Int J Legal Med (2016) 130:1265–1280
DOI 10.1007/s00414-016-1401-9

ORIGINAL ARTICLE

Early markers for myocardial ischemia and sudden cardiac death

Sara Sabatasso[1,2] · **Patrice Mangin**[1] · **Tony Fracasso**[1] · **Milena Moretti**[1,2] · **Mylène Docquier**[3] · **Valentin Djonov**[2]

Received: 2 February 2016 / Accepted: 13 June 2016 / Published online: 8 July 2016

Abstract The post-mortem diagnosis of acute myocardial ischemia remains a challenge for both clinical and forensic pathologists. We performed an experimental study (ligation of left anterior descending coronary artery in rats) in order to identify early markers of myocardial ischemia, to further apply to forensic and clinical pathology in cases of sudden cardiac death. Using immunohistochemistry, Western blots, and gene expression analyses, we investigated a number of markers, selected among those which are currently used in emergency departments to diagnose myocardial infarction and those which are under investigation in basic research and autopsy pathology studies on cardiovascular diseases. The study was performed on 44 adult male Lewis rats, assigned to three experimental groups: control, sham-operated, and operated. The durations of ischemia ranged between 5 min and 24 h. The investigated markers were troponins I and T, myoglobin, fibronectin, C5b-9, connexin 43 (dephosphorylated), JunB, cytochrome c, and TUNEL staining. The earliest expressions (≤30 min) were observed for connexin 43, JunB, and cytochrome c, followed by fibronectin (≤1 h), myoglobin (≤1 h), troponins I and T (≤1 h), TUNEL (≤1 h), and C5b-9 (≤2 h). By this investigation, we identified a panel of true early markers of myocardial ischemia and delineated their temporal evolution in expression by employing new technologies for gene expression analysis, in addition to traditional and routine methods (such as histology and immunohistochemistry). Moreover, for the first time in the autopsy pathology field, we identified, by immunohistochemistry, two very early markers of myocardial ischemia: dephosphorylated connexin 43 and JunB.

Keywords Early markers · Myocardial ischemia · Forensic pathology · Clinical pathology · Sudden cardiac death

Introduction

Ischemic heart disease (IHD) remains the leading cause of death worldwide and is the primary cause of sudden cardiac death (SCD) [1, 2]. Although about 50 % of cardiac arrests occur in individuals with a known heart disease, many people suffer from concealed IHD and SCD is the first manifestation of the disease [1, 2]. The more recurrent substrate of IHD is atherosclerotic coronary artery disease [3]. In the latter condition, the triggering factor for arrhythmia responsible for SCD is a transitory (coronary spasm) or prolonged (occlusive coronary stenosis) myocardial ischemia [4, 5].

In SCD cases, the post-mortem diagnosis of acute myocardial ischemia represents a current challenge for both clinical and forensic pathologists, especially when death occurs within a short period of time (minutes to a few hours) after the onset of the ischemic injury. The earliest light microscopy changes (wavy fibers, coagulative necrosis including cytoplasmic homogenization and hypereosinophilia, nuclear alterations) might be subtle and non-obvious. A most conclusive histologic change, the neutrophils infiltration, becomes apparent as early as 6 to 12 h [6–8]. The well-known features of chronic

Electronic supplementary material The online version of this article (doi:10.1007/s00414-016-1401-9) contains supplementary material, which is available to authorized users.

✉ Sara Sabatasso
sara.sabatasso@hcuge.ch

[1] University Center of Legal Medicine Lausanne-Geneva, Rue Michel-Servet 1, CH 1211 Geneva, Switzerland

[2] Institute of Anatomy, University of Bern, CH 3000 Bern, Switzerland

[3] Genomics platform, Centre Médical Universitaire, University of Geneva, CH 1211 Geneva, Switzerland

inflammation, granulation tissue formation, and fibrosis occur later, in terms of weeks [7–10].

At present, there is no highly specific and sensitive "gold standard" for the diagnosis of early myocardial ischemia, i.e., occurring before the evolution of the histologic changes. Several attempts, mainly at the morphological level, have been made in order to find out the proper methodology for this diagnosis, with no significant results in terms of detection of truly early myocardial ischemia.

Recent works on post-mortem diagnosis of early myocardial ischemia have investigated, at the immunohistochemical level, some markers that accumulate in (such as fibronectin) or leak from (such as troponins, myoglobin, and S100A1) the human cardiomyocyte when the ischemic injury occurs [5, 11–13]. The rationale behind immunohistochemical investigations is that ischemia damages the cellular membrane, contractile proteins, cytoskeleton, and subcellular organites in a relatively short time, so antibodies against various components of the cardiomyocyte can be used. However, the time needed to observe such a depletion or accumulation in the cardiomyocyte is still too long: at least 1 h should have elapsed from the onset of symptoms to death to see some histochemical modifications, even using a combination of various markers. The diagnostic potential of markers/mediators of early inflammation (CD15, Il-1ß, IL-6, TNF-α, IL-15, IL-8, MCP-, ICAM-1, CD18, and tryptase) during myocardial infarction has also been extensively investigated and discussed [14–17].

In forensic and clinical pathology, the role of cardiomyocyte apoptosis as a diagnostic tool in cases of early myocardial injury has been investigated by TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP biotin nick end-labeling) assay, biochemistry (DNA laddering method), and immunohistochemistry [18–22]. TUNEL assay is sensitive for the detection of ischemia, but not specific, as apoptosis can be triggered also by non-ischemic events. Moreover, it allows the detection of ischemia not earlier than 2–4 h in human post-mortem material and as early as 45 min in experimental models [18–22].

Gene expression studies on rodent myocardium undergoing ischemia in experimental conditions seem to be more promising. Harpster et al. have shown the early induction of some genes as soon as 15 min after left anterior descending (LAD) coronary artery ligation in mice. These genes mainly encode for the AP-1 (activator protein 1) [23].

In this investigation, we wanted to test some markers of early myocardial ischemia, selected among those which are currently used in emergency departments to diagnose myocardial infarction (namely, troponins I and T and myoglobin) [24] and those which are under investigation in basic research and forensic pathology studies on cardiovascular diseases (fibronectin, C5b-9, connexin 43 (Cx43), JunB, TUNEL staining [5, 10, 11, 18–23, 25–28], and cytochrome c). In order to do this, we performed immunohistochemistry and gene expression analysis (using the NanoString nCounter® gene expression system [29]). The protein expression of Cx43 has been evaluated by Western blots as well.

We used a rat model of myocardial ischemia (ligation of LAD [left anterior descending] coronary artery).

Materials and methods

A detailed description is available in Supplementary materials and methods (see Supplementary material).

Animal model of myocardial ischemia

Permanent occlusion of the LAD coronary artery provides a model for acute myocardial ischemia/infarction, whereby the development of an ischemic/infarcted zone in the left ventricle mimics intra-arterial blockage (e.g., atherogenic thrombosis) [30]. All applicable international, national, and institutional guidelines for the care and use of animals were followed and all procedures were performed in compliance with the Swiss veterinary law regulations. The study protocol was approved by the Institutional Animal Care and Use Committee (license FR 21/09, delivered by the Swiss Federal Veterinary Office).

Forty-four adult male Lewis rats (weighing 250–300 g) were used for the experiment and assigned to three experimental groups: control (5 rats), sham-operated (12 rats, i.e., 3 rats per 4 time points), and operated (27 rats, i.e., 3 rats per 9 time points). The operated animals underwent complete LAD ligation. The time points after LAD ligation were $t = 5$', $t = 15$', $t = 30$', $t = 1$ h, $t = 2$ h, $t = 4$ h, $t = 7$ h, $t = 12$ h, and $t = 24$ h. For the sham operations, we chose only four time points: 30', 1, 4, and 12 h.

Tissue processing for light microscopy and immunohistochemistry

At each time point after LAD ligation and for each experimental group, the hearts were harvested and cut in two halves at the level of ligation. The slices cut just below the ligation (about 3–4 mm thick, including the ischemic part, the border zone ischemic-free wall, and the free wall) were fixed in paraformaldehyde 2 % (for 2 h) and then in alcohol 70 %. Tissue was then embedded in paraffin. Four to five micrometer sections were prepared from paraffin blocks and either stained with hematoxylin-eosin (H&E) or with different antibodies for immunohistochemistry.

Immunohistochemistry

For the antibodies anti-fibronectin, C5b-9, troponin I and T, and JunB, we used the streptavidin/biotin immunoperoxidase detection system (Vector labs) and DAB (3,3′-

Diaminobenzidine) as a chromogenic substrate, with hematoxylin counter-staining. For the anti-myoglobin antibody, a HRP conjugated secondary antibody was used for reaction and DAB for its detection. For the anti-unphosphorylated Cx43 antibody, we used immunofluorescence with a Cy3 conjugated secondary antibody.

For the TUNEL assay, we used the in situ cell death detection kit, POD, from Roche.

Semi-quantitative evaluation of immunohistochemistry and TUNEL

Each slide was observed by two examiners. The distribution of the staining reactions was scored semi-quantitatively in the ischemic (antero-lateral wall of the left ventricle) and non-ischemic (right ventricle) regions of the same heart.

Antigens with accumulation into the cardiomyocyte (fibronectin, C5b-9) or with an expression within the gap junctions (Cx43) or the nucleus (JunB) were graded as 0 = negative, 1 = staining of single cells, 2 = staining of cell groups, and 3 = large/diffuse staining. Similarly, the TUNEL assay was graded as 0 = negative, 1 = staining of single cells, 2 = staining of cell groups, and 3 = large/diffuse staining.

Antigens with loss/depletion from the cardiomyocyte (troponin I and T, myoglobin) were graded as 0 = negative, 1 = single cell loss of the reaction, 2 = cell groups loss, and 3 = diffuse loss.

For myoglobin, the staining could not be assessed in 4 rats (1 at 30', 2 at 1 h, and 1 at 2 h of ischemia).

Statistical analyses

When comparing marker values between early (30'–2 h) and later (4–12 h) ischemia, differences were assessed using Fisher's exact tests. For each marker, the values from 0 to 3 of the semi-quantitative evaluation were compared between 9 hearts (rats) in the early (30'–2 h) ischemia and 9 hearts (rats) in the later (4 h–12 h) ischemia. Statistical significance was assessed at the 0.05 level for all analyses.

