test was performed. Statistically significant differences in the number of positively stained vessels, leukocytes and cardiomyocytes were found, as reported in Table 4.

3.3. RT-PCR (mRNA-210)

mRNA-210 levels were investigated through RT-PCR. Results normalization was achieved using cel-miR-39, an exogenous miRNA. mRNA-210 was not detectable in 10 cases belonging to group 2 and in 3 cases belonging to group 1. In Group 1 (n = 19), a mean value of mRNA-210 expression (RT-PCR) of 0.345 was found, ranging from a minimum of 0 to a maximum of 2.6. In Group 2 (n = 16), a mean value of mRNA-210 expression (RT-PCR) of 0.013 was found, ranging from a minimum of 0 to a maximum of 0.124. Data are shown in Graph 1 and Graph 2.

First, Shapiro-Wilk test was performed, as reported in Table 5.

The ANOVA test documented statistically significant differences in mRNA-210 levels between the two groups, as reported in Table 6.

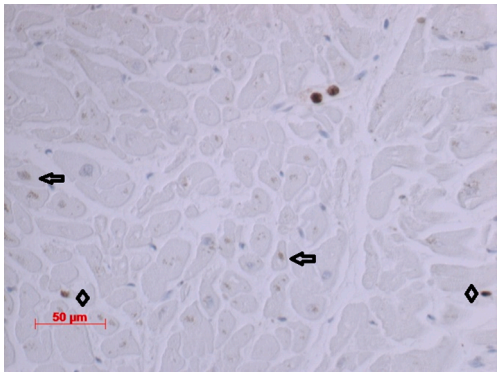


Fig. 2b. Anti-hif-1α (ihc) – group 1: positive leucocytes indicated by rhombuses, positive cardiomyocytes by arrows.

Table 2
Anti-hif-1α positivity (ihc).

anti-HIF-1α positivity (IHC)		Group 1	Group 2
Vessels	N° cases	19	16
	Mean	9.7	3.4
	Minimum	0	0
	Maximum	14	11
Leukocytes	N° cases	19	16
	Mean	19.7	3
	Minimum	0	0
	Maximum	27	13
Cardiomyocytes	N° cases	19	16
	Mean	9.4	0.8
	Minimum	0	0
	Maximum	25	7

Table 3
Shapiro-wilk normality test, data regarding IHC.

Shapiro-Wilk normality test			
		W	**p-value**
Group 1	Vessels	0.92876	0.1643
	Leukocytes	0.84839	0.006316
	Cardiomyocytes	0.78425	0.000681
Group 2	Vessels	0.41355	4.415e-07
	Leukocytes	0.27265	4.553e-08
	Cardiomyocytes	0.27265	4.553e-08

Table 4
Kruskal-Wallis rank sum test, data regarding IHC.

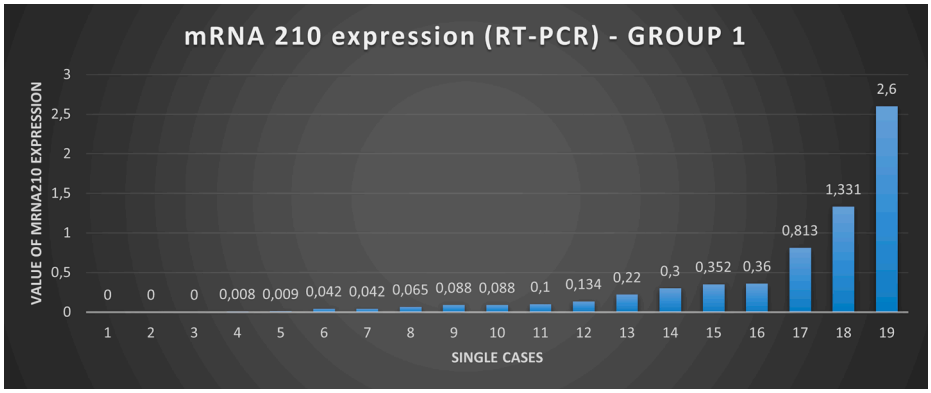
Kruskal-Wallis rank sum test			
	chi-squared	**df**	**p-value**
Vessels	21.396	1	3.735e-06
Leukocytes	23.473	1	1.267e-06
Cardiomyocytes	14.109	1	0.0001725

