Myocardial Injury in Post Mortem Biopsies of Patients with COVID-19

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Case Report

Keywords: Cardiac injury , COVID-19 , endothelium , fibrosis , pathology

DOI: https://doi.org/10.21203/rs.3.rs-45192/v1

Abstract

Background: Myocardial injury is significantly and independently associated with mortality in COVID-19 patients. However, the pathogenesis of myocardial injury in COVID-19 is still not clear, and cardiac involvement by SARS-CoV-2 remains a major challenge worldwide.

Aim: This histopathological and immunohistochemical study seeks to clarify the pathogenesis of myocardial injury in COVID-19.

Methods: Postmortem minimally invasive autopsies were performed in two patients who died from COVID-19, and the myocardium samples were compared to a control patient. Immunohistochemistry (IHC) staining was performed using monoclonal antibodies against the following targets: caspase-1, ICAM-1, TNF-α, IL-4, IL-6, CD163, TGF-β, MMP-9, type 1 and type 3 collagen.

Results: The histopathological analysis showed severe pericellular interstitial edema surrounding each of the cardiomyocytes. The IHC analysis showed increased expression of caspase-1, ICAM-1, IL-4, IL-6, CD163, MMP-9 and type 3 collagen in the COVID-19 patients compared to the control. On the other hand, no difference from the control was observed in expression of TNF-α, TGF-β and type 1 collagen.

Conclusion: Our findings point to a pathogenesis related with pyroptosis leading to endothelial disfunction. The subsequent inflammation with associated interstitial edema could explain the myocardial disfunction and arrythmias in COVID-19 patients. The presence of Th2 response, MMP-9 and type-3 collagen suggests progression to myocardial fibrosis in the long term.

Introduction

Since the first cases in December 2019 [1], the coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to grow despite unprecedented worldwide efforts in search of treatments and vaccines. COVID-19 is mainly a respiratory disease, causing viral pneumonia and acute respiratory distress syndrome. However, in most critical cases, cardiovascular manifestations have been related to poor outcomes [2]. Myocardial injury (defined by troponin blood levels above the 99th-percentile upper reference) was observed in 7 to 17% [3] of patients and was significantly and independently associated with mortality [4]. Common cardiac complications among hospitalized patients with COVID-19 include myocarditis, arrhythmias and acute heart failure. The heart failure may contribute up to 40% of deaths, and circulatory failure may be the death cause even without respiratory failure [3]. Prothrombotic coagulopathy have been also described in 25% of patients resulting in venous and arterial thromboembolic events [5].

Recently, the evidence of SARS-CoV-2 genome detection in endomyocardial biopsies proved that SARS-CoV-2 infection directly impair the heart [6]. However, the mechanism of cardiac damage by SARS-CoV-2 is not clear and remains a major challenge worldwide. Autopsies of patients with COVID-19 revealed

infiltration of the myocardium by interstitial mononuclear inflammatory cells [7]. SARS-CoV-2 particles have been already observed in myocardial interstitial cells [8] and endothelial cells [9] by electron microscopy. Varga et al.[9] suggested that pyroptosis might have an important role in endothelial cell injury in patients with COVID-19. Pyroptosis is a programmed cell death characterized by caspase-1 activation and subsequent cellular signaling that culminates in inflammasome pathway activation, secretion of interleukin-6 (IL-6) and endothelial disfunction [10]. This activation could be the initial pathway for myocardial injury and could also explain the involvement of various organs and tissues that has been described in COVID-19 [11].

Interstitial myocardial has been described as a possible mechanism for myocardial injury [6,12] in a process initiated by intercellular edema, which is accompanied by endothelial stress and consequent innate immune system recruitment through endothelial ICAM-1 expression [13]. Once the immune cells, notably Th2 type cells, have migrated to the site of the injury or aggression, they start secreting interleukins and pro-fibrotic chemokines as well as inducing matrix metalloproteinases and subsequent type 1 and type 3 collagen secretion by surrounding tissue fibroblasts [14]. Myocardial fibrosis changes the cytoarchitecture and extracellular environment of the myocardium and may lead to both systolic and diastolic dysfunction, and also arrhythmias [15].