Protein extraction and Western blotting for Cx43

Proteins were extracted from ischemic and non-ischemic ventricular myocytes exposed to the various durations of myocardial ischemia in modified RIPA buffer, as previously described [31]. Western blotting was performed using antibodies against all Cx43 species irrespective of the phosphorylation sites (BD Transduction Laboratories), phosphorylated-Cx43 (Ser 368, from Cell Signaling), and GAPDH (Chemicon).

Gene expression analysis

To perform the gene expression analysis, we used the NanoString nCounter® gene expression system [29].

Individual Teg and gene sequences (or gene ID) were scanned to design a multiplexed probe library, named CodeSet. These sequences were sent to the NanoString company, which performed design of optimal oligonucleotides, and the NanoString nCounter assays were performed as previously described [32].

As target genes to test in the CodeSet, we chose Junb (JunB), as it was among the earliest markers to be expressed at the immunohistochemical level, and Cycs (cytochrome c), in order to see whether a decrease in induction is observable as well after myocardial ischemia. Moreover, Cycs is one of the factors which has been shown to be downregulated after myocardial infarction [33]. Four genes for the normalization process were included in the CodeSet.

Results

H&E staining of ischemic hearts

By H&E staining, the first histological changes were evident already by 30' after LAD ligation, in terms of hypereosinophilia of some cardiomyocytes (Fig. 1a, b). The changes were more evident by 1 h after LAD ligation, when wavy fibers, interstitial edema, and hypereosinophilia of some cardiomyocytes were observed (Fig. 1c, d). By 2 h after LAD ligation, a more pronounced interstitial edema and patchy hypereosinophilic cardiomyocytes were observed (Fig. 1 e, f). The non-ischemic zone (right ventricle) is illustrated in Fig. 1g–l and shows no evident changes at 30' and 1 h. By 2 h, a slight ondulation of some fibers was seen in the non-ischemic (right ventricle) heart (Fig. 1k, l).

Biomarkers in the interval 15–30 min after LAD ligation: Cx43 and JunB

A very early immunohistochemical expression was shown for the antibodies anti-Cx43 (dephosphorylated) and anti-JunB. In fact, for anti-Cx43, for which we performed immunofluorescence in addition to chromogenic detection, we observed, as soon as 15' after LAD ligation, a gap junctional expression localized in a small ischemic region (Figs. 2b, d and 3a), with lateralisation (localization of the marker along the long axis of the cardiomyocyte) in some cardiomyocytes (Fig. 2d). A slightly more intense expression was observed at 30' after LAD ligation (Fig. 3b). A larger involved zone and a more intense fluorescence signal were observed, proportionally, with increasing durations of ischemia, as shown in Fig. 3c–

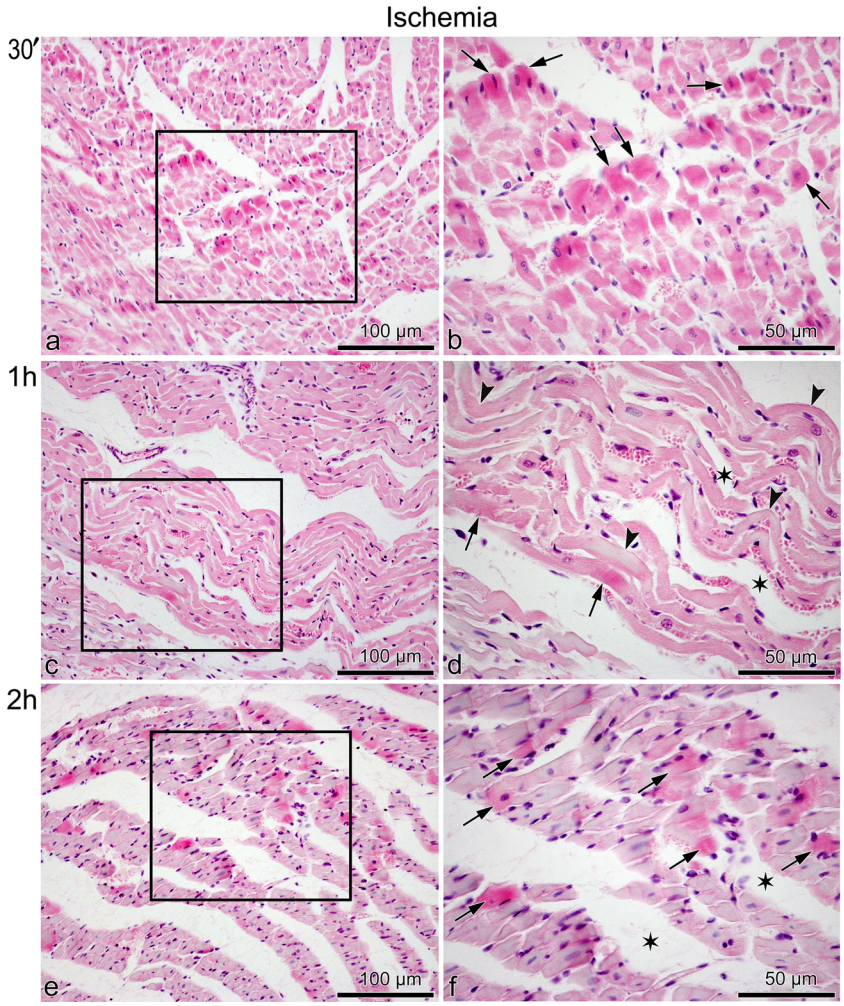


Fig. 1 H&E staining of ischemic (**a**–**f** antero-lateral wall of left ventricle) and non-ischemic (**g**–**l** right ventricle), control hearts. **a**, **b** (30 min after LAD ligation) Hypereosinophilia of some cardiomyocytes (*arrows*); **b** (×40) is a higher magnification from the rectangle in **a** (×20). **c**, **d** (1 h after LAD ligation) Wavy fibers (*arrowheads*), interstitial edema (*stars*), and hypereosinophilia of some cardiomyocytes (*arrows*); **d** (×40) is a higher magnification from the rectangle in **c** (×20). **e**, **f** (2 h after LAD ligation) More pronounced interstitial edema (*stars*) and patchy, hypereosinophilic cardiomyocytes (*arrows*); **f** (×40) is a higher magnification from the rectangle in **e** (×20). **g**–**l** The non-ischemic zone (right ventricle), with no evident changes at 30' and 1 h. By 2 h, a slight ondulation of some fibers (*arrowheads* in **l**) was seen (**k**, **l**). **h**, **j**, **l** (×40) are higher magnifications of the rectangles in **g**, **i**, and **k** (×20)

d. The non-ischemic zone (right ventricle) is illustrated in Figs. 2a, c and 3e and shows only very few positive cardiomyocytes, which are scattered in the subendocardium.

For JunB, the expected nuclear staining (as JunB is a transcription factor), limited to some nuclei, was observed as soon as 30' after LAD ligation in the subendocardial region (Fig. 4b, e). A gradient of expression towards the epicardium was evident with increasing duration of ischemia, as shown in Fig. 4c, f. The non-ischemic zone (right ventricle) is illustrated in Fig. 4a, d and shows no staining.

Biomarkers in the interval 1–2 h after LAD ligation: fibronectin, cardiac troponins I and T, myoglobin, C5b-9

In the ischemic zone (antero-lateral wall of the left ventricle), the antibody anti-total fibronectin showed accumulation into the cardiomyocytes as early as 1 h after LAD ligation (Fig. 5b, d). The non-ischemic zone (right ventricle) is illustrated in Fig. 5a, c and shows no intracellular staining.

The antibodies anti-cTnI, cTnT, and myoglobin showed depletion from the cardiomyocytes as early as 1 h after LAD ligation as well (Figs. 6b, d; 7b, d; and 8b, d, respectively). The non-ischemic zones (right ventricles) are illustrated in Figs. 6a, c; 7a, c; and 8a, c, respectively; any depletion of the three makers was observed in these zones.

The staining with the antibody anti-C5b-9 showed scattered accumulations in single cardiomyocytes as soon as 2 h after LAD ligation (Fig. 9b, d). No accumulation was seen in the non-ischemic zone (right ventricle), as shown in Fig. 9a, c.

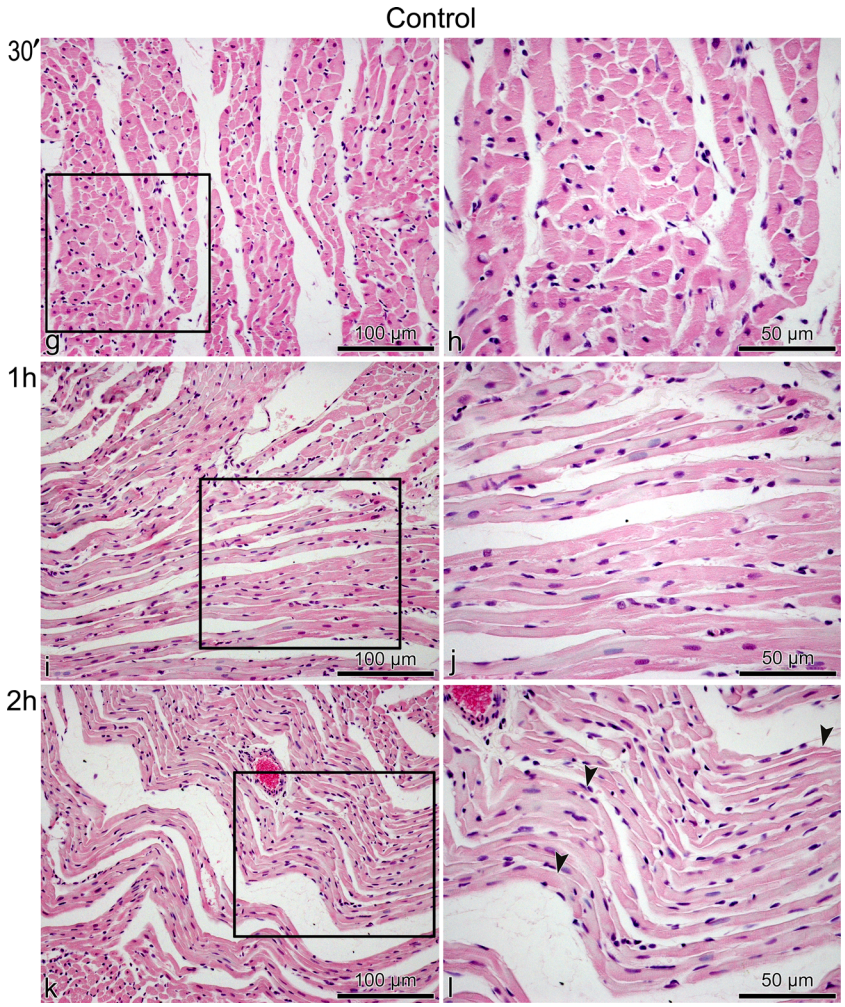


Fig. 1 continued.