4. Discussion and Conclusions

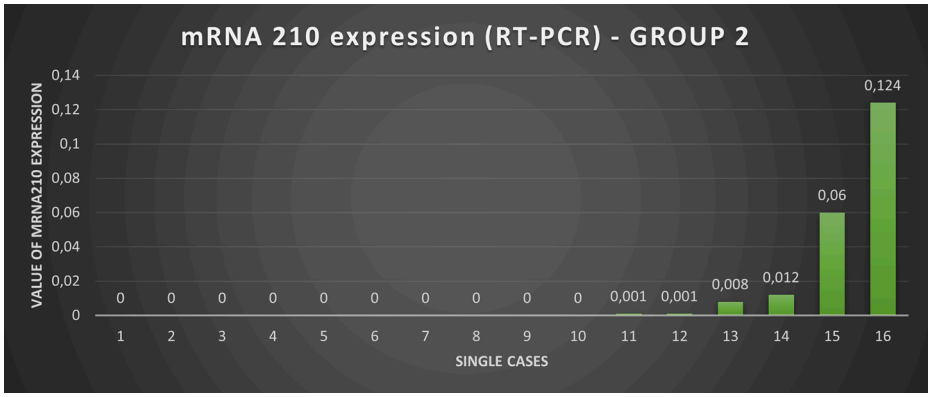
Cellular hypoxia refers to a condition in which the demand for molecular oxygen, necessary to produce the levels of ATP required to perform physiological functions, exceeds the vascular supply. As a result, a state of oxygen depletion occurs. To protect against hypoxia, eukaryotic cells are able to elicit oxygen- sensitive adaptive transcriptional responses driven primarily by the hypoxia- inducible factor (HIF) pathway [30].

In normoxic conditions, HIF-α subunits are hydroxylated by oxygen-dependent prolyl-4-hydroxylases (PHDs). Von Hippel–Lindau protein, an E3 ubiquitin ligase, binds to the hydroxylated HIF-α and acts as a substrate recognition component of the E3 ubiquitin ligase complex, which leads to the proteosomal degradation of HIF protein. The asparagine residues of HIF-α subunits are also hydroxylated by factors inhibiting HIFs (FIHs). Thus, under normoxic conditions, although the HIF-1α subunit is expressed, it degrades quickly, so it does not accumulate. Under hypoxia, the activity of PHDs and FIHs are suppressed, so HIF-α subunits translocate into the nucleus to bind with HIF-1β. The heterodimeric HIF-α:HIF-1β transcription factor complex locates to the hypoxia-responsive elements of its target genes, resulting in their transcriptional upregulation [31,32].

Liu et al (2020) reported that HIF-1α is involved in many signalling pathways, like the phosphatidylinositol-3kinase signalling pathway, the SENP1/HIF-1α signalling pathway, the HIF-1α /BNIP3/Bcline-1 signalling pathway, the Bal-2/adenovirus E1B 19kD-related protein 3 signalling pathway, and the MAPK/ HIF-1α signalling pathway [33].



Graph 1. mRNA expression (RT-PCR) in group 1.



Graph 2. mRNA expression (RT-PCR) in group 2.

Table 5
Shapiro-wilk normality test, miR-210 values.

Shapiro-Wilk normality test		
	W	**p-value**
Group 1	0.57276	2.381e-06
Group 2	0.46068	1.019e-06

Table 6
ANOVA test, miR-210 values.

ANOVA test					
	Df	Sum Sq	Mean Sq	F value	**Pr (>F)**
miR-210 values					
Group	1	0.957	0.9572	4.273	**0.0466**
Residuals	33	7.393	0.2240		

Given that miRNAs reduce the protein output from existing transcripts, they are the perfect candidates for controlling HIF expression during hypoxia. Thus, MicroRNAs (miRNAs) are small molecules of 20–26 nucleotides with a highly conserved sequence of single chain-encoded RNA. miRNAs regulate various biological processes such as cell proliferation, differentiation, apoptosis, autophagy, mitochondrial metabolism, angiogenesis, and haematopoiesis. These effects are largely achieved by destabilizing target mRNAs or inhibiting translation [34].

Hence, during early hypoxia, specific temporal changes of miRNA levels may contribute to HIF-1 accumulation; moreover, during prolonged hypoxia, the miRNA expression is changed to help to maintain low HIF-1 function. 40 miRNAs capable of modulating HIF expression have been identified. Conversely, HIF-1 promotes the expression of several hypoxamiRs including miR-210. In particular, Kelly et al. (2011) identified a regulator of HIF-1, glycerol-3-phosphate dehydrogenase 1-like (GPD1L), which is regulated by the HIF-1-inducible mRNA-210. Induction of mRNA-210 by HIF-1 during hypoxia causes a decreased GPD1L protein expression, which in turn results in increased HIF-1 stability. Conversely, when HIF-1 expression is low, mRNA-210 level is low as well, and, as a consequence, GPD1L expression is turned-on [30].

