Opinion paper of the ESC Working Group on Cellular Biology of the Heart:

COVID-19 related cardiac complications – from clinical evidences to basic mechanisms

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Abstract - The pandemic of Coronavirus disease (COVID)-19 is a global threat, causing high mortality, especially in the elderly. The main symptoms and the primary cause of death are related to interstitial pneumonia. Viral entry also into myocardial cells mainly via the angiotensin converting enzyme type 2 (ACE2) receptor and excessive production of pro-inflammatory cytokines, however, also make the heart susceptible to injury. In addition to the immediate damage caused by the acute inflammatory response, the heart may also suffer from long-term consequences of COVID-19, potentially causing a post-pandemic increase in cardiac complications.

Although the main cause of cardiac damage in COVID-19 remains coagulopathy with micro- (and to a lesser extent macro-) vascular occlusion, open questions remain about other possible modalities of cardiac dysfunction, such as direct infection of myocardial cells, effects of cytokines storm, and mechanisms related to enhanced coagulopathy. In this opinion paper, we focus on these lesser appreciated possibilities and propose experimental approaches that could provide a more comprehensive understanding of the cellular and molecular bases of cardiac injury in COVID-19 patients. We first discuss approaches to characterize cardiac damage caused by possible direct viral infection of cardiac cells, followed by formulating hypotheses on how to reproduce and investigate the hyperinflammatory and pro-thrombotic conditions observed in the heart of COVID-19 patients using experimental in vitro systems. Finally, we elaborate on strategies to discover novel pathology biomarkers using omics platforms.

Keywords: SARS-CoV-2; COVID-19; myocardial injury; disease modelling; infection; inflammation.
Introduction

Since the onset of the COVID-19 pandemic outbreak in Wuhan, China, patients with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection exhibited signs of severe acute myocardial injury, proven by significantly elevation in circulating cardiac troponin (cTn) T and I levels, occasional heart failure with acute decrease in ejection fraction, arrhythmias, and high in-hospital mortality\(^1\)-\(^5\). It is well known that elderly patients presenting with comorbidities or cardiovascular risk factors are more prone to cardiac complications of SARS-CoV-2 infection\(^6\). There are several possible links between COVID-19 and cardiac dysfunction. These include diffuse coagulopathy causing micro/macro-vascular occlusions and hypoxia, which may unmask underlying coronary artery disease; reduced lung compliance (the ‘stiff lung’\(^7\)), which impairs right and left ventricular function\(^8\); direct cytotoxicity due to infection of myocardial and/or endothelial cells or exposure to the so-called cytokine storm. Despite the prevalent causes of cardiac injury appear to be well characterized at present\(^9\), the cause-effect relationships existing between the severity of COVID-19 cardiac injury and cardiovascular risk factors remains elusive\(^\text{10}\). Indeed, the expression of various viral entry receptors (the so-called coronavirus-associated receptors and factors – SCARFs) in myocardial cells\(^11\) suggests that exposure of the heart to the virus might, directly or indirectly, determine cytopathic effects even in healthy individuals. In line with this hypothesis, retrospective analyses in cohorts of COVID-19 patients have shown that the impact of infection on myocardial damage is not limited only to patients with pre-existing risk conditions (e.g., ischemic heart disease, heart failure), but it is also relevant in individuals with apparently healthy hearts suffering potentially persistent consequences\(^\text{12-14}\). Furthermore, in various autopsy reports\(^\text{15-20}\) SARS-CoV-2 infection has been associated with signs of cardiomyocyte toxicity either directly, i.e., associated with the presence of viral particles, or indirectly, without detection of viral particles and apparently mediated by systemic inflammation\(^21,\ 22\). Finally, the first prospective study aimed at identifying potential long-term cardiopulmonary damage after acute COVID-19 has described a high rate of diastolic dysfunction in moderate-to-severely ill COVID-19 patients, persisting months after the infection, similar to what has been observed after infection with the phylogenetically related SARS-CoV-1\(^\text{14}\). Together, this evidence suggests that SARS-CoV-2 damages myocardial cells by direct infection and that, for a better understanding of the relationships between cardiovascular risk factors, comorbidities and COVID-19-related cardiac complications, it is critical also to consider direct cytotoxicity effects\(^23\).

This Opinion Paper from the Working Group on Cellular Biology of the Heart of the European Society of Cardiology will highlight possible experimental approaches that may be implemented to understand the direct and indirect modalities of cardiac damage due to SARS-CoV-2, to unravel the short- and long-term effects of the virus on myocardial cells, and discuss potential biomarkers to stage the degree of cardiac damage. This contribution parallels and integrates recent reports.
focusing on other aspects of the pathophysiology of COVID-19, such as endothelial dysfunction\textsuperscript{24, 25}, to which interested readers are referred for more extensive information.