Given that the cardiac manifestations play a major role in adverse outcomes and that there is lack of pathological studies showing myocardial injury in COVID-19, we investigated myocardial samples in a histopathological and immunohistochemical study to help clarify the pathogenesis of COVID-19 myocardial injury in lethal cases.

Methods

Postmortem minimally invasive autopsies were performed in two patients who died from COVID-19 in Marcelino Champagnat Hospital, Brazil. This study was approved by the National Council of Ethics in Research – CONEP (No. 3.944.734). Patients' families authorized the autopsies and signed the informed consent form before the procedures. Both patients tested positive for SARS-CoV-2 on nasopharyngeal swabs (RT-PCR). Myocardial tissue was collected by left anterior mini thoracotomy for direct access to the left ventricle. The pericardium was sectioned and a fragment of myocardial tissue with dimensions approximately 75x75 mm was obtained. The tissues from the myocardial biopsies were fixed in neutral buffered formalin for over 24 hours, and then processed under standard biosafety measures. Hematoxylin and eosin-stained sections were prepared, and slides were examined by two pathologists.

Immunohistochemistry (IHC) staining was performed in the myocardium samples using monoclonal antibodies against the following targets: caspase-1, intercellular adhesion molecule-1 (ICAM-1), tumor necrosis factor alpha (TNF-α), interleukin-4 (IL-4), interleukin-6 (IL-6), CD163 (macrophage-specific protein), transforming growth factor (TGF-β), matrix metalloproteinase-9 (MMP-9), type 1 and type 3 collagen. Table 1 summarizes the specifications of the antibodies used to investigate the formalin-fixed,

paraffin-embedded myocardial tissues. Scores of biomarker expression according to the IHC staining were given independently by each of the pathologists. Any disagreements between the results would be resolved by a third pathologist.

The postmortem myocardium biopsies of the two patients with COVID-19 were then compared to a myocardium sample from a control patient. The control had similar age (80 years), similar underlying conditions (type 2 diabetes mellitus, systemic arterial hypertension and coronary artery disease) and died of acute pulmonary thromboembolism after hip arthroplasty surgery.

Results

Clinical data from the baseline of COVID-19 patients is presented in Table 2. Both patients 1 and 2 were elderly over 70 years (73 and 80, respectively), had history of coronary artery disease and needed invasive ventilation during their hospitalization. The first patient remained on mechanical ventilation for 10 days, and the second, for 21 days. Also, both patients had myocardial injury demonstrated by high levels of troponin on the blood tests. In the echocardiogram, patient 1 had mid-range ejection fraction (43%) and mild eccentric hypertrophy before the onset of COVID-19 symptoms and maintained the pattern during hospitalization. Patient 2 had preserved ejection fraction (60%) and preserved dimensions of the left ventricle.

The sample tissue from the COVID-19 patients were compared to the control and severe pericellular interstitial edema surrounding each of the cardiomyocytes was observed in the former patients. Lipofucsin pigment and mild signs of cardiomyocyte hypertrophy were seen in COVID-19 patients and control. The IHC analysis showed an increase in staining, and therefore increased expression, for caspase-1, ICAM-1, IL-4, IL-6, CD163, MMP-9 and type 3 collagen in the COVID-19 patients compared to the control. No difference from the control was observed in expression of TNF-α, TGF-β and type 1 collagen. Scores of biomarker expression according to the IHC analysis are shown in Table 3. Images of the caspase-1, IL-4, MMP-9 and type 3 collagen slides, for both COVID-19 and control, are shown in Figure 1.

A few other aspects of topography in the samples are worth noting. Firstly, the caspase-1 and IL-6 were present in the cytoplasm, whereas ICAM-1 was present in the membrane of endothelial cells. Secondly, the MMP-9 and type 1 collagen were observed in large quantities in the interstitial and perivascular spaces. All the results were analyzed and integrated to the previous pathological knowledge. Then, our proposed mechanism is shown in Figure 2 with possible pathways involved in COVID-19 myocardial injury.