MicroRNA-210 is significantly upregulated during hypoxia. Under hypoxic conditions microRNA-210 is involved in cell growth arrest, stem cell survival, repression of mitochondrial respiration, angiogenesis, and arrest of DNA repair [35,36]. The expression of this miRNA is regulated by HIF-1α. Serocki et al (2018) hypothesised that, during acute hypoxia, miR-210 levels are induced by HIF-1/HIF-2 to prevent HIF-3 accumulation, while during chronic hypoxia HIF-1/HIF-2 levels decline and lead to the reduction of miR-210 and subsequent HIF-3 signaling [35].

Takai et al (2020) investigated the histopathological expression of microRNA-210 under hypoxic conditions using a femoral artery ligation model established in C57BL/6J mice; the Authors concluded that in situ hybridization of microRNA-210 could potentially be used for the detection of hypoxia as a histological marker in addition to the IHC of HIF-1α. [36].

Indeed, it is well known that MicroRNA-210 expression is altered in cardiovascular diseases such as atherosclerosis, AMI, aortic stenosis, and heart failure; furthermore, overexpression of microRNA-210 in some of these diseases exerts protective effects on target organs [34,37]. Emerging evidence suggests that miR-210 can be considered an independent indicator of the extent of cellular damage and for severity assessment of various clinical CVDs. miR-210 can also predict the prognosis of diseases such as atherosclerosis and can guide the development of follow-up treatment strategies [37].

Giving these premises, the role of HIF-1α and mRNA-210 as possible markers of AMI in clinical and forensic pathology remains elusive, even though immunohistochemical and gene expression methods have recently investigated various candidates [23]. As a results, this study comprises, to the best of our knowledge, the first attempt to assess and correlate HIF-1α and mRNA-210 in post-mortem cardiac samples in order to examine a possible role in the diagnosis of EMI.

Thus, in the present study, we analysed postmortem cardiac samples. We evaluated HIF-1α expression using IHC staining, and mRNA-210 level through RT-PCR, and compared against each other, in order to define if HIF-1α and mRNA-210 might represent reliable markers of EMI.

Exclusion criteria applied in the study comprised subjects whose specimens tested positive for drugs because of possible alterations in myocardial tissue (certain toxic substances clearly proved to be cardiotoxic: chronic and excessive alcohol consumption is one of the main causes of dilated cardiomyopathy, while cocaine use is associated with intramyocardial small-vessel disease, cardiomegaly with left ventricular hypertrophy, myocardial fibrosis, and eosinophilic myocarditis; cases of methamphetamine-induced cardiomyopathy have also been described [38,39]), cases of traumatic cardiac and pericardial injuries (traumatic injuries are related to histological alterations such as haemorrhagic infiltration), cases of asphyxial deaths (some markers are expressed both in AMI and in global hypoxia [24]).

4.1. IHC staining (HIF-1α marker) – group 1

Group 1 was characterized by cases of sudden, natural deaths caused by acute myocardial infarction (n = 19). In 10 out of 19 cases, immunostaining positivity was observed only in myocardium and sub-endocardium samples, confirming that the sub-endocardium is more vulnerable than the epicardium to the effects of ischemia and hypoxia [40]. In the single case in which immunohistochemical staining positivity was detected exclusively in the myocardium, a viral myocarditis was diagnosed. Since it is known that viral myocarditis can cause cardiomyocyte degeneration, necrosis, and immune-inflammatory response in the myocardium [41], the pattern of IHC positivity could be explained by this pathological process.

In cases with absence of histological aspects of coronary artery disease (n = 6), the majority of IHC positive leukocytes were represented by tissue-resident macrophages. Indeed, previous research has documented that HIF-1α and HIF-2α are up-regulated by human macrophages exposed to hypoxia in vitro [42]. In cases characterized by coronary artery disease diagnosed through histological observation (n = 13), the majority of positively stained leukocytes showed intravascular location, suggesting a HIF-1α-induced margination, and comparative observation on H&E-stained samples demonstrated that the majority of IHC positive leukocytes were associated to microhemorrhages. Indeed, it has been demonstrated that leukocyte hypoxia induces increased adhesion to endothelia, as well as leukocyte CD18 mRNA and surface protein expression. Moreover, HIF-1 binds to the proximal 166-bp CD18 promoter in a region that bears a classic hypoxia response element, and this binding is induced by hypoxia [43].

In 9 out of 19 cases, no positive cardiomyocytes were found. It might be related to an immediately fatal arrythmia and a consequently short agonal period [44], resulting in no accumulation of HIF-1α in cardiomyocytes. In 3 out of 19 cases, wavy cardiomyocytes showed immunohistochemical staining positivity, suggesting that wavy cells underwent hypoxic injury.