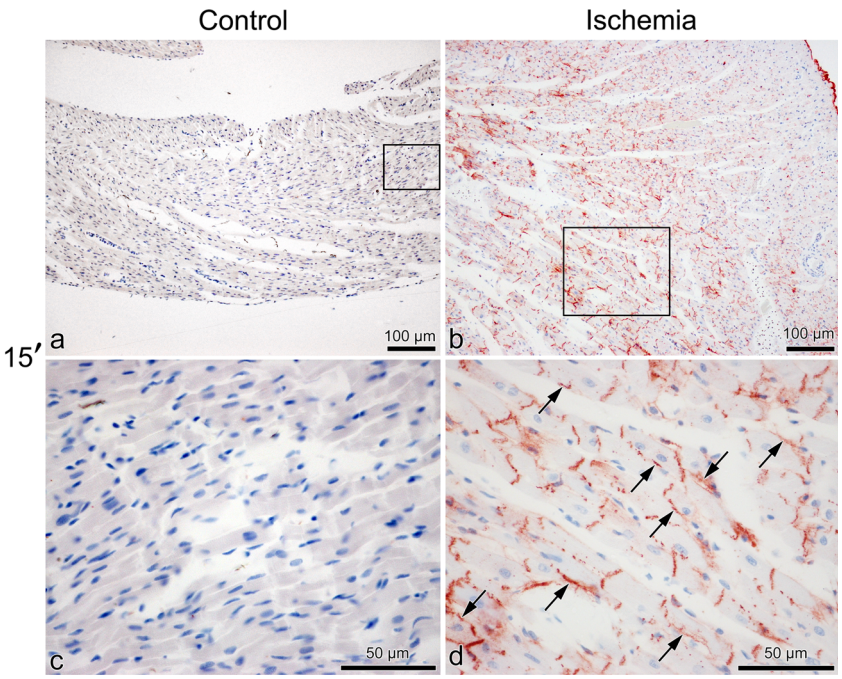


Fig. 2 Cx43 staining (chromogenic detection) of ischemic (**b**, **d** antero-lateral wall of left ventricle) and non-ischemic (**a**, **c** right ventricle) hearts. **b** (15 min after LAD ligation) Gap junctional expression of Cx43 in an intramyocardial zone (×10). **d** Higher magnification from the rectangle in **b** (×40). *Arrows* indicate cardiomyocytes with lateralisation of Cx43. **a**, **c** No expression of Cx43 is seen in the non-ischemic (right ventricle) part of the same heart. **c** (×40) is a higher magnification of the rectangle in **a** (×10)

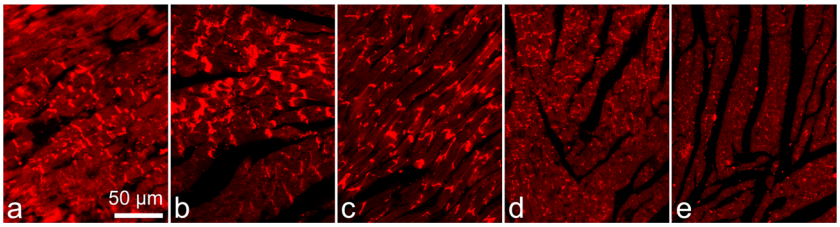


Fig. 3 Cx43 immunofluorescence. **a** (15 min after LAD ligation) Gap junctional expression in a small ischemic region. **b** (30 min after LAD ligation) Slightly more intense expression. A larger involved zone and a more intense fluorescence signal were observed, proportionally, at 1 h (**c**) and 2 h (**d**) after LAD ligation. No signal was detected in the non-ischemic zone (right ventricle, **e**)

TUNEL assay

The TUNEL assay showed nuclear positivities as soon as 1 h after LAD ligation, as shown in Fig. 10b, d. Surprisingly, we observed a slight cytoplasmic staining as well by TUNEL assay, which is apparently in contradiction with most of the literature reports and with the nuclear localization of the apoptotic process. Nevertheless, this contemporary nuclear and cytoplasmic TUNEL staining has already been described and the cytoplasmic localization has been interpreted as a manifestation of a simultaneous necrotic process [20]. No signal was detected in the non-ischemic zone (right ventricle), as illustrated in Figs. 10a, c and 11.

Semi-quantitative evaluation of immunohistochemistry and TUNEL

The scores of the staining reactions in the ischemic (MI) and non-ischemic (NI) regions of the same heart for each time point (3 hearts/rats per time point) are illustrated in Table 1. No reaction was observed (immunohistochemistry by chromogenic detection) at 15 min of ischemia. For the other time points, the results for all markers were consistent with the tendency described in the previous paragraphs.

When analyzing the very early phase of ischemia (ranging from 30' to 2 h), at 30' of ischemia, the only marker for which all 3 hearts had a score of 3 was Cx43. At 30' of ischemia, 2 hearts had a score of 2 and 1 heart had a score of 1 for JunB. For all the other markers, any heart had a score > 1 at 30' of ischemia. This confirms Cx43 and JunB as early markers of myocardial ischemia. By 1 h of ischemia, all 3 hearts still had a score of 3 for Cx43, and 2 out of 3 hearts had a score of 3 and 1 heart had a score of 2 for JunB, thus indicating a slightly more pronounced reaction for JunB. By 2 h of ischemia, 2 out of 3 hearts had a score of 3 and 1 heart had a score of 2 for Cx43, and for JunB, the 3 hearts had scores of 1, 2, and 3, respectively. This indicates a slightly less pronounced reaction for both markers with increasing durations of ischemia.

The graphical representation of the semi-quantitative evaluation in the ischemic myocardium is shown in Table 2. The results of the comparison of marker values between early (30'–2 h) and later (4–12 h) ischemia are illustrated in Table 3. The observed differences between markers were statistically significant for fibronectin ($p = 0.002$), C5b-9 ($p = 0.001$), cTnT ($p = 0.047$), and myoglobin ($p = 0.014$), meaning that

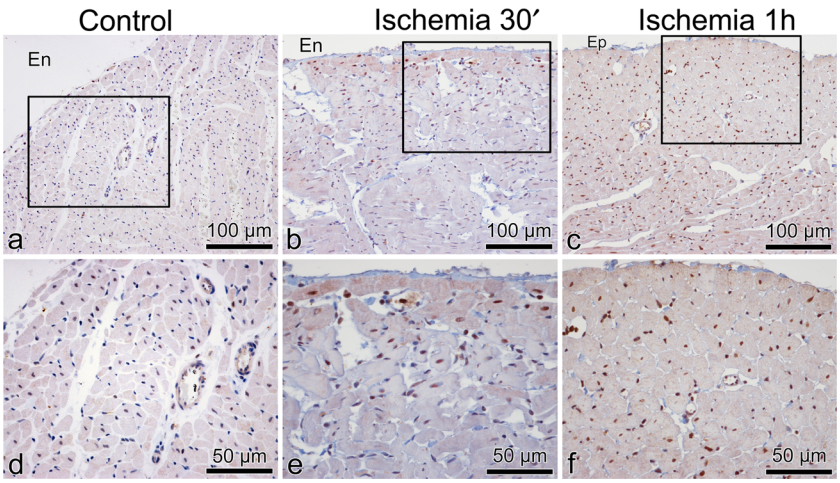


Fig. 4 JunB staining of ischemic (**b**, **c**, **e**, **f**: antero-lateral wall of left ventricle) and non-ischemic (**a**, **d**: right ventricle) hearts. **b** (30 min after LAD ligation) Expression of JunB in some nuclei (brown-stained) in the subendocardial region (×20). *En* endocardium. **e** Higher magnification from the rectangle in **b** (×40). **c** (1 h after LAD ligation) A gradient of expression towards the epicardium is shown (×20). *Ep* epicardium. **f** is higher magnification from the rectangle in **c** (×40). **a**, **d** Only very few, positive cardiomyocytes are seen in the non-ischemic (right ventricle) part of the same heart, scattered in the subendocardium. **d** (×40) is a higher magnification from the rectangle in **a** (×20)

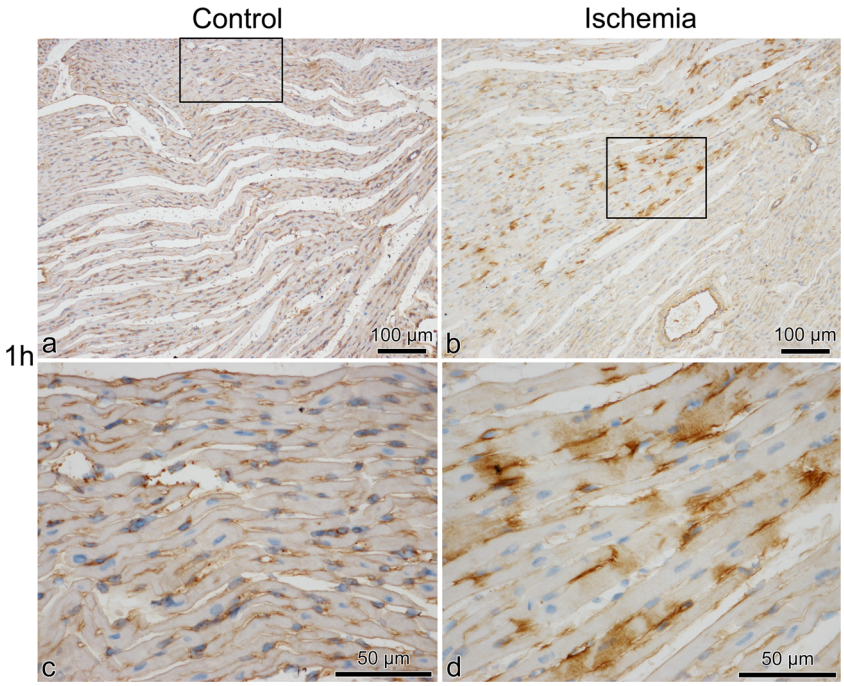


Fig. 5 Fibronectin staining of ischemic (**b**, **d** antero-lateral wall of left ventricle) and non-ischemic (**a**, **c** right ventricle) hearts. **b** (1 h after LAD ligation) Accumulation of fibronectin in single cardiomyocytes or in small groups of them (×10). **d** Higher magnification from the rectangle in **b** (×40). **a**, **c** No intracellular accumulation of fibronectin is seen in the non-ischemic (right ventricle) part of the same heart. **c** (×40) is a higher magnification of **a** (×10)

these markers are more expressed in the later phase of ischemia (by 4 h). Immunohistochemistry was negative (no ischemia was detected) in all control hearts for each marker.