Discussion

Pyroptosis is an inflammatory form of programmed cell death that occurs most frequently upon infection by intracellular pathogens, and requires the function of the enzyme caspase-1 [16]. Caspase-1 is activated

during pyroptosis as part of a multiprotein signaling platform, the inflammasome complex, and subsequently mediates the activation and secretion of various interleukins, such as IL-1β, IL-4 and IL-11, as well as the rupture of the cell membrane [17]. We observed higher levels of caspase-1 adjacent to endothelial cells in the COVID-19 samples demonstrating endothelial infection, pyroptosis and injury in these patients. Moreover, SARS-CoV-2 particles have been described in endothelial cells by electron microscopy [9] and the caspase-1 identification is in accordance with Varga et al. [9], who suggested that pyroptosis might have an important role in endothelial cell injury in patients with COVID-19.

The IHC analysis also showed increased expression of ICAM-1, IL-6, IL-4, CD163, MMP-9 and type 3 collagen in the COVID-19 samples when compared to the control. The first two biomarkers, ICAM-1 and IL-6, were present in large quantities in the endothelial cells and indicate endothelial activation as well as immune cell recruitment and response. These findings are in line with the observations on caspase-1, and with previous biopsies studies which had already shown that the inflammatory process in cardiac tissue permeates the vascular wall [6,10]. SARS-CoV-2 potentially causing endotheliitis [9], which is determinant of microvascular dysfunction by shifting the vascular equilibrium towards more vasoconstriction with subsequent organ ischemia, inflammation with associated tissue edema, and a procoagulant state [18].

The endothelial injury in COVID-19, which was demonstrated to occur via pyroptosis and interleukin secretion, would increase capillary permeability and cause tissue edema [15,17,18]. When comparing the sample tissues from the COVID-19 patients to the control, we observed severe pericellular interstitial edema in between the cardiomyocytes, causing them to separate. The maintenance of cytoarchitecture and extracellular environment of the myocardium is fundamental for the electrical and contractile function of the heart [15]. Thus, the cardiac dysfunction and arrhythmias associated with myocardial injury in COVID-19 may be related to the myocardial interstitial edema and consequent loss of structure of the syncytium [19].

Although studies including post mortem biopsies are still scarce, the literature converges into a few key aspects regarding the lesions caused by SARS-CoV-2, those being mainly the hyaline membrane formation alongside diffuse alveolar damage findings, the myocardial interstitial edema and the endothelium inflammation [20–22]. Our results show myocardial interstitial edema, which alongside the endothelial inflammation caused by SARS-CoV-2, may progress to a later fibrotic myocardial reorganization, as evidenced by the presence of IL-4, ICAM-1, MMP-9 and type 3 collagen expression. These findings may indicate an early stage myocardial fibrotic response as opposed to a pre-existing fibrosis, that would be marked by a higher level of type 1 collagen expression and little IL-4 and MMP-9 expression.

The high levels of MMP-9, CD163, IL-4 and IL-6 demonstrate myocardial inflammatory stress in the COVID-19 tissue. SARS-CoV-2 particles have been already observed in a cytopathic interstitial inflammatory cell in myocardial tissue [8] and other autopsies of patients with COVID-19 also revealed infiltration of the myocardium by interstitial mononuclear inflammatory cells [6,8]. MMP-9 is a

endopeptidase which cleaves structural elements of the extracellular matrix and also plays important roles in immune cell function [20]. MMP-9 promotes Th2 cells recruitment and it has been shown to be significantly increased during several cardiovascular diseases, including hypertension, atherosclerosis and myocardial infarction [20]. This, along with the high CD163 expressing macrophages on the tissue samples, are signs of cell recruitment, which is characteristic of immune inflammatory response [21]. Monocytes and macrophages recruited are capable of producing and secreting large amounts of pro-inflammatory mediators and pro-fibrotic growth factors [22].

The persisting Th2 (IL-4) cytokine-driven immune mechanism is relevant to the process of myocardial fibrosis [23]. In fact, IL-6 and IL-4 have already been shown to be two profibrotic cytokines, as they induce MMP-9 expression and collagen synthesis through gene transcription modulation [23–25]. MMP-9 also stimulates cardiac fibroblast migration, increases collagen synthesis, upregulates angiogenic factors, and induces the transition of cardiac fibroblasts to myofibroblasts [14,22].