The mean value of IHC positive cardiomyocytes in cases characterized by coronary artery disease diagnosed through histological observation was about five times as high as in cases characterized by the absence of histological aspects of coronary artery disease (12.7 vs 2.4). These results suggest that cardiomyocytes HIF-1α positivity in cases with coronary artery disease could be largely attributed to a chronic hypoxic damage.

4.2. IHC staining (HIF-1α marker) – group 2

Group 2 was composed of 16 control cases. Comparative observation on H&E-stained samples demonstrated that the majority of IHC positive leukocytes were represented by tissue-resident macrophages.

IHC positivity was found only in 3 out of 16 cases. In 2 cases of them, a relevant number of IHC positive cells was found in myocardium and in sub-endocardium. In both cases the cause of death was identified as lethal traumatic brain injury related to road accident. In one case, the subject was found dead the morning after the accident, while in the second case the subject died about 2 h later the accident. In the light of these elements, it seems reasonable to assume that the agonal period caused a hypoxic condition which produced an accumulation on HIF-1α in sub-endocardium and in myocardium, but not in sub-epicardium, which is less vulnerable to hypoxic injury [40].

In the third case characterized by IHC positivity, the immunostaining positive pattern involves sub-endocardium, myocardium, and sub-epicardium. In this case the cause of death was identified as lethal traumatic brain injury related to road accident. The subject died about 11 h later the accident. Thus, we can hypothesize that the prolonged agonal period caused a protracted hypoxic condition which produced an accumulation on HIF-1α not only in sub-endocardium and in myocardium, but also in sub-epicardium.

Beside these three cases, the mean value of IHC positive cells for each case in the remaining 13 cases was zero.

Statistical evaluation demonstrated statistically significant differences in terms of number of IHC positive vessels, leukocytes, and cardiomyocytes in group 1, so our data indicate that HIF-1α could be reliably used in the diagnostic process of AMI in forensic practice.

4.3. RT- PCR (mRNA-210)

In Group 1, there was detectable evidence of mRNA-210 in the majority of cases (16 out of 19 cases). In Group 2, there was detectable evidence of mRNA-210 only in 6 out of 16 cases. 3 of these are the cases in which IHC positivity was found.

Statistical analysis documented statistically significant differences in the expression of mRNA-210 through RT-PCR between the two groups, suggesting that mRNA-210 could represent a promising additional tool in the diagnosis of EMI.

Our data seem to confirm an over-expression of mRNA-210 in response to hypoxic damage in cardiomyocytes, as reported by Boštjančič et al. (2009) [45]. Furthermore, Fasanaro et al (2008) found that mRNA-210 up-regulation is a crucial element of endothelial cell response to hypoxia, affecting cell survival, migration, and differentiation [46].

Given the paucity of the cohort examined, this study can be considered as a pilot study in the assessment of the role of HIF-1α and mRNA-210 in postmortem cardiac samples. Although it does not claim to be an experimental study, the results of our study make a compelling argument to pursue more rigorous assessments.

4.4. Limitations

Limitations in the present study might be attributed mainly to the small sample size and to the different age ranges of the two groups. There was a relatively narrow spectrum of cases, which affected the stated results. Because the sample size was small, cases were not stratified by post-mortem interval, although a relationship between the extent of HIF-1α and HIF-1α mRNA expression and the time elapsed from death has been recently suggested in gingival tissues [47].

5. Conclusions

The present study indicates that HIF-1α and mRNA-210 in post-mortem cardiac specimens could represent appropriate biomarkers in

the diagnosis of AMI. Interestingly, the structures that documented the most relevant results were the vessels. The results of this study corroborate the use of both the two techniques used: immunohistochemistry and gene expression studies. Declined in a forensic context, the use of microRNAs appears promising because it provides quantitative results, and therefore potential numerical data to be brought to court. On the other hand, immunohistochemistry may be attractive because of the possibility of showing microscopic images that provide qualitative information.

Since mRNAs do not undergo major changes after death and various mRNAs are invoked in the increase or decrease of HIF-1α, more meaningful results will be obtained by performing mRNA-seq in addition to mRNA-210. Furthermore, by finding combinations of molecules that specifically increase or decrease in acute myocardial infarction obtained from mRNA-seq results, it will be possible to contribute to the further development of forensic diagnosis.

Notwithstanding the limitations, these results do confirm the growing role of immunohistochemical and gene expression studies and the correlation between immunohistochemistry and genetics in forensic pathology. Further research is required to collect a higher number of samples and to stratify cases according to parameters such as post-mortem interval and age range.

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All the authors contributed to the design and implementation of the research, to the analysis of the results and to the writing of the manuscript.

Disclaimers.

The views expressed in the submitted article are our own and not an official position of the institution.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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