Western blots

Regarding the Western blots for Cx43, we observed a temporary early induction (at 15' and 30' after LAD ligation) of phosphorylation on Serine 368 in the C-terminal tail of Cx43 (Fig. 11).

Gene expression analysis

The results of the gene expression analysis were analyzed using Student's t test. Statistical significance was set at $p < 0.05$.

Using a cutoff value of a fold change in expression > 1.5, we observed that JunB was significantly induced by 120' of myocardial ischemia with a fold change of 9.22. On the other hand, a significant decrease in induction was observed for the

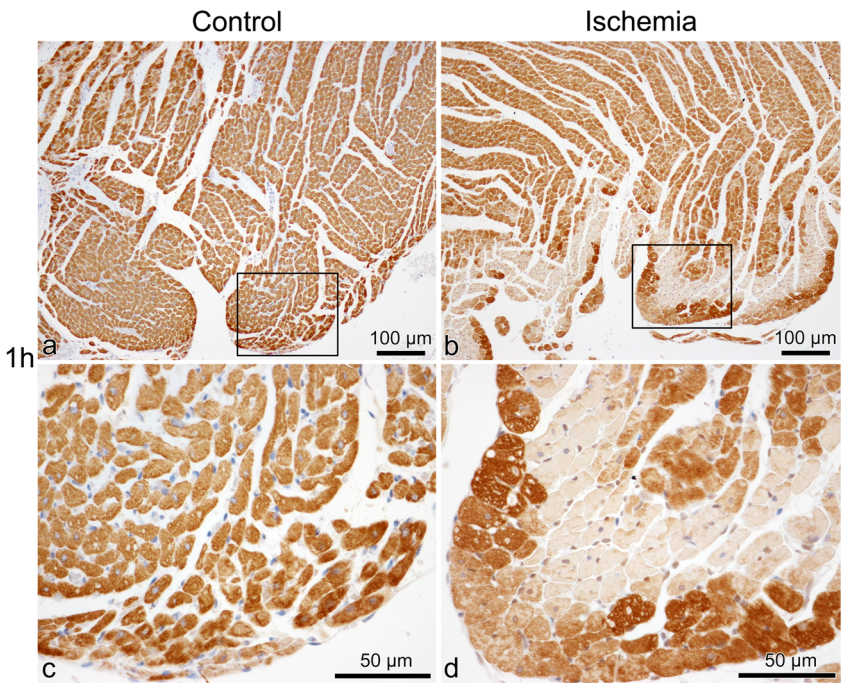


Fig. 6 cTnI staining of ischemic (**b**, **d** antero-lateral wall of left ventricle) and non-ischemic (**a**, **c** right ventricle) hearts. **b** (1 h after LAD ligation) Zones of depletion of cTnI in groups of cardiomyocytes, mainly in the subendocardial region (×10). **d** Higher magnification from the rectangle in **b** (×40). **a**, **c** No depletion of cTnI is seen in the non-ischemic (right ventricle) part of the same heart. **c** (×40) is a higher magnification from the rectangle in **a** (×10)

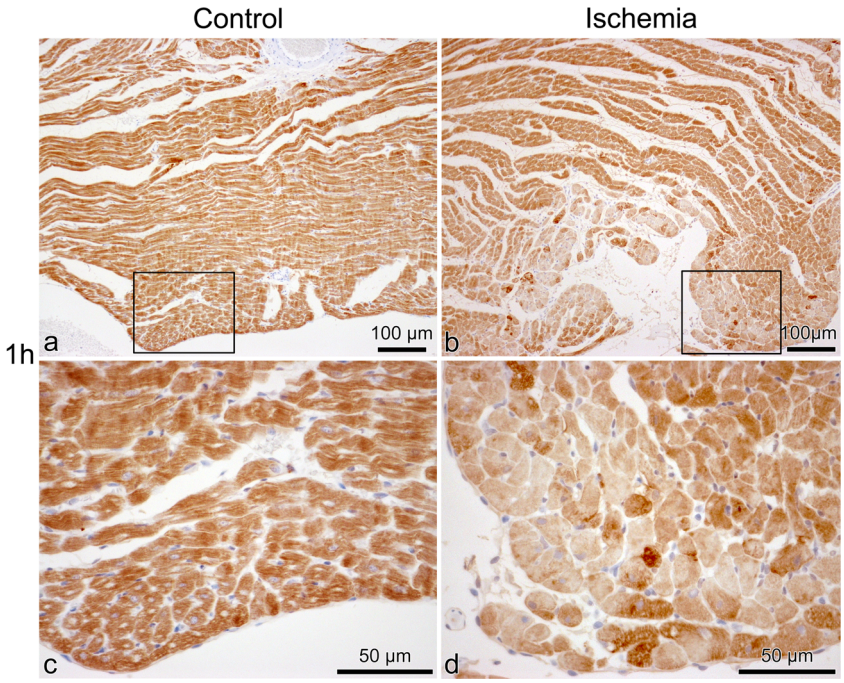


Fig. 7 cTnT staining of ischemic (**b**, **d** antero-lateral wall of left ventricle) and non-ischemic (**a**, **c** right ventricle) hearts. **b** (1 h after LAD ligation) Zones of depletion of cTnT in groups of cardiomyocytes, mainly in the subendocardial region (×10). **d** Higher magnification from the rectangle in **b** (×40). **a**, **c** No depletion of cTnI is seen in the non-ischemic (right ventricle) part of the same heart. **c** (×40) is a higher magnification from the rectangle in **a** (×10)

cytochrome c at 30' of myocardial ischemia with a fold change of 0.62.

Discussion

Our results confirm the limits of morphology (H&E staining) for the detection of very early myocardial ischemic damage [2, 7, 8, 34, 35]: in fact, in the first minutes/h following LAD ligation in rats, only subtle and non-specific modifications have been detected. On the other hand, immunohistochemical stainings with the antibodies we tested have given more satisfactory and robust results and have allowed us to select a panel of markers of early myocardial ischemia to further apply to autopsy cases of SCD. The markers we have studied are fibronectin, cTnI, cTNT, myoglobin, C5b-9, JunB, and Cx43. Moreover, we performed the TUNEL assay for apoptosis detection.

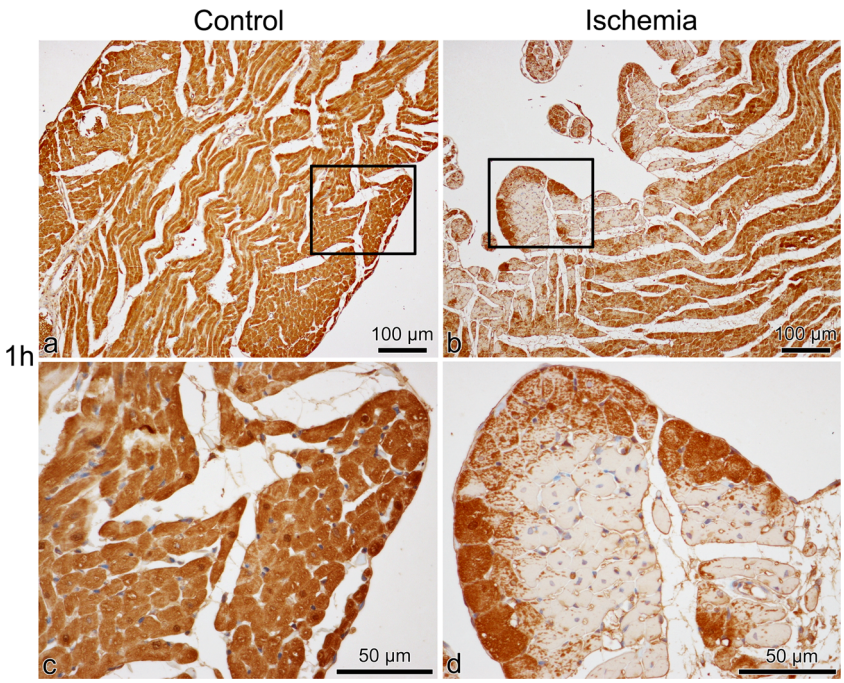


Fig. 8 Myoglobin staining of ischemic (**b**, **d** antero-lateral wall of left ventricle) and non-ischemic (**a**, **c** right ventricle) hearts. **b** (1 h after LAD ligation) Zones of depletion of myoglobin in groups of cardiomyocytes, mainly in the subendocardial region (×10). **d** Higher magnification from the rectangle in **b** (×40). **a**, **c** No depletion of myoglobin is seen in the non-ischemic (right ventricle) part of the same heart. **c** (×40) is a higher magnification from the rectangle in **a** (×10)