As expected, we found no difference in TNF-α between cases and control. We also observed no difference in TGF-β. This is a cytokine with major roles in cardiac fibrogenesis [22,26,27] which activates SMAD2/3 pathways, stimulating alternative pathogenetic pathways and regulating cell synthesis and differentiation, promoting fibrogenesis [27]. We hypothesize that the TGF-β pathway was still not activated in these cases. If not TGF-β, an alternative pathway for myocardial fibrosis, such as the activation of macrophages via IL-4 [22,27] or mast cell degranulation [22], might be involved in the pathophysiology of COVID-19.

Myocardial fibrosis is characterized by a dysregulated collagen turnover and excessive fibrillar collagen accumulation in the interstitial and perivascular spaces [22,26]. Synthesis of both type 1 and type 3 collagen is markedly increased in the remodeling fibrotic heart regardless of the etiology of fibrosis [22]. In our study, type 3 collagen was observed in large quantities in the interstitial and perivascular spaces in the COVID-19 samples when compared to the control. Type 1 collagen, in contrast, showed no difference between cases and control.

Type 1 collagen cross links with type 3 collagen to form the final fibers in myocardial fibrosis which is primarily associated with thick fibers that confer tensile strength, and because of that, takes longer to form [22,28]. A genetic response study on experimental autoimmune myocarditis showed that myocardial fibrosis had formed on day 21, but not before [29]. In addition, advanced age and chronic illnesses are known to lead to myocardial fibrosis, and both our control and COVID-19 samples were patients well over 70 years old.

Type 3 collagen, on the other hand, typically forms thin fibers and, because of that, it takes less time to build [22,28]. Our observation of type 3 collagen in the COVID-19 samples, but not in the control, along with the increased expression of CD163, IL-4, IL-6 and MMP-9, is consistent with the hypothesis that COVID-19 acute myocardial injury may cause myocardial fibrosis in the long term. In addition, formation of chronic

myocardial interstitial edema, observed in the COVID-19 samples but not in the control, also results in deposition of interstitial collagen, which causes interstitial fibrosis [13].

An important finding is that the echocardiogram from our COVID-19 patients 24 hours before death showed no changes in ejection fraction or in left ventricle before or after the disease. Patient 1 had mid-range ejection fraction (43%) and mild eccentric hypertrophy before the onset of COVID-19 symptoms and maintained the pattern during hospitalization. Patient 2 had preserved ejection fraction (60%) and preserved dimensions of the left ventricle. At that time, the IHC biomarkers had probably already been activated. Our findings could mean that biomarker changes appear much earlier than echocardiographic changes and, therefore, we should not wait for alterations in echocardiogram to infer cardiac involvement of COVID-19.

The microvascular dysfunction may lead to thrombosis and justifies the rational use of anticoagulant and anti-aggregating therapy [30]. The myocardial interstitial edema presented here may be one explanation for the high prevalence of cardiac arrhythmia in COVID-19 patients despite other drug factors that may be involved, such as the use of hydroxychloroquine and azithromycin[31]. Furthermore, our findings suggest that COVID-19 myocardial injury may cause myocardial fibrosis in the long term. Based on laboratory tests, individualized cardiac magnetic resonance could be useful to assess patients' cardiac involvement, and thus guide treatment. Additionally, drugs which act in cardiac remodeling, such as angiotensin-converting enzyme inhibitors or mineralocorticoid receptor antagonists, could be useful in a long-term myocardial protective effect[32,33]. However, further studies evaluating cardiac sequelae and mortality following hospital discharge are needed.

We present a panel of immunohistochemical markers showing different and intricated mechanisms of myocardial injury. However, our study has a few limitations. The two COVID-19 patients were elderly with more than 70 years of age and had underlying conditions that could be confounders. Also interpretation of our findings should take into account that autopsies do not allow the observation of the entire pathological process, and cannot predict the evolution of the disease.