1. Modelling cardiac damage due to direct susceptibility of cardiomyocytes/non-cardiomyocyte cells to SARS-CoV-2 infection

Despite the multiple clinical manifestations and the complexity of the underlying mechanisms, which are still not completely understood\textsuperscript{26-28}, myocardial injury in COVID-19 could be also mediated by direct infection of myocardial cells. In fact, according to recent data produced with single-cell RNA sequencing, various cardiac cell types (cardiomyocytes, endothelial cells, smooth muscle cells, and fibroblasts) have been found to express the angiotensin converting enzyme-2 (ACE2) transmembrane protein\textsuperscript{29, 30}, one of the two major host cell receptors that mediate SARS-CoV-2 infection via interaction with the viral spike protein\textsuperscript{31}. Although the Transmembrane Serine Protease 2 (TMPRSS2), crucial for spike protein priming and viral entry, is not expressed at significant levels in cardiac cells\textsuperscript{32}, other receptors and peptidases, such as cathepsin-L (CTSL), furin and SCARFs are expressed in cardiomyocytes\textsuperscript{11, 33}, and neuropilin-1 [a vascular endothelial growth factor (VEGF) receptor] is expressed in endothelial cells\textsuperscript{34, 35}. These can compensate for the lack of TMPRSS2 and facilitate SARS-CoV-2 infection and replication in the myocardium. This picture is further complicated in case of concomitant cardiac diseases, such as aortic stenosis and heart failure, or in patients treated with antihypertensive therapies affecting the renin-angiotensin system, such as ACE-inhibitors, which have been shown to increase myocardial susceptibility to the virus due to ACE2 upregulation\textsuperscript{29, 30, 36-39}. Collectively, these findings prompt the modelling of SARS-CoV-2 infection using primary or stem cell-derived myocardial cells with genetically-controlled levels of ACE2 and other SARS-CoV-2 co-receptors in order to reveal the likely complex interaction of the different molecular pathways involved in acute cardiac injury and, potentially, in post-infection myocardial fibrosis.

Suggested pathways for SARS-CoV-2 direct damage to the heart

The myocardium may be directly affected by SARS-CoV-2 by various modalities that might occur separately or in concert\textsuperscript{40}. These different modalities are represented in Figure 1.

a) The cytotoxic effect of SARS-CoV-2 on the endothelium\textsuperscript{41} (Figure 1, panel a) may result in a pro-thrombotic status leading to diffuse micro-thrombosis in the heart, and conditions resembling type 2 myocardial infarction (MI)\textsuperscript{26}, or the Takotsubo syndrome\textsuperscript{42, 43}. The hypothesis on the role of endothelial dysfunction and SARS-CoV-2 infection has been extensively discussed elsewhere\textsuperscript{24} and is supported, at least in part, by the direct observations of thrombi in the microcirculation of myocardial tissues in patients with COVID-19\textsuperscript{19, 44}.

b) Although there are relatively few reports of confirmed cardiomyocyte infection and myocarditis in COVID-19 patients\textsuperscript{40}, ultrastructural and molecular studies have shown the
presence of SARS-CoV-2 in the myocardial tissue of patients with COVID-19\textsuperscript{17} (Figure 1, panel b). On the other hand, exposure of induced pluripotent stem cell (iPSCs)-derived cardiomyocytes to the virus showed the ability of SARS-CoV-2 to cause cytotoxicity, cell death and cessation of cell contraction due to the ability of the virus to bind cardiomyocytes \textsuperscript{20, 33, 39, 45}. Of course, \textit{in vitro} uptake does not imply \textit{in vivo} entrance of the virus into cardiomyocytes. Even more important, although the use of the anti-viral remdesivir was able to block damage to cardiomyocytes in culture\textsuperscript{20}, the efficacy of the drug for treating COVID-19 patients has been questioned\textsuperscript{46, 47}. This is a clear caveat in extending the implications of \textit{in vitro} results to the clinical scenario.

c) A third, relatively unexplored hypothesis is that, besides being exposed to viral cytotoxicity, the myocardium is a site for SARS-CoV-2 infection and replication within multiple non-contractile myocardial cell types such as endothelial cells, fibroblasts and pericytes (Figure 1, panels c, d). This aspect is particularly relevant for a systematic modelling of cardiac damage determined by infection, given the relevance of these cell types for myocardial inflammation\textsuperscript{49-51}. In support of this hypothesis, a recently published report has suggested that cardiac fibroblasts can be infected by the virus and increase its replication with an efficiency related to the level of expression of the ACE2 receptor. On the other hand, exposure to the virus also resulted in an ACE2-independent, sustained pro-inflammatory response, leading to upregulation of genes encoding inflammatory cytokines and extracellular matrix components involved in cardiac fibrosis\textsuperscript{52}. More controversial is, to date, the possibility that endothelial cells become infected by SARS-CoV-2. In fact, while initial reports showed the presence of the virus in the endothelial cells of COVID-19 patients\textsuperscript{41}, very recently the direct exposure of primary vascular endothelial cells to the virus did not give rise to productive infection, likely correlated to the absence of ACE2 expression in the tested cell lines\textsuperscript{53}.