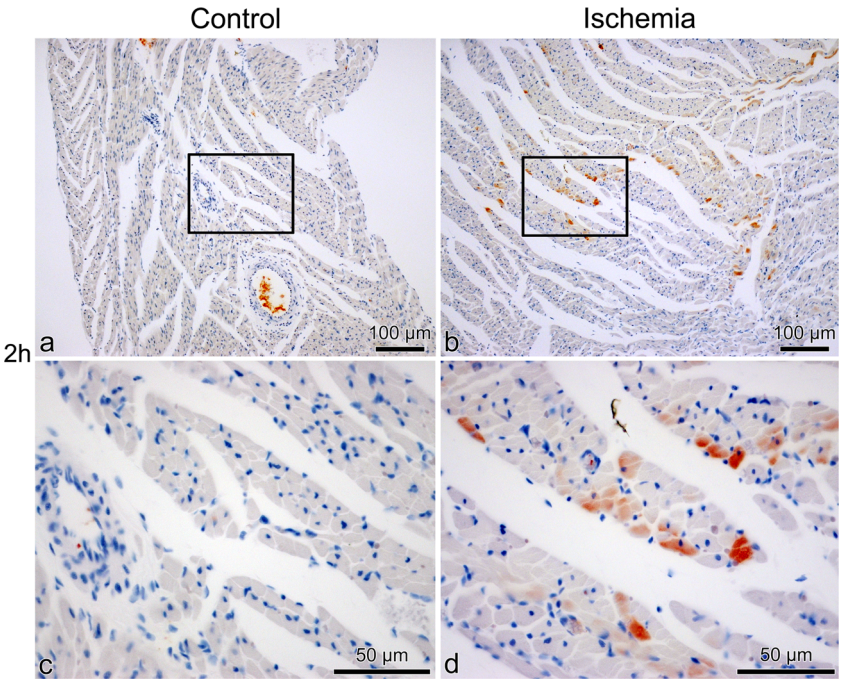


Fig. 9 C5b-9 staining of ischemic (**b**, **d** antero-lateral wall of left ventricle) and non-ischemic (**a**, **c** right ventricle) hearts. **b** (2 h after LAD ligation) Scattered accumulations in single cardiomyocytes. **d** Higher magnification from the rectangle in **b** (×40). **a**, **c** No accumulation of C5b-9 is seen in the non-ischemic (right ventricle) part of the same heart. **c** (×40) is a higher magnification from the rectangle in **a** (×10)

It has already been shown that no a single marker but a panel of markers can be useful for the detection of myocardial ischemia [5]. Immunohistochemical investigations have been made on various components of the cardiomyocyte, such as the cytoskeleton [36], the contractile proteins cTnI, cTnT [37, 38], and cTnC [39], and other proteins such as myoglobin [39–41], heart fatty acid-binding protein (H-FABP) [39], and desmin [36]. Other antigens have been investigated which accumulate into the cardiomyocyte after the ischemic damage of the cell membrane, such as fibronectin [5, 11, 12, 42–45] and C5b-9 [38–41, 46–48], or leak from it after the ischemic damage, such as S100A1 protein [13]. Most of these studies have been performed on human (autopsy) material, and their common findings were that no single marker is sensitive and specific enough to detect early myocardial ischemia, and that, even using a panel of markers, immunohistochemistry does not allow for the diagnosis of a very early myocardial ischemia, i.e., of duration of less than 1 h.

Fibronectin has been shown to be highly expressed in human [11, 12, 44, 45, 49] and experimental myocardial

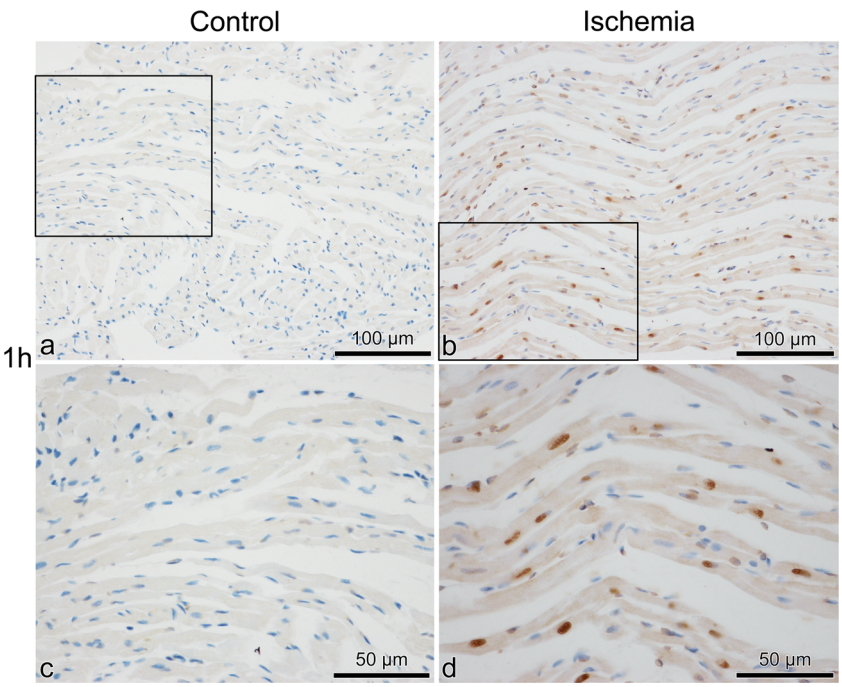


Fig. 10 TUNEL assay of ischemic (**b**, **d** antero-lateral wall of left ventricle) and non-ischemic (**a**, **c** right ventricle) hearts. **b** (1 h after LAD ligation) Nuclear positivities in groups of cardiomyocytes. Some signal is seen in the cytoplasm as well. **d** Higher magnification (×40) from the rectangle in **b** (×20). **a**, **c** No signal is seen in the non-ischemic (right ventricle) part of the same heart. **c** (×40) is a higher magnification from the rectangle in **a** (×20)

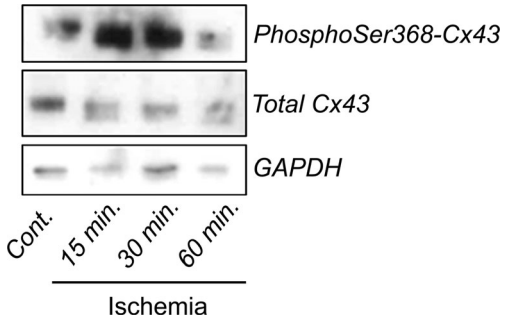


Fig. 11 Western blots for Cx43: temporary early induction (at 15 and 30 min after LAD ligation) of phosphorylation on Serine 368 in the C-terminal tail of Cx43

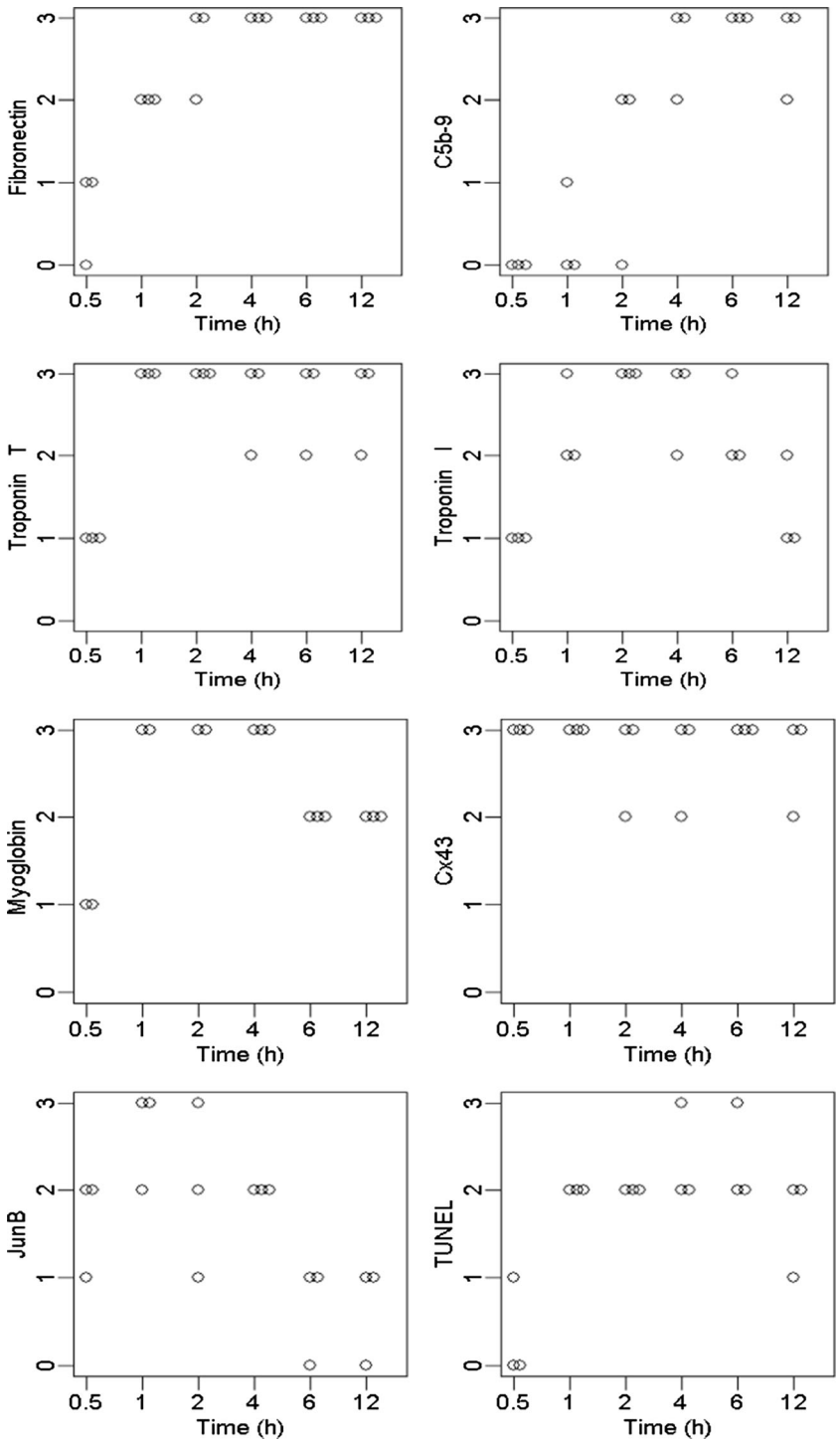
infarction [42–45, 50]. It is produced in a cellular and a plasma form [28]. During myocardial ischemia, the plasma fibronectin is most likely to accumulate in the cardiomyocyte, through loss of integrity of the plasma membrane [28].

The results of the immunohistochemical investigations we performed in our study show an earlier expression (1 h after LAD ligation) for the antibody anti-total fibronectin in comparison to what is reported in the literature concerning experimental models [42, 43]. On the other hand, our results are more consistent with what is reported in the literature concerning human (autopsy) material, as a deposition of fibronectin into ischemic cardiomyocytes has been described as early as 30 min to 1 h from the onset of symptoms to death [11, 12, 39]. However, our results are in contrast with those of Campobasso et al., who reported a significant expression of fibronectin only in cases with longer survival, i.e., with death occurring, according to witnesses, 8 to 10 h after the onset of symptoms [5].