Conclusion

In conclusion the pathogenesis of COVID-19 myocardial injury seems to be related with pyroptosis leading to endothelial cell injury and disfunction, which explains the impaired microcirculatory function. Subsequently, the inflammation with associated interstitial edema would explain the myocardial disfunction and arrythmias in COVID-19 patients. These patients could be more prone to thrombotic diseases, heart failure and even death. Our hypothesis seems to be a plausible explanation as to why patients with underlying heart conditions are predisposed to developing more severe cases of COVID-19. Finally, the presence of Th2 response, MMP-9 and type 3 collagen suggests that COVID-19 myocardial injury may cause myocardial fibrosis in the long term. Therefore, these patients should be monitored for systolic and diastolic dysfunction and arrythmias leading to heart failure after the acute phase of COVID-19.

Declarations

The authors declare no competing interests.

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Tables

Table 1 – Specifications of the antibodies used to investigate the formalin-fixed, paraffin-embedded myocardial tissues

Antibody	*Type*	*Clone/Code*	*Dilution*	*Source*
Anti-Casp 1	Polyclonal/Rabbit	ab189796	1:200	Abcam
Anti-CD163	Monoclonal/Mouse	Ab9324	1:400	Abcam
Anti-Col 1	Polyclonal/Rabbit	ab34710	1:200	Abcam
Anti-Col 3	Polyclonal/Rabbit	Ab 7778	1:200	Abcam
Anti-Col 4	Monoclonal/Mouse	CIV22	1:100	Dako
Anti-ICAM 1	Monoclonal/Mouse	23G12	1:100	Novocastra
Anti-IL-4	Polyclonal/Rabbit	PA525165	1:200	Thermo Fisher
Anti-IL-6	Monoclonal/Mouse	Ab9324	1:400	Abcam
Anti-MMP 9	Monoclonal/Mouse	EP1254	1:200	Abcam
Anti-TGF-β	Polyclonal/Rabbit	E11262	1:200	Spring
Anti-TNF-α	Polyclonal/Rabbit	Ab6671	1:100	Abcam