d) An important point that must be addressed concerns the possible modality of transmission of the virus in the heart. This aspect appears particularly relevant considering that viral infection could progress through the mode of direct diffusion from cell to cell (the so-called 'viral synapse'\textsuperscript{54}) other than the release of the virus in the extracellular compartment. It should also be considered that cells exposed to the virus could raise or induce a strong innate immune response through the activation of the nuclear factor-κB (NF-κB) pathway, due to the interaction of the spike protein with cellular receptors such as the Toll-like receptors (TLRs)\textsuperscript{55, 56}. Whether a synaptic-like intercellular viral propagation exists in the heart (Figure 1, panels c, d) and infection independent activation of pro-inflammatory pathways occurs in the myocardium\textsuperscript{57} (see also section below) is thus far only a matter of speculation and should be further investigated \textit{in vitro} and \textit{in vivo}. 

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e) A final possibility, so far only speculative, but supported by evidence emerged from the autopsy reports on the lung as the primary target organ of SARS-CoV-2, and from in vitro reports on cell lines, is that expression of the viral Spike protein on the surface of infected cells might promote cell fusion of contractile and non-contractile cells (Figure 1, panel e) and the formation of syncytia with extensive cardiac damage. If a similar pathologic mechanism is experimentally confirmed in cardiac cells, the use of anti-syncytial drugs could be an option to prevent cardiac damage in COVID-19.

Towards a further systematic understanding of direct myocardial injury by SARS-CoV-2

In order to explore how SARS-CoV-2 directly damages the myocardial tissue, we suggest the use of advanced in vitro systems, in which virus-exposed myocardial cells are mixed with non-exposed cells, both in physical connection and separated by barriers permeable to the virus and/or secreted factor (Figure 2). Using this setting, the ability of infected cells to ‘pass’ the virus or viral particles to neighbouring uninfected cells via membrane contacts, such as adherens junctions-associated pores and/or tunneling nanotubes (TNT), might be assessed and quantified using methods for re-isolation of cells from mixed cultures, followed by analysis of the intracellular viral bioprocess with conventional or single cell transcriptomics and/or proteomics. This approach is ideally tackled within an ‘organoid’ system, in which primary cells (e.g., fibroblasts, pericytes) from human cardiac explants are mixed with human iPSC-derived cardiomycocytes after in vitro infection, or vice-versa (Figure 2). A similar approach has been successfully applied to demonstrate the cellular effects of SARS-CoV-2 infection in human enterocytes, or iPSC-derived hepatic/pancreatic organoids, and at least partially in cardiac organoids. With the latter approach, infected and uninfected cells are co-cultured in bioreactors enabling medium exchange by fluidic connections. These systems, set to assess and quantify the delivery of drugs and/or metabolites to cells, enable monitoring of trans-infection between different types of myocardial cell, or even in co-cultured lung-cardiac organoids. The proposed systems can also be exploited to screen drugs that inhibit viral entry and intracellular replication directly into cardiac cells, thus contributing to assess the efficacy of cardioprotective strategies (Figure 2).

Finally, since post-acute evolution of the infection may also result in chronic activation of pro-fibrotic pathways in the heart, it is of importance to assess matrix remodelling activity (e.g., expression of matrix metalloproteinases) and the electromechanical coupling of cells exposed to the virus (e.g., cardiac fibroblasts and myocytes) alone or within organoids. This approach enables evaluation of the impact of the viral infection on matrix compaction, composition and remodelling, and their readout on the propagation of the action potentials that are required to maintain a synchronized heartbeat. In this regard, the combination of matrix components with cardiac cells exposed to virus or to recombinant SARS-CoV-2 proteins likely represent highly standardisable approaches, also amenable to high-throughput screening of drugs with antiviral action or antifibrotic/antiarrhythmic effects. This approach can optimally benefit from the use of biological material (cardiomyocytes,
fibroblasts) obtained by iPSC reprogramming of cells from patients carrying genetic mutations for arrhythmic syndromes such as the long-QT syndrome, one of the conditions associated with severe arrhythmias caused by various COVID-19 medications, i.e. hydroxychloroquine with azithromycin, and the use of conventional or pathological experimental settings mimicking some common effects of the disease, such as electrolyte imbalance, altered pH, or hypoxemia.