Table 1 The scores of the staining reactions in the ischemic (MI) and non-ischemic (NI) regions of the same heart/rat at each time point (from 30 min to 12 h) after LAD ligation

Fibronectin 30’	30’			1 h			2 h			4 h			6 h			12 h		
	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3
MI	1	0	1	2	2	2	2	3	3	3	3	3	3	3	3	3	3	3
NI	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
C5b-9	30’			1 h			2 h			4 h			6 h			12 h		
	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3
MI	0	0	0	0	1	0	0	2	2	3	2	3	3	3	3	3	2	3
NI	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Troponin T	30’			1 h			2 h			4 h			6 h			12 h		
	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3
MI	1	1	1	3	3	3	3	3	3	2	3	3	3	2	3	2	3	3
NI	0	0	0	0	1	0	1	0	1	0	1	1	1	1	1	1	1	0
Troponin I	30’			1 h			2 h			4 h			6 h			12 h		
	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3
MI	1	1	1	3	2	2	3	3	3	3	3	2	2	2	3	1	1	2
NI	0	0	0	0	0	0	0	0	1	0	1	1	1	1	1	0	0	0
Myoglobin	30’			1 h			2 h			4 h			6 h			12 h		
	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3
MI	1	1		3	3		3	3		3	3	3	2	2	2	2	2	2
NI	0	0		0	0		0	1		0	1	1	0	1	1	0	0	1
Cx43	30’			1 h			2 h			4 h			6 h			12 h		
	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3
MI	3	3	3	3	3	3	3	3	2	2	3	3	3	3	3	3	2	3
NI	0	1	1	0	0	0	0	1	0	0	0	0	0	0	1	1	0	0
JunB	30’			1 h			2 h			4 h			6 h			12 h		
	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3
MI	1	2	2	2	3	3	1	2	3	2	2	2	1	1	0	1	1	0
NI	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
TUNEL	30’			1 h			2 h			4 h			6 h			12 h		
	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3	Rat 1	Rat 2	Rat 3
MI	1	0	0	2	2	2	2	2	2	3	2	2	2	2	3	2	2	1
NI	0	0	1	0	0	0	0	1	2	1	1	1	0	0	2	0	1	0

Table 2 Semi-quantitative evaluation of staining results for fibronectin, C5b-9, cTnT, cTnI, myoglobin, Cx43, JunB, and TUNEL at 0.5, 1, 2, 4, 6, and 12 h duration of ischemia. 0 = negative, 1 = staining/loss of staining of single cells, 2 = staining/loss of staining of cell groups, 3 = large/diffuse staining/loss of staining. *Empty dots* represent the number of hearts evaluated. For myoglobin, the staining was non-assessable in four rats (one at 0.5 h, two at 1 h, and one at 2 h of ischemia)



Concerning cTnI and cTnT, their measurement in serum is considered the gold standard test for the clinical diagnosis of acute myocardial infarction [51]. In post-mortem samples, they have shown good sensitivity and specificity as immunohistochemical markers (depletion from the cardiomyocyte) in cases of myocardial ischemia lasting for a few hours [5, 39]. In our investigation, we were able to show a depletion of both cTnI and cTnT from small groups of

Table 3 Marker values between early (30'–2 h) and late (4–12 h) ischemia. Differences were assessed using Fisher's exact test

	A	B 0–2 h	C 4–12 h	*p* value[a]
Fibronectin	0	1	0	0.002
	1	2	0	
	2	4	0	
	3	2	9	
C5b-9	0	6	0	0.001
	1	1	0	
	2	2	2	
	3	0	7	
Troponin T	0	0	0	0.047
	1	3	0	
	2	0	3	
	3	6	6	
Troponin I	0	0	0	0.712
	1	3	2	
	2	2	4	
	3	4	3	
Myoglobin	0	0	0	0.014
	1	2	0	
	2	0	6	
	3	4	3	
Cx43	0	0	0	1
	1	0	0	
	2	1	2	
	3	8	7	
JunB	0	0	2	0.150
	1	2	4	
	2	4	3	
	3	3	0	
TUNEL	0	2	0	0.457
	1	1	1	
	2	6	6	
	3	0	2	

Statistical significance was assessed at the 0.05 level. The scores of the semi-quantitative immunohistochemical evaluation are indicated in column A (0 = negative, 1 = staining/loss of staining of single cells, 2 = staining/loss of staining of cell groups, 3 = large/diffuse staining/loss of staining). Column B is the number of hearts with a given score in the time interval 30 min–2 h. Column C is the number of hearts with a given score in the time interval 4–12 h

[a] Fisher's exact test

cardiomyocytes as early as 1 h after LAD ligation. This is in accordance with the results of another experimental study, in which loss of cTnI and cTnT was shown to occur as early as 1 h after LAD ligation in rats [36]. Cardiac troponin T was also investigated by Campobasso et al., and a depletion limited to small groups of cells was described in cases of coronary deaths, with less than 1 h from the onset of symptoms to death [5], similarly to our observations. Cardiac troponin I was investigated in human ischemic myocardia by Jasra et al. [38], who showed a clear negative staining only in cases where infarction occurred for more than 6 h, which was much later than what our results showed.

Another marker of early myocardial ischemia we tested is myoglobin, which can be detected in serum as early as 2 h after the onset of myocardial necrosis [52]. In forensic and clinical pathology investigations, myoglobin has shown good sensitivity and specificity for myocardial ischemia lasting for a few hours [5, 39–41]. We have shown a depletion of myoglobin from groups of cardiomyocytes as soon as 1 h after LAD ligation. This is consistent with the results of the study of Campobasso et al. on human autopsy material, as diffuse areas of myoglobin depletion were shown in cases in which death occurred less than 1 h after the onset of symptoms according to witnesses. Ortmann et al. showed an even earlier depletion of myoglobin from cardiomyocytes of a person who died within 30 min from prodromi [39]. The loss of staining with the antibody anti-myoglobin has been reported in canine myocardium as soon as 30 min after LAD ligation as well [41].

C5b-9 is the final product of the complement cascade and causes direct cellular damage by opening the pores of the cell membrane [46]. It can detect myocardial injury as early as 40 min after the beginning of ischemia [47, 53]. In rats, complement activation has been shown to be initiated about 2 h after LAD ligation, and deposits of C8 and C9 were evident at 3 h [54]. In ischemic human myocardium, C5b-9 has shown a later expression in comparison to fibronectin [11, 12, 39], but in one study, it has been demonstrated to react earlier than fibronectin [5]. Our results are more concurrent with the majority of other studies, as we have shown an accumulation of C5b-9 in rat cardiomyocytes as early as 2 h after LAD ligation and thus later than fibronectin. The reason why C5b-9 reacts later than all the other markers we studied, including fibronectin, is not known. One explanation could be that assembly and activation of the complement complex from C3b and C5 to form C5b-9, then the activation of C5b-9, and finally its deposition on the cell membrane first and into the cytoplasm later, may require more time than the "simple" accumulation of the circulating fibronectin into the cardiomyocyte through the cell membrane disrupted by ischemia. Another hypothesis is that C5b-9 deposition occurs as a secondary reaction on an already irreversibly necrotic cardiomyocyte [48]. The cause of complement activation on ischemic cardiomyocytes has not yet been elucidated. One verisimilar hypothesis is that ischemia provokes the loss of the cell membrane's capacity to inactivate spontaneously deposited C3b, leading to C5 activation and C5b-9 self-attack of the cell [48].

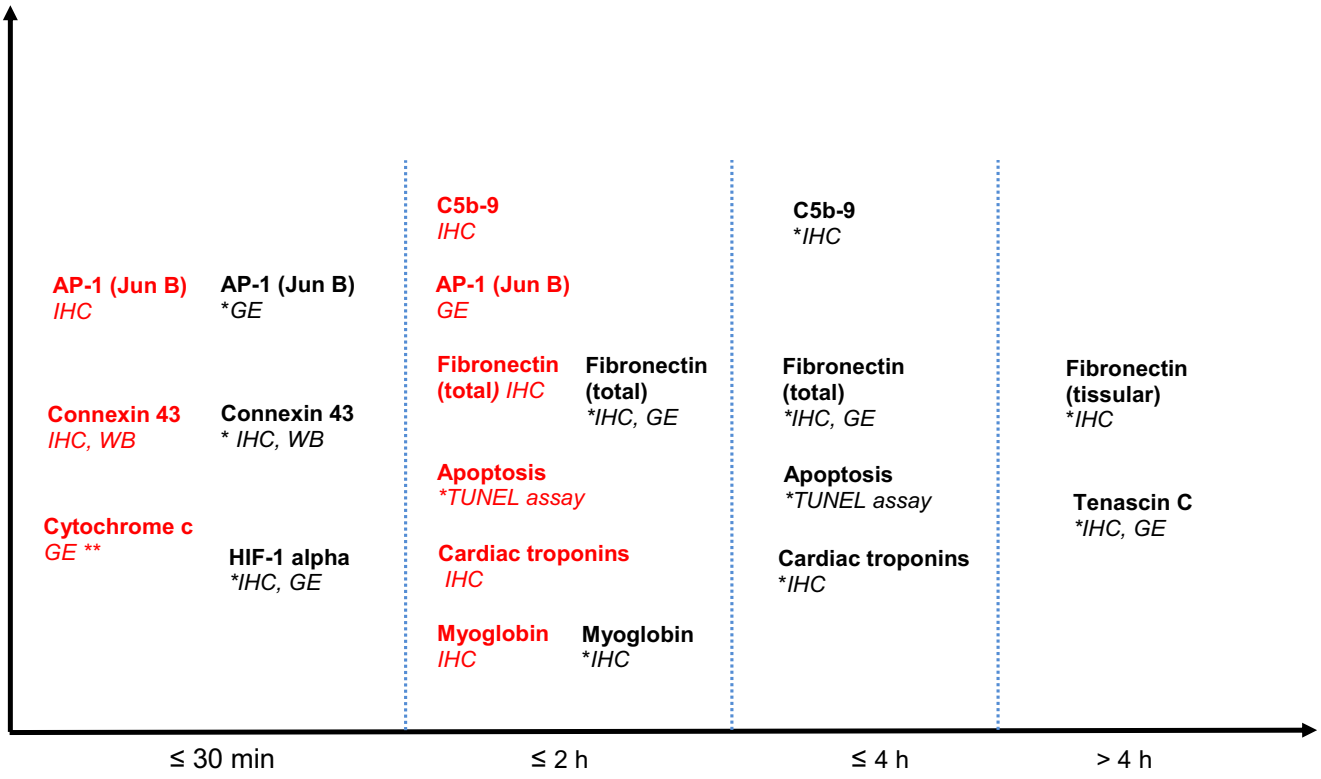
Another early marker of myocardial ischemia we tested is the gap junctional protein Cx43. Uncoupling induced