Table 2– Clinical data from the baseline of patients

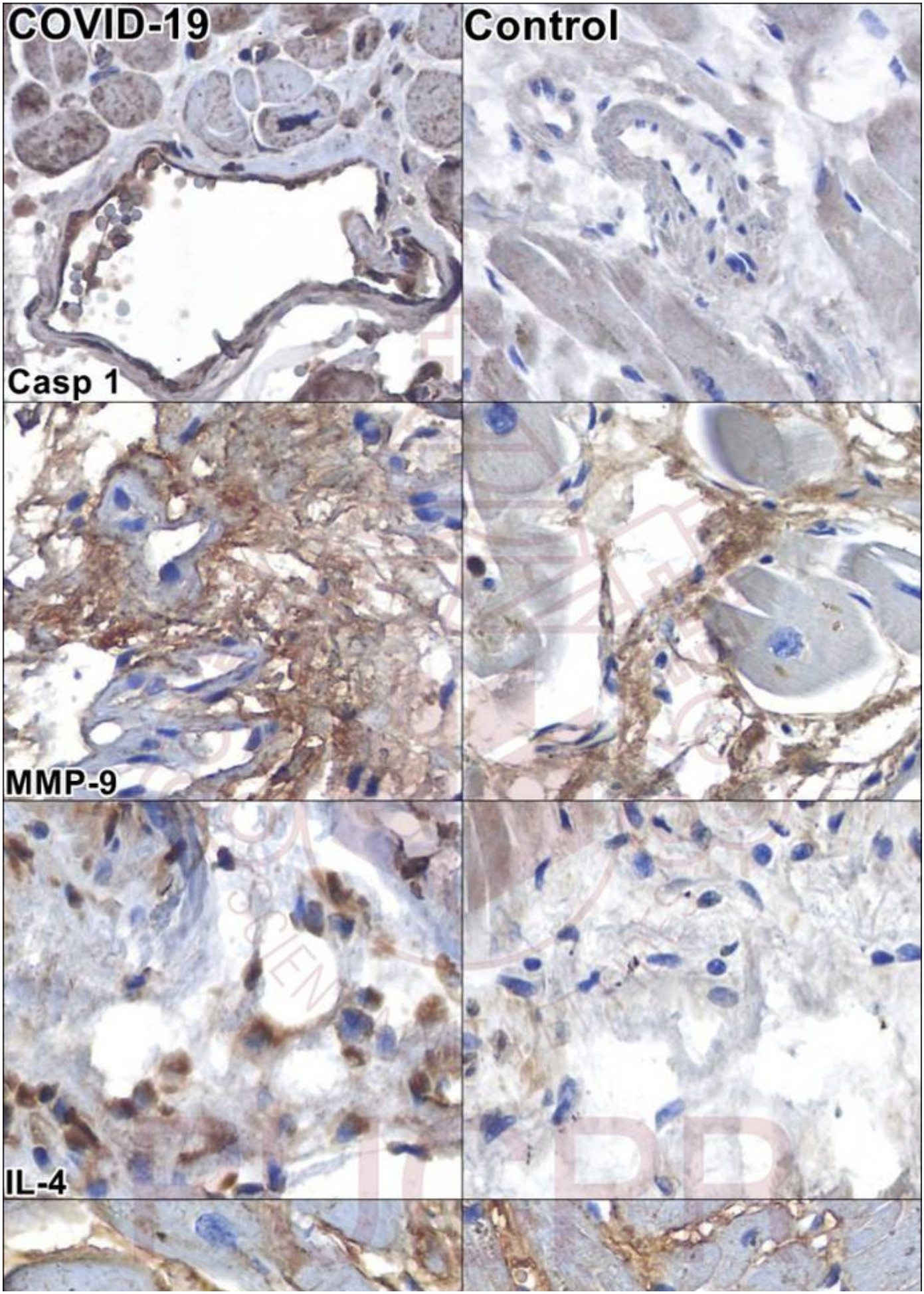
	Patient 1	Patient 2
Gender, Age (years)	Male, 73	Male, 80
Underlying Conditions	Type 2 Diabetes Mellitus Chronic Kidney Disease Dialysis Atrial Fibrillation Coronary Artery Disease Heart Failure Peripheral Obstructive Artery Disease	Systemic Arterial Hypertension Coronary Artery Disease Heart Failure Class III obesity
Medications	Insulin NPH 20UI/day Losartan 100 mg/day Hydrochlorothiazide 25 mg/day Metoprolol 50 mg/day Acetylsalicylic Acid 100 mg/day Clopidogrel 75 mg /day Rosuvastatin 10 mg/day Cilostazol 100 mg/day Erythropoietin 4000UI 48/48h	Acetylsalicylic Acid 81 mg/day Metoprolol 50 mg/day Rivaroxaban 20 mg/day Ezetimibe 10 mg/day Pitavastatin 4 mg/day Trimetazidine 70 mg/day Carbamazepine 400 mg/day Trazodone 150 mg/day Inhaled Beclomethasone 200 µg/day Inhaled Formoterol 12 µg/day
Length of stay on Mechanical Ventilation	10 days	21 days
Chest Computed tomography at admission	Diffuse and bilateral “opacities with ground-glass attenuation”, suggestive of viral pulmonary infection	Diffuse and bilateral “opacities with ground-glass attenuation”, suggestive of viral pulmonary infection
Relevant initial laboratory tests	C-Reactive Protein = 83 mg/L; D-dimer = 3436 µg/mL hs-Troponin I = 12,6 pg/mL Globular volume = 25% Hemoglobin = 8.6 g/dL Total leukocytes = 9,200 / band cells = 1,932 (21%) and lymphocytes = 552 (6%).	C-Reactive Protein = 52 mg/L; D-dimer = 816 µg/mL hs-Troponin I = 10.9 pg/dL Globular volume = 37.5% Hemoglobin = 12.8 g/dL Total leukocytes = 4,700 / band cells = 188 (4%) and lymphocytes = 423 (9%).
Laboratory tests 24 hours before death	C-Reactive protein = 270 mg/dL; D-dimer = 4,858 µg/mL Troponin = 56.4 pg/dL Globular volume = 23% Hemoglobin = 8.0 g/dL Leukocytes = 22,000 Lymphocytes = 440 (2%).	C-Reactive protein = 407 mg/dL; D-dimer = 4,507 µg/mL Troponin = 32.7 pg/dL Globular volume = 29.4% Hemoglobin = 9.7 g/dL Leukocytes = 9,400 Lymphocytes = 1,316 (14%).
Echocardiogram 24 hours before death	Ejection fraction = 43% Left ventricle = mild eccentric hypertrophy	Ejection fraction = 60% Left ventricle = preserved dimensions
Therapeutic drugs	Hydroxychloroquine 800 mg/day on the 1st day and 400 mg/day on the other days + Azithromycin 500 mg/day for 5 days; Oseltamivir 150 mg/day for 5 days; Metronidazole 1,5g/day, Meropenem 1g/day, Linezolid 1,2g/day	Hydroxychloroquine 800 mg/day on the 1st day and 400 mg/day on the other days + Azithromycin 500 mg/day for 5 days; Oseltamivir 150 mg/day for 5 days; Ceftriaxone 2g / day
Invasive procedure	Hemodialysis three times a week	Tracheostomy