2. Indirect effects of SARS-CoV-2 infection on pro-inflammatory and pro-fibrotic pathways in the cardiovascular system

Several mechanisms have been proposed for how SARS-CoV-2 could indirectly affect the cardiovascular system, i.e., without involving direct infection of cardiovascular cells (Figure 1, panels a, b). These include ACE2 downregulation/shedding, a SARS-CoV-2-elicited cytokine storm, activation of thrombotic mechanisms, i.e., the activation of platelets and of the so-called “neutrophil extracellular traps” (NETs), and profound changes in the immune profile. These phenomena triggered by the virus can occur in parallel in case of viral damage, and interact with each other, exacerbating their effect.

ACE2 downregulation and shedding

After the initial interaction between SARS-CoV-2 spike protein and ACE2, the expression of ACE2 in the epithelial cells of the lung alveoli is strikingly reduced. Loss of ACE2 leads to the accumulation of angiotensin (Ang) II in the circulatory system, which plays a central role in the activation of the interleukin (IL)-6 amplifier, with the coactivation of NF-κB and the Janus kinases (JAK)-signal transducer and activator of transcription Signal Transducer And Activator Of Transcription 3 (STAT3) pathways. Therefore, SARS-CoV-2-infected patients fail to exert a robust, interferon (IFN)-mediated antiviral response, and exhibit exuberant inflammatory cytokine production. Ang II may further induce tumour necrosis factor (TNF) convertase (ADAM17), which leads to shedding of membrane-bound ACE2 and the release of soluble ACE2. Of interest, the expression of ADAM17 is negatively regulated by microRNA-145 and the administration of specific antagonists targeting microRNA-145 has been found to increase the level of circulating ACE2, thereby reducing viral entry into cells. Beyond the local renin angiotensin aldosterone system (RAAS) activation in the lung, there is evidence that patients suffering from a severe course of SARS-CoV-2 infection have elevated levels of plasma Ang II, which correlate with total viral load and the degree of lung injury. Loss of ACE2 and activation of the RAAS also result in a widespread endothelial dysfunction and multiple organ injury, including the heart, the kidney and the lung.

In summary, the susceptibility of the heart and the vasculature to the hyperactive RAAS and pro-inflammatory cytokines appears to be the prevalent modality by which SARS-CoV-2 can indirectly affect the cardiovascular system.

Cytokine storm
SARS-CoV-2 infection induces a strong activation of the innate immune system, leading to elevated levels of several pro-inflammatory cytokines, including IL-6, IL-1, IL-2, TNF-α and IFN-γ. Besides a direct impact of SARS-CoV-2 on ACE2 and Ang II, the activation of the innate immune system is in part due to the activation of the IL-6 amplifier via TLR4\textsuperscript{79}. The resulting “cytokine storm-related hyper-inflammation syndrome” underlies many of the severe manifestations of COVID-19 and is suggested to contribute to COVID-19-associated cardiovascular disease and death. IL-6 appears to play a central role here, with increased serum levels correlating with the onset of acute respiratory distress syndrome (ARDS) and with adverse clinical outcome. Besides elevated circulating IL-6, COVID-19 patients exhibit increased plasma levels of the soluble IL-6 receptor (sIL-6R) in plasma, reflecting its enhanced cleavage from the cell surface during infection\textsuperscript{80}. By binding to ubiquitously expressed cellular gp130, circulating IL-6/sIL-6R complexes can directly activate JAK-STAT signalling throughout the body. Such activation in endothelial cells may cause secretion of VEGF, a reduction of E-cadherin expression, and defective pericyte coverage\textsuperscript{81}, contributing to vascular permeability and leakage\textsuperscript{82}. Beyond these effects, which participate in the pathophysiology of hypotension and pulmonary dysfunction in ARDS, IL-6 induces oxidative stress and endothelial dysfunction through overexpression of the Ang II type-1 receptor\textsuperscript{83}. In a highly interdependent relationship with Ang II signalling, IL-6 further promotes vascular hypertrophy, vascular inflammation and stiffness, involving induction of matrix expansion\textsuperscript{84, 85}. In the heart, both protective and harmful effects of IL-6 have been reported\textsuperscript{86}. Myocarditis is a striking example of the dysregulation of the IL-6 response leading to a detrimental outcome. IL-6 is protective in the heart as far as it limits viral replication and thus cardiac damage\textsuperscript{87}, but long exposure to IL-6 can contribute to heart failure\textsuperscript{88}. IL-6 receptor antagonism with tocilizumab in experimental myocarditis has been shown to reduce cardiac inflammation and cardiac fibrosis, and to improve cardiomyocyte titin phosphorylation and thus myocardial stiffness\textsuperscript{89}. In summary, IL-6 appears to be a central player in the hyperinflammatory response to SARS-CoV-2. The efficacy of anti-IL-6R agent tocilizumab for the treatment of severe cases of COVID-19 is debated, due to the lack of confirmation of the efficacy of this drug in three randomized trials\textsuperscript{90}, initially demonstrated in small observational studies\textsuperscript{91, 92}, and two large positive trials\textsuperscript{93, 94}. The effect of anti-IL-6R agents is likely to depend on the severity of the inflammatory/cytokine storm response. The key factors that could play an important role in the effect of anti-IL-6R agents may be the timing [given before admission to the Intensive Care Unit (ICU)], and also the combination with other drugs, such as high doses corticosteroid, which is being evaluated in several ongoing trials\textsuperscript{95} (Figure 2).