by ischemia has been shown to be associated with dephosphorylation of Cx43, accumulation of non-phosphorylated Cx43 within gap junctions, and "lateralisation" (i.e., translocation of Cx43 to the lateral plasma membranes and eventually translocation from gap junctions into intracellular pools) [27, 31, 55]. In our investigation, we used an antibody recognizing unphosphorylated Cx43 by immunofluorescence. Our results, showing a local expression of dephosphorylated Cx43 by 15 min of myocardial ischemia, confirm that this is the time point by which cell-to-cell uncoupling starts in the heart, with a signal becoming more and more diffuse and intense with increasing durations of ischemia, corresponding to more advanced and fully developing uncoupling [27]. Western blots for total and phosphorylated (Ser 368) Cx43 showed that acute ischemia (15 and 30') was associated with a marked increase of the phosphorylation on Serine 368 of the C-terminal part of Cx43, whereas total Cx43 did not markedly change. This is consistent with another study showing the maximal phosphorylation of Cx43, involving the Serine 368 in the C-terminal part, after 5' of incubation of neonatal rat cardiomyocytes with HDL (high-density lipoprotein) [56].

When comparing our marker values between early (30'–2 h) and later (4–12 h) ischemia, we observed statistically significant differences for fibronectin, C5b-9, cTnT, and myoglobin, meaning that these markers are more expressed in the later phase of ischemia (by 4 h). This is not surprising for C5b-9, as it is known to react later than other markers, as already discussed. Concerning fibronectin, myoglobin, and cTnT, we observed, respectively, accumulation and depletions starting by 1 h, but the signals became more pronounced with increasing durations of ischemia, thus making their scores much higher in the later phase, reflecting the more severe cell membrane damage.

The TUNEL assay was positive by 1 h after LAD ligation in our samples. In humans, DNA fragmentation has been detected by TUNEL assay in myocardial infarctions after 2 to 4 h [57, 58] and by 45 min after LAD ligation in rats [21]. The process of apoptosis is rapid in its onset (within 2 h) and usually completed within 6 h [21]; therefore, the TUNEL assay could be considered an early detection method of myocardial ischemia. For this reason, we wanted to test it in our study, and we have shown an expression which is comparable to what is reported in the literature for the same animal model (45 min) [21]. The role of apoptosis in myocardial ischemic cell death is quite controversial; it is still debated whether myocardial cells die during ischemia via apoptosis or oncosis. It seems that the severity of ischemia determines whether

Table 4 Time clusters of detection of different markers since myocardial ischemia (in *black*: data from the literature, in *red*: data from the results of the present study). *Asterisk* represents methods by which the investigations were performed. *IHC* immunohistochemistry, *WB* Western blots, *GE* gene expression, *BC* biochemistry. *Double asterisks* decrease in induction



the cardiomyocytes die via apoptosis or oncosis [58]. Many authors agree that both apoptosis and oncosis are responsible for acute ischemic death of cardiomyocytes [59].

One of the most promising markers of early myocardial ischemia that we investigated is JunB, which is a protein (transcription factor) encoded in humans by the JunB gene. It belongs to the AP-1 (activator protein 1) family, a heterodimeric protein which regulates gene expression in response to a variety of stimuli and cellular processes, including differentiation, proliferation, and apoptosis [60]. Presently, JunB has not been investigated at the immunohistochemical level. As immunohistochemistry is a fundamental method in autopsy pathology, we wanted to study the expression of JunB in ischemic myocardium of rats at the immunohistochemical level, in addition to the gene expression analysis. We found a very early nuclear expression, i.e., as early as 30 min after LAD ligation. Very interestingly, by 30 min, this expression was limited to the subendocardial layer, and it gradually became diffused to the myocardium towards the epicardium with the increasing durations of ischemia. This is in accordance with the observation that ischemia/necrosis in the myocardium follows a "wavefront" from the endocardium towards the epicardium [8]. Why JunB is expressed in the nuclei of ischemic myocardial cells and why its expression, almost in parallel with that of Cx43, becomes slightly less pronounced by 2 h of ischemia (as we have shown in our results) remains to be explained. What is known is that AP-1 transcription factors are involved in both the induction and inhibition of apoptosis and that the exact result is highly tissue and cell-cycle phase specific [61] and depends also on environmental factors. Whether JunB in ischemic myocardium has a pro- or anti-apoptotic role or both is not known. It could be that it acts as a rescue mechanism in inhibiting the apoptosis of ischemic cells and, at the same time, in promoting the proliferation of viable cardiomyocytes [62, 63]. From this point of view, JunB could be investigated not only as an early marker of myocardial ischemia but also as a therapeutic target to enhance or inhibit in patients affected by acute cardiac events. This deserves further investigations in the basic research field.

In this investigation, besides the study of early tissular changes following myocardial ischemia, we wanted to explore the phenomenon at the degree of cell activation as well, and we did so for two selected markers: JunB (as it was one of the earliest to be expressed at the immunohistochemical level), and cytochrome c (as it is one of the factors that has been shown to be downregulated after myocardial infarction [33]). Therefore, we performed a gene expression investigation on those markers by using the NanoString nCounter® gene expression system.

By this analysis, we observed a significant induction of JunB at 120 min after LAD ligation. This result is an apparent contradiction with the early immunohistochemical expression, which has been shown at 30 min after LAD ligation. Yet, it can be explained by the fact that the few positive nuclei showed by immunohistochemistry represent only a small percentage of the tissue that has been analyzed by NanoString and that can obviously have been "overlooked" by the system. Nevertheless, this analysis allowed us to confirm JunB as a reliable marker of early myocardial ischemia. Moreover, we showed a significant, very early (as soon as 30 min after LAD ligation) decrease in induction of the Cycs gene (cytochrome c). This result can be explained by the fact that the production of reactive oxygen species during ischemia impairs the expression of genes encoded by the mitochondrial DNA, with a downregulation of the expression of factors of the mitochondrial respiratory chain, such as cytochrome c [64].

In order to better illustrate our results and, at the same time, to compare them with data obtained from the literature, we created a table of the temporal distribution of the different markers. We subdivided them into clusters of time of detection since myocardial ischemia: within 30 min (≤30 min), within 2 h (≤2 h), within 4 h (≤4 h), and beyond 4 h (>4 h) (Table 4).

In conclusion, we underline the advantages of this study, which encourage us to set up the next investigations. In fact, (i) this is a purely experimental study, which allowed us, in this phase, to identify a panel of true early markers of myocardial ischemia (JunB, Cx43, TUNEL assay, fibronectin, C5b-9, myoglobin, cTnT, cTnI, and, for its downregulation, cytochrome c), not influenced by factors such as agony, resuscitation, or autolysis that normally challenge the investigation of SCD at post-mortem examination; (ii) in order to achieve our results, we employed traditional and routine methods, namely histology and immunohistochemistry, thus confirming their relevance in forensic and clinical pathology investigations; (iii) we explored new frontiers by gene expression analysis, which allowed us to identify the earliest upregulated (JunB) and downregulated (cytochrome c) markers of myocardial ischemia and which encourage us to apply the NanoString technology to a larger number of genes of interest.

In the perspective to apply the results of this study to the investigation of SCD at post-mortem examination, two aspects have to be considered: first, in the post-mortem setting, the morphological, immunohistochemical, and molecular biology results can be challenged by a lot of factors such as autolysis, pre-existing ischemic events, myocardial damage (due to cardiogenic shock, for example), long agony, resuscitation, injection of catecholamines and/or drug consumption as cocaine or ecstasy. Second, in the post-mortem setting, it is often impossible to identify, macroscopically, the ischemic

site to investigate. Therefore, sampling at different myocardial regions will be recommended.