* Reference values: hs-Troponin I < 19,8 pg/mL, D-dimer < 500 µg/mL. The choice of the antibiotics was done according to the diagnosis and protocol for the patient's profile.

Table 3 – Scores of biomarker expression in the myocardium samples according to the immunohistochemistry analysis

	Patient 1 (COVID-19)	Patient 2 (COVID-19)	Control
Caspase-1	+++	+++	+
CD 163	++	++	+
Collagen 1	++	++	++
Collagen 3	+++	+++	+
Collagen 4	++	++	++
ICAM-1	+++	+++	+
IL-4	+++	+++	+
IL-6	+++	+++	+
MMP-9	+++	+++	++
TGF-β	+	+	+
TNF-α	++	++	++

Figures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Control
Casp 1
MMP-9
IL-4

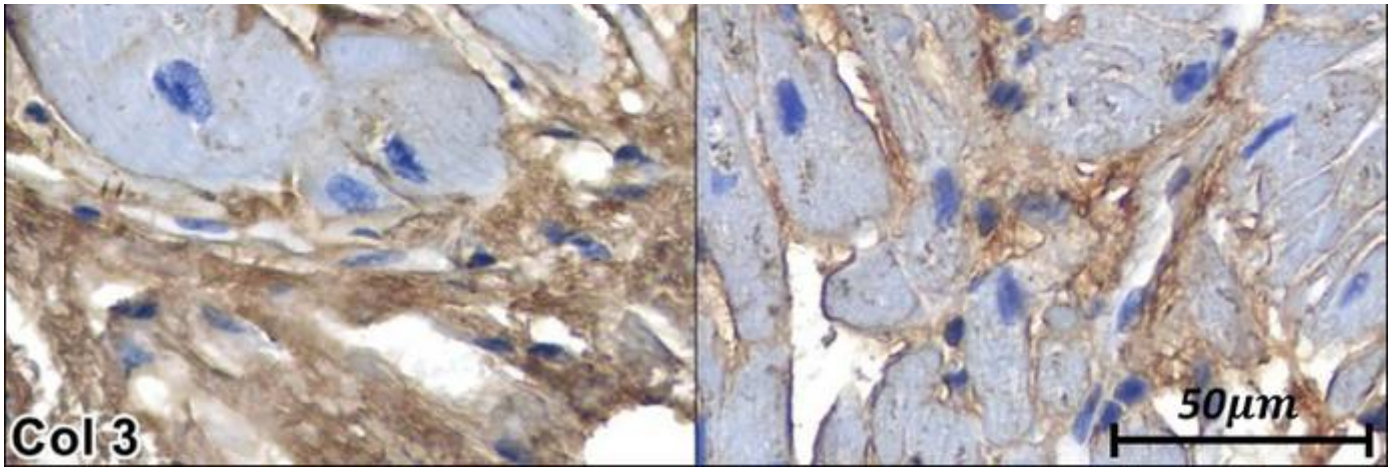

Figure 1

Immunohistochemistry analysis of the myocardial tissue with staining for Caspase-1, IL-4, MMP-9 and type 3 collagen