**Immunothrombosis**

Negative COVID-19 outcomes are associated with increased levels of fibrin degradation products (D-dimers) and lower platelet counts, which are markers for an activation of hemostatic pathways\textsuperscript{90}. Hypercoagulation is considered the main cause of organ failure in severe cases of COVID-19, supported by recent observations of micro-thrombi in the lungs, brain, heart and other organs\textsuperscript{96-99}. Not only is the endothelium damaged in response to viral infection, but hyperactivated monocytes,
platelets and neutrophils may also play a pathophysiological role in this process. The coagulation cascade is induced by tissue factor, which is mainly expressed by circulating monocytes, but also exposed on activated endothelial cells, leading to fibrin deposition and blood clotting. Neutrophils are recruited from activated endothelial cells and release NETs, consisting of DNA, histones, and granule protein. NETs may serve as a scaffold for thrombus formation by capturing and activating platelets, red blood cells and procoagulant molecules. COVID-19 patients have elevated circulating levels of NETs, measured as myeloperoxidase-DNA complexes, and their levels correlate with both the severity of disease and the occurrence of myocardial infarction. The presence of platelets, neutrophils and NET-like structures in the lung and in cardiac microthrombi has been confirmed in COVID-19 autopsies, and potentially contributes to organ fibrosis. In support of enhanced immunothrombotic status in severe COVID-19 patients, serum or plasma from COVID-19 patients have been shown to trigger excessive NET formation in neutrophils in vitro, with enhanced NETosis found in neutrophils from COVID-19 patients. Finally, NETs released by SARS-CoV-2-activated neutrophils have been shown to promote lung epithelial cell death in vitro. Also, autoantibodies that recognize phospholipids could trigger NETs activation, thus exposing patients to risks of hyper-coagulation, similar to that occurring in the antiphospholipid antibody syndrome. Taken together, these data support NETs as potential effectors of thrombosis in organs affected by SARS-CoV-2, including the heart, where NETs activation could affect the microcirculation, determining diffuse ischemic conditions. So far, NETs formation can only be assessed indirectly, based on the detection of circulating DNA, histone H3, and myeloperoxidase. In this regard, while neutrophil activation can be monitored using conventional techniques, such as antibody-based multiparametric flow cytometry, there is still a lack of validated assays to reveal the activity of enzymes linked to NET release (e.g., elastase, myeloperoxidase) directly on the cell surface. The use of enzymatic activity-sensitive probes would be an advantage to unravel the relationships between neutrophil activation and NETs release upon stimulation of the cells with, for example, COVID-19 patient sera, or to readily detect circulating activated neutrophils at different times after infection or during recovery. This approach could finally benefit from the adoption of microfluidic devices to unravel whether the excessive NETosis in the microcirculation observed in several organs, including the heart, depends on an exacerbation of the shear forces action on hyper-activated neutrophils (Figure 2). This can be useful also for testing the efficacy of conventional and experimental strategies to reduce neutrophil activation and NETs release during the acute phase of infection, as well as the emerging post-COVID-19 syndrome.

