**Introduction**

Coronavirus disease 2019 (Covid-19), with common symptoms of fever, headache, fatigue, myalgia, dry cough, and in the advanced form of the disease increasing respiratory distress [1], is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a single-stranded RNA virus, belonging to the Betacoronavirus genus [2]. SARS-CoV-2 has been reported to infect several organs in the human body by binding to its extracellular protease receptor e.g. the angiotensin-converting enzyme 2 (ACE2) and type II transmembrane serine protease (TMPRSS2) [3,4]. The SARS-CoV-2 infection leads to the activation of both the innate and adaptive immune systems [5,6].

The heart is often affected in Covid-19 patients. Studies have shown that patients with previous cardiovascular diseases such as cardiomyopathy, hypertension, coronary heart disease, and arrhythmia have a higher risk of being critically ill when infected with SARS-CoV-2 [7]. Direct viral infection of the myocardium could lead to myocarditis. Covid-19 leads to rising concentrations of cardiac biomarkers such as Troponin, lactate dehydrogenase, high sensitivity amino-terminal B-type natriuretic peptide, creatinine kinase, and creatinine kinase myocardial band indicating myocardial damage [8–10]. To date, tens of studies have reported myocarditis/myopericarditis secondary to Covid-19 infection in living patients with a male predominance (58%), and a median age of ~50 years [11]. On conventionally stained heart-tissue sections, inflammatory cellular infiltration with or without concomitant myocyte necrosis is required by the Dallas pathological criteria for the diagnosis of myocarditis [12]. Electrocardiogram findings are variable, and troponin is elevated in 91% of cases. Glucocorticoids are the most commonly used in the treatment of myocarditis (58%). However, our knowledge of myocarditis associated with Covid-19 is incomplete; thus, guidelines for diagnosis and management of Covid-19 associated myocarditis have not been established yet [11].

It has been suggested that Covid-19 may cause acute myocardial infarction (AMI), a heart attack [13]. AMI causes myocardial damage through ischemia followed by necrosis [14].

The Covid-19 disease is associated with hypoxemia caused by acute respiratory distress syndrome (ARDS) [15]. The hypoxia-induced systemic response is regulated by the
Hypoxia-Induced Factor (HIF) containing subunits HIF-α, responsible for the hypoxia regulation, and HIF-b, responsible for the transcription. Hypoxia also induces neo-angiogenesis creating new blood vessels from an already existing blood vessel. Neo-angiogenesis is stimulated by the HIF pathway through the production of vascular growth endothelial factors (VEGFs) which activate resting endothelial cells and induce cell migration, cell division, and vacuolization [16].

We already reported that the patients who had ARDS suffering from left ventricular hypertrophy of the heart are overrepresented among those who died of Covid-19 [17]. We showed generalized hypoxic damage to the myocardium. However, the endothelium damage in various tissues of the Covid-19 victims was not associated with the presence of replicating viruses, therefore, the reason for cardiac involvement has not yet been explained [17]. As a follow-up to our previous research, this current study examines the histomorphological changes of the myocardium in Covid-19 ARDS deceased patients based on histological and immunohistochemical (IHC) studies and RNA in situ hybridization. We aimed to characterize myocardial involvement by using a panel of antibodies to detect hypoxic and inflammatory changes and the presence of SARS-CoV-2 proteins. In addition, the integrity of the walls of small vessels was analyzed. We hypothesize that virus or coded mediators originating from the virus-infected lung tissues (affecting small vessel endothelium integrity) are responsible for systemic organ damage e.g. the damage to the myocardium [18].

**Material and methods**

**Sampling and tissue preparation**

During the autopsy, cardiac tissue samples (>140) were collected from thirty-seven (n = 37) individuals of Covid-19 deceased patients (The reason for death was ARDS in all cases) in addition to twenty-one (n = 21) unrelated control cases. Following accredited laboratory procedure at Karolinska University Hospital, Huddinge; the tissues were formalin-fixed, paraffin-embedded, stained with hematoxylin-eosin (HTX), and sectioned (~3.5 μm thickness). Subsequently, all slides were examined microscopically and scanned digitally using Nanozoomer S360 (Hamamatsu, Japan) at high resolution (40× objective). The