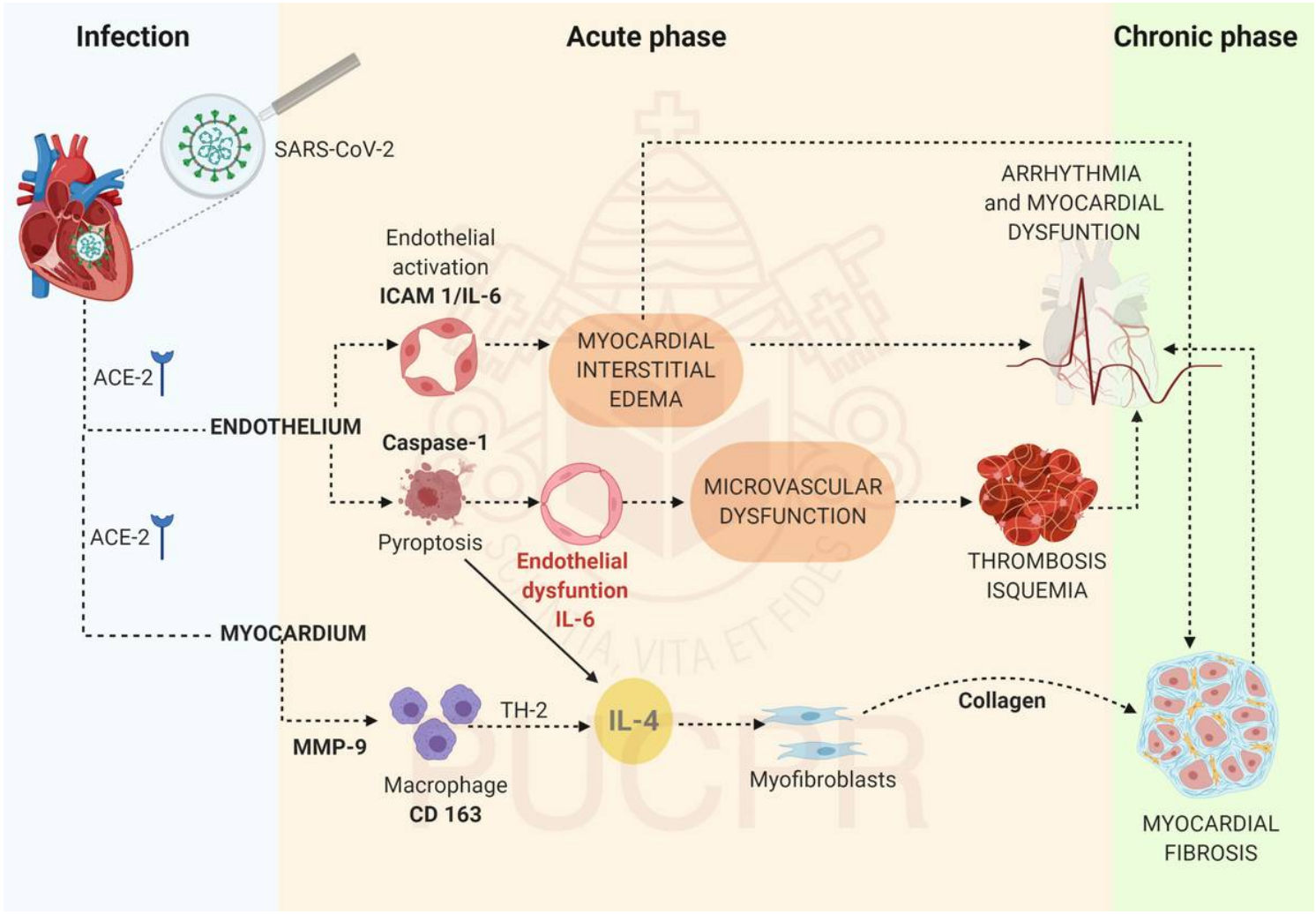

Figure 2

The possible mechanism of myocardial injury in COVID-19