**Altered immune cell profile**

Emerging findings from the peripheral blood mononuclear cell immune profile comparing mild and moderate versus severe COVID-19 reveal profound changes in innate and adaptive immune cell compartments. Regardless of the severity of the disease, COVID-19 is associated with increased numbers of neutrophils and reduced number of T-lymphocytes, as well as a selective depletion of...
non-classical monocytes\textsuperscript{116}. Since patrolling, non-classical monocytes play a crucial role in endothelial cell homeostasis and repair, the loss of this monocyte population may contribute to microthrombosis and associated complications. Moreover, a COVID-19-specific alternative activation pattern of classical monocytes correlating with disease severity has been identified\textsuperscript{116}. It remains merely speculative, however, whether an altered monocytes phenotype promotes enhanced cardiac infiltration of this leukocyte subset and subsequent cardiac damage. Severe COVID-19 is associated with emergency granulopoiesis and increased frequency of immature and dysfunctional neutrophils. These immature myeloid cells may have immunosuppressive functions, as previously observed in cancer and sepsis\textsuperscript{117-119}. Moreover, a cluster of mature neutrophils expressing CD274 [Programmed Death-Ligand 1 ((PDL1)] was only detected in severe COVID-19 cases, suggesting that this receptor acts as an immune ‘checkpoint’, blocking T-cell activation\textsuperscript{116}. Additional studies are necessary to clarify the role of these subsets of immature, potentially immunosuppressive neutrophils in cardiac inflammation following COVID-19. It has been recognized that life-threatening COVID-19 conditions can result from the presence of anti-IFN autoantibodies, which trigger reduction of innate immune response to SARS-CoV-2\textsuperscript{120}. Since neutralization by autoantibodies in response to SARS-CoV-2 infection affects IFN-α but not the IFN-β subtypes, it is tempting to speculate that treating patients with IFN-β may ameliorate the disease. This latter conclusion also bears an important cardioprotective readout, given the beneficial effect of IFN-β on reduction of fibrosis-associated factors in cardiac fibroblasts\textsuperscript{121}.

3. Novel biomarkers associated to SARS-Cov-2 infection
Since the beginning of the pandemic, several reports have investigated the important prognostic value of markers of acute cardiac injury [mainly cTn, pro-thrombotic state (D-dimer, fibrinogen), increased inflammatory response (C-reactive protein, lactate dehydrogenase, IL-6, procalcitonin, ferritin) and heart failure [brain natriuretic peptide (BNP) and its N-terminal pro-peptide, NT-proBNP] in patients with COVID-19\textsuperscript{1, 4, 6, 27, 122, 123}. Although multiple studies consistently demonstrate that several cardiac biomarkers correlate with the severity and prognosis of COVID-19 infection in critically ill patients, whether established cardiovascular biomarkers might provide additional prognostic information over clinical or physiological information in unselected patients hospitalized for COVID-19 with various degrees of disease severity has been recently questioned\textsuperscript{124}. Indeed, the levels of biomarker and the interpretation of the data depend on the severity of COVID-19, sex, age and the condition of new versus pre-existing cardiac disease\textsuperscript{124}. Importantly, the prognostic value of some of these blood-based biomarkers in the context of COVID-19 might differ from commonly used reference standards\textsuperscript{125}. Additional circulating biomarkers potentially associated with SARS-CoV-2 infection have been identified and proposed for future clinical use in COVID-19. For example, it has been recently shown that growth differentiation factor 15 (GDF-15) may represent a potential biomarker. GDF-15 is a member of the transforming growth factor β superfamily, which is induced by aging and several
diseases, including cardiovascular diseases, sepsis and cancer. GDF-15 has improved prognostic value compared to various cardiovascular and inflammatory biomarkers in unselected patients hospitalized with COVID-19. GDF-15 levels were elevated in the majority of COVID-19 hospitalized patients, and higher concentrations were associated with ICU admission and death during hospitalization, as well as SARS-CoV-2 viremia and hypoxemia. Although the precise mechanisms underlying the latter association are not yet completely understood, these results suggest that GDF-15 may provide important pathophysiological information in hospitalized patients with COVID-19 while contributing to risk stratification. SARS-CoV-2 binding to ACE2 leads to its internalization and cleavage to soluble ACE2 (sACE2), decreasing ACE2 tissue levels. It has been proposed that sACE2 levels might reflect a higher cellular content of ACE2 and thus greater susceptibility to COVID-19. In addition, sACE2 might be a marker of the RAAS dysregulation. Consistent with this possibility, in two large, independent cohorts of elderly patients with atrial fibrillation and increased risk of stroke, higher levels of sACE2 were associated with male sex, cardiovascular disease, diabetes, and older age, which are also the main risk factors for complications and mortality of COVID-19 patients. Interestingly, levels of GDF-15, NT-proBNP and high-sensitivity cTn had the strongest associations with sACE2 levels and the risk of death and cardiovascular complications. More recently, in a large, prospective, global, community-based cohort of patients, increased levels of circulating sACE2 have been associated with a higher increased risk of total death, myocardial infarction, incident heart failure, stroke and diabetes. However, to what extent sACE2 levels in blood samples of COVID-19 patients reveal a prognostic role remains to be established. Also, further research is warranted to dissect the relationships between circulating sACE2 levels and ACE2 expression in various organs, and whether ACE2 and/or sACE2 levels might affect the risk of SARS-CoV-2 infection or severity. Large-scale targeted/untargeted molecular screening technologies can also help to find novel measurable markers of cardiovascular risk in COVID-19 patients. For example, genome-wide association studies (GWAS) and Mendelian randomization can help identify host genetic variants associated with critical illness that enable identification of novel mechanistic targets for therapeutic development. Very recently, the Genetics Of Mortality In Critical Care (GenOMICC) study has discovered new variants in patients admitted to ICUs in the United Kingdom, plausibly associated with the immune-mediated phase of COVID-19, such as activated IFN signalling, monocyte activation and infiltration. Moreover, transcriptomic studies have identified a specific transcriptional signature induced by viral infection in cardiomyocytes, characterized by the induction of genes involved in IFN signalling, apoptotic cell death, reactive oxygen species production and disruption of structural proteins associated with myofibrillar fragmentation. In this regard, non-coding RNA expression (especially miRNAs) may have prognostic value for their involvement in the regulation of the replication cycle/viral genome translation of RNA viruses, including COVID-19, and in the control of risk conditions associated with a worse COVID-19 prognosis. Finally, COVID-19 may alter
the expression pattern of circulating miRNAs\textsuperscript{137}. If confirmed, this will suggest the existence of specific miRNA signatures characterizing the disease and possibly correlated to its severity in the heart and other organs\textsuperscript{138} (Figure 2). On the other hand, it should be noted that there are currently substantial diagnostic challenges with miRNA expression pattern analysis, e.g., non-standardized test formats, lack of knowledge about normal miRNA levels, and how they may be affected by confounding factors\textsuperscript{139}.

Proteomic profiling of sera from COVID-19 patients is of interest as it provides valuable and novel information on disease progression and prognosis, leading to the identification of novel biomarkers or targets discriminating cardiovascular involvement\textsuperscript{140-142}. In addition, the definition of SARS-CoV-2-encoded proteome in relation to human genetics enables the unmasking of risk factors for adverse outcome in patients, as well as possible therapeutic interventions that may prevent infection\textsuperscript{143}. Untargeted proteomics may be utilized to identify key molecular effectors associated with viral infection in cells exposed to the virus \textit{in vitro}. By using proteomics, an exhaustive map of the cellular pathways affected by the virus can be derived and crucial components of mRNA maturation, protein translation and metabolic control machineries can be identified, producing useful information for potential targeting key intracellular cascades implicated in viral replication\textsuperscript{144}. In line with these results, metabolomic and lipidomic serum profiling has shown that COVID-19 exerts remarkable effects on the metabolism by increasing, for example, the levels of ketone bodies and 2-hydroxybutyric acid, indicating altered hepatic glutathione synthesis and oxidative stress, and promoting a redistribution of serum lipoproteins potentially enhancing atherosclerotic risk\textsuperscript{145, 146}.

Finally, it has been suggested that COVID-19 may also exacerbate age-related mitochondrial dysfunction with detrimental effects on inflammation, oxidative phosphorylation metabolism and antiviral response\textsuperscript{147}. Exposure of myocardial-derived cells to sera of patients, ideally sampled at various stages of the disease, could be an answer to the problem of potential alterations in cardiac metabolism leading to damage and cytotoxicity. Considering that molecular targets identified by omics analyses are an integral part of the pathophysiology in several diseases, the use of omics in COVID-19 can help in the identification of new possible therapeutic strategies for targeting, for example, \textit{i}) viral proteins essential for virus entry into the host cell, \textit{ii}) proteins involved in virus-host interaction, and \textit{iii}) pathways involved in intracellular signalling elicited by virus entry or virus/cell interactions (Figure 2).

**Conclusions**

The quest for immediate answers to SARS-CoV-2 pandemic outbreak has prompted scientists around the world to make an unprecedented effort to understand the pathophysiological basis of the infection and its consequences for a systemic disease affecting various organs and organ systems. According to the fast growing availability of scientific literature on this topic, with unprecedented speed, and daily clinical experience, the heart appears as one of the elective targets of the virus, even if, to date, the nature of the damage and the persistence of long-term complications are unclear.
A possible consequence of COVID-19 in the post-pandemic period could be an increase in heart failure\textsuperscript{148}, with social costs additive to those already sustained for combatting the impact of the disease.

The introduction of SARS-CoV-2 experimental model systems may help understand the interplay between systemically acting factors (e.g., the cytokine storm) and tissue-specific responses that determine the multi-organ failure often observed in most severe cases\textsuperscript{149,150}. Finally, an aspect that should be taken into account in this scenario is the relevance, in addition to age, of risk factors, including sex and frequently associated co-morbidities, in ACE2 regulation, inflammatory responses and thrombosis\textsuperscript{151-158}. In line with our recent recommendations for ischemic heart disease\textsuperscript{159}, it will be thus important to address the interaction of confounders such as sex and comorbidities in COVID-19 experimental settings, by including, for example, individuals and cells from both sexes. Moreover, in the event that sexual dimorphic phenotypes are observed, it should be determined experimentally whether they are dependent on the hormonal status, and if they are specific, or modified by genetics and sex. In this regard, in vivo preclinical models will be extremely helpful for addressing the role of sex by combining COVID-19 experimental conditions with specific comorbidity models in male and female animals, also including the most prevalent and relevant risk conditions for COVID-19, and the effects of their co-medications (Figure 2). Together with the results from in vitro modelling with human cells, integrated with unbiased multi-omics approaches and molecular network analyses, these efforts will contribute to a decisive advancement in precision medicine and genetic surveillance to predict new genetic variants of SARS-CoV-2, to redesign second generation vaccines for new variants and to improve prevention, diagnosis and treatment of COVID-19 cardiac complications more effectively. In this regard, the creation of a global genetic surveillance system for the virus appears necessary for the effective control of the pandemic. This will be key in the years to come to ensure that we will not be in a position where, at the time of herd immunity, the virus escapes from immunological control, and it becomes necessary to redesign vaccines for a new variant.

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Conflict of interest

PF is the founder and CEO of Pharmahungary Group, a group of R&D companies.
References


38. Oz M, Lorke DE, Kabbani N. A comprehensive guide to the pharmacologic regulation of angiotensin converting enzyme 2 (ACE2), the SARS-CoV-2 entry receptor. *Pharmacology & Therapeutics* 2021;**221**:107750.


What’s Sex Got to Do With COVID-19? Gender-Based Differences in the Host Immune Response to Coronaviruses. *Frontiers in Immunology* 2020;11.

Genes encoding ACE2, TMPRSS2 and related proteins mediating SARS-CoV-2 viral entry are upregulated with age in human cardiomyocytes. *Journal of Molecular and Cellular Cardiology* 2020;147:88-91.

Figures

Figure 1. Proposed mechanisms of myocardial damage by SARS-CoV-2. (a) Infection of endothelial cells and exposure to circulating cytokines may cause increased platelet activation and micro-thrombosis. In the heart, this can lead to diffuse clotting, causing conditions similar to type-2 myocardial infarction. (b) Direct damage of cardiomyocytes may occur as a result of ACE2 receptor expression. Direct infection of these cells may cause decreased cardiac contractility and cell death due to cardiotoxicity. (c, d) Infection of stromal cells, recently demonstrated in vitro, could damage the heart through infection-independent differentiation into myofibroblasts and ACE2-dependent intracellular replication. In addition to the release of the virus in the intercellular space by infected and death cells, the possibility of spreading the virus to adjacent cells by the so-called `viral synapse" is discussed as a further modality of virus propagation in cardiac tissue and myocardial
injury. (e) Formation of multinucleated syncytia might cause extensive cardiac damage due to the fusion of contractile and non-contractile myocardial cells, both of which express ACE2 receptor. This is still an experimentally unsupported mechanism of cardiac damage that could be addressed with in vitro studies using patient-derived and iPSCs-derived cardiac cells.

Figure 2. Experimental algorithm and bottom-up approach proposed to limit cardiac damage induced by SARS-CoV-2 infection. The disease caused by SARS-CoV-2 infection leads to a sharp elevation in the level of circulating inflammatory cytokines and to an increase in thrombotic events, especially in the microcirculation. The left box describes materials that could be combined in vitro to systematically approach the problem of cardiac damage from direct infection of myocardial cells, or by mimicking the pro-inflammatory effects of the cytokine storm. This approach aims to i) clarify
the cardiotoxicity and intra-cardiac viral replication of the virus, \textit{ii) assess} the pro-inflammatory/pro-fibrotic responses in the heart due to direct/indirect effects and, \textit{iii) dissect} cardiac remodelling and arrhythmic events. The box on the right indicate materials and tools that could be used to investigate in vitro the problem of the hypercoagulation found in COVID-19 patients with reference to \textit{i) neutrophil activation and release of the neutrophil extracellular traps, ii) mechanisms of platelet and coagulation cascades activation and, iii) the effects of shear forces in COVID-19-dependent diffuse cardiac thrombosis. The results emerging from these two experimental research areas should be finally integrated (arrows) with results from 'omics research performed directly with patient samples, and with results of epidemiologic/genetic studies and clinical trials. The aims of this bottom-up approach are, \textit{i) to define} new markers to assess the severity of cardiac damage, \textit{ii) to understand the relevance of modifiable/non-modifiable risk conditions, comorbidities and drugs in the severity of cardiac damages and iii) to validate treatments reducing the impact of COVID-19 on cardiac health.