Acknowledgments We are grateful to Prof Brenda Kwak, Dr Sandrine Morel, and Mrs Regula Buergy for their expertise, assistance, and performance of Western blots for Cx43.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Priori SG, Blomström-Lundqvist C, Mazzanti A et al (2015) 2015 ESC Guidelines for the management of patients with ventricular arrhythmias and the prevention of sudden cardiac death: The Task Force for the Management of Patients with Ventricular Arrhythmias and the Prevention of Sudden Cardiac Death of the European Society of Cardiology (ESC). Eur Heart J 36(41):2793–2867
2. Hayashi M, Shimizu W, Albert CM (2015) The spectrum of epidemiology underlying sudden cardiac death. Circ Res 116(12):1887–1906
3. Saukko P, Knight B (2004) Knight's forensic pathology, 3rd edn. Edward Arnold, London, pp 492–494
4. Priori SG, Aliot E, Blomstrom-Lundqvist C et al (2001) Task force on sudden cardiac death of the European Society of Cardiology. Eur Heart J 22:1374–1450
5. Campobasso CP, Dell'Erba AS, Addante A et al (2008) Sudden cardiac death and myocardial ischemia indicators: a comparative study of four immunohistochemical markers. Am J Forensic Med Pathol 29:154–161
6. Bouchardy B, Majno G (1974) Histopathology of early myocardial infarcts: a new approach. Am J Pathol 74:301–330
7. Fishbein MC, Maclean D, Maroko PR (1978) The histopathologic evolution of myocardial infarction. Chest 73:843–849
8. Vargas SO, Sampson BA, Schoen FJ (1999) Pathologic detection of early myocardial infarction: a critical review of the evolution and usefulness of modern techniques. Mod Pathol 12:635–645
9. Buja LM (1998) Modulation of the myocardial response to ischemia. Lab Invest 78:1345–1373
10. Cowan MJ, Reichenbach D, Turner P et al (1991) Cellular response of the evolving myocardial infarction after therapeutic coronary artery reperfusion. Hum Pathol 22:154–163
11. Fracasso T, Karger B, Pfeiffer H et al (2010) Immunohistochemical identification of prevalent right ventricular ischemia causing right heart failure in cases of pulmonary fat embolism. Int J Legal Med 124:537–542
12. Fracasso T, Pfeiffer H, Sauerland C et al (2011) Morphological identification of right ventricular failure in cases of fatal pulmonary thromboembolism. Int J Legal Med 125:45–50
13. Haitao B, Ying Y, Jianye H et al (2013) Immunohistochemical detection of S100A1 in the postmortem diagnosis of acute myocardial infarction. Diagn Pathol 8:84
14. Turillazzi E, Di Paolo M, Neri M et al (2014) A theoretical timeline for myocardial infarction: immunohistochemical evaluation and western blot quantification for Interleukin-15 and Monocyte chemotactic protein-1 as very early markers. J Transl Med 12:1–10
15. Turillazzi E, Pomara C, Bello S et al (2015) The meaning of different forms of structural myocardial injury, immune response and timing of infarct necrosis and cardiac repair. Curr Vasc Pharmacol 13:6–19
16. Fineschi V (2015) Measuring myocyte oxidative stress and targeting cytokines to evaluate inflammatory response and cardiac repair after myocardial infarction. Curr Vasc Pharmacol 13:3–5
17. Neri M, Fineschi V, Di Paolo M et al (2015) Cardiac oxidative stress and inflammatory cytokines response after myocardial infarction. Curr Vasc Pharmacol 13:26–36
18. Piro FR, di Gioia CR, Gallo P et al (2000) Is apoptosis a diagnostic marker of acute myocardial infarction? Arch Pathol Lab Med 124:827–831
19. Abbate A, Melfi R, Patti G et al (2000) Apoptosis in recent myocardial infarction. Clin Ter 151:247–251
20. Rodríguez-Calvo MS, Tourret MN, Concheiro L et al (2001) Detection of apoptosis in ischemic heart: usefulness in the diagnosis of early myocardial injury. Am J Forensic Med Pathol 22:278–284
21. Edston E, Gröntoft L, Johnsson J (2002) TUNEL: a useful screening method in sudden cardiac death. Int J Legal Med 116:22–26
22. Nakatome M, Matoba R, Ogura Y et al (2002) Detection of cardiomyocyte apoptosis in forensic autopsy cases. Int J Legal Med 116:17–21
23. Harpster MH, Bandyopadhyay S, Thomas DP et al (2006) Earliest changes in the left ventricular transcriptome postmyocardial infarction. Mamm Genome 17:701–715
24. Thygesen K, Alpert JS, White HD (Joint ESC/ACCF/AHA/WHF task force for the redefinition of myocardial infarction) (2007) Universal definition of myocardial infarction. JAAC 50:2173–2195
25. Melanson SF, Tanasijevic MJ (2005) Laboratory diagnosis of acute myocardial infarction. Cardiovasc Pathol 14:156–161
26. Lampe PD, Cooper CD, King TJ et al (2006) Analysis of phosphorylation of connexin43 at S325/328/330 in normoxic and ischemic heart. J Cell Sci 119:3435–3442
27. Beardslee MA, Lerner DL, Tadros PN et al (2000) Dephosphorylation and intracellular redistribution of ventricular connexin43 during electrical uncoupling induced by ischemia. Circ Res 87:656–662
28. Willems I, Arends JW, Daemen M (1996) Tenascin and fibronectin expression in healing human myocardial scars. J Pathol 179:321–325
29. Geiss GK, Bumgarner RE, Birditt B et al (2008) Direct multiplexed measurement of gene expression with color-coded probe pairs. Nat Biotechnol 26(3):317–325
30. Bhindi R, Witting PK, McMahon AC et al (2006) Rat models of myocardial infarction: pathogenic insights and clinical relevance. Thromb Haemost 96(5):602–610
31. Morel S, Burnier L, Roatti A et al (2010) Unexpected role for the human Cx37 C1019T polymorphism in tumor cell proliferation. Carcinogenesis 31:1922–1931
32. Beaume M, Hernandez D, Docquier M et al (2011) Orientation and expression of methicillin-resistant *Staphylococcus aureus* small RNAs by direct multiplexed measurements using the nCounter of Nanostring technology. J Microbiol Methods 84:327–334
33. Ide T, Tsutsui H, Hayashidani S et al (2001) Mitochondrial DNA damage and dysfunction associated with oxidative stress in failing hearts after myocardial infarction. Circ Res 88:529–535
34. Yoshida K (2009) Pursuing enigmas on ischemic heart disease and sudden cardiac death. Leg Med (Tokyo) 11(2):51–58
35. Basso C, Rizzo S, Thiene G (2010) The metamorphosis of myocardial infarction following coronary recanalization. Cardiovasc Pathol 19(1):22–28
36. Zhang JM, Riddick L (1996) Cytoskeleton immunohistochemical study of early ischemic myocardium. Forensic Sci Int 80(3):229–238
37. Fishbein MC, Wang T, Matijasevic M et al (2003) Myocardial tissue troponins T and I: an immunohistochemical study in

experimental models of myocardial ischemia. Cardiovasc Pathol 12(2):65–71
38. Jasra SK, Badian C, Macri I et al (2012) Recognition of early myocardial infarction by immunohistochemical staining with cardiac troponin-I and complement C9. J Forensic Sci 57(6):1595–1600
39. Ortmann C, Pfeiffer H, Brinkmann B (2000) A comparative study on the immunohistochemical detection of early myocardial damage. Int J Legal Med 113(4):215–220
40. Brinkmann B, Sepulchre MA, Fechner G (1993) The application of selected histochemical and immunohistochemical markers and procedures to the diagnosis of early myocardial damage. Int J Legal Med 106(3):135–141
41. Leadbeatter S, Wawman HM, Jasani B (1989) Immunocytochemical diagnosis of early myocardial ischaemic/hypoxic damage. Forensic Sci Int 40(2):171–180
42. Shekhonin BV, Guriev SB, Irgashev SB et al (1990) Immunofluorescent identification of fibronectin and fibrinogen/fibrin in experimental myocardial infarction. J Mol Cell Cardiol 22(5):533–541
43. Casscells W, Kimura H, Sanchez JA et al (1990) Immunohistochemical study of fibronectin in experimental myocardial infarction. Am J Pathol 137(4):801–810
44. Hu BJ, Chen YC, Zhu JZ (1996) Immunohistochemical study of fibronectin for postmortem diagnosis of early myocardial infarction. Forensic Sci Int 78(3):209–217
45. Hu BJ, Chen YC, Zhu JZ (2002) Study on the specificity of fibronectin for post-mortem diagnosis of early myocardial infarction. Med Sci Law 42(3):195–199
46. Piercecchi-Marti MD, Lepidi H, Leonetti G et al (2001) Immunostaining by complement C9: a tool for early diagnosis of myocardial infarction and application in forensic medicine. J Forensic Sci 46(2):328–334
47. Edston E, Kawa K (1995) Immunohistochemical detection of early myocardial infarction: an evaluation of antibodies against the terminal complement complex (C5b-9). Int J Legal Med 108(1):27–30
48. Schäfer H, Mathey D, Hugo F et al (1986) Deposition of the terminal C5b-9 complement complex in infarcted areas of human myocardium. J Immunol 137(6):1945–1949
49. Ortiz-Rey JA, Suarez-Penaranda JM, Da Silva EA et al (2002) Immunohistochemical detection of fibronectin and tenascin in incised human skin injuries. Forensic Sci Int 126(2):118–122
50. Knowlton AA, Connelly CM, Romo GM et al (1992) Rapid expression of fibronectin in the rabbit heart after myocardial infarction with and without reperfusion. J Clin Invest 89(4):1060–1068
51. Collinson PO, Gaze DC (2007) Biomarkers of cardiovascular damage. Med Princ Pract 16(4):247–261
52. Kavsak PA, MacRae AR, Newman AM et al (2007) Effects of contemporary troponin assay sensitivity on the utility of the early markers myoglobin and CKMB isoforms in evaluating patients with possible acute myocardial infarction. Clin Chem Acta 380(1–2):213–216
53. Thomsen H, Schulz A, Bhakdi S (1990) Immunohistochemical C5b-9-complement complex demonstration in early stages of myocardial necroses using paraffin sections. Z Rechtsmed 103(3):199–206
54. Väkevä A, Morgan BP, Tikkanen I et al (1994) Time course of complement activation and inhibitor expression after ischemic injury of rat myocardium. Am J Pathol 144(6):1357–1368
55. Saffitz JE, Kléber AG (2012) Gap junctions, slow conduction, and ventricular tachycardia after myocardial infarction. J Am Coll Cardiol 60(12):1111–1113
56. Morel S, Frias MA, Rosker C et al (2012) The natural cardioprotective particle HDL modulates connexin43 gap junction channels. Cardiovasc Res 93(1):41–49
57. Bardales RH, Hailey LS, Xie SS et al (1996) In situ apoptosis assay for the detection of early acute myocardial infarction. Am J Pathol 149(3):821–829
58. Jugdutt BI, Idikio HA (2005) Apoptosis and oncosis in acute coronary syndromes: assessment and implications. Mol Cell Biochem 270(1–2):177–200
59. Takemura G, Fujiwara H (2006) Morphological aspects of apoptosis in heart diseases. J Cell Mol Med 10(1):56–75
60. Shaulian E, Karin M (2002) AP-1 as a regulator of cell life and death. Nat Cell Biol 4(5):E131–E136
61. Piechaczyk M, Farras R (2008) Regulation and function of JunB in cell proliferation. Biochem Soc Trans 36(Pt5):864–867
62. Kajstura J, Urbanek K, Perl S et al (2010) Cardiomyogenesis in the adult human heart. Circ Res 107:305–315
63. Bergmann O, Bhardwaj RD, Bernard S et al (2009) Evidence for cardiomyocyte renewal in humans. Science 324:98–102
64. Wu C, Yan L, Depre C et al (2009) Cytochrome c oxidase III as a mechanism for apoptosis in heart failure following myocardial infarction. Am J Physiol Cell Physiol 297:C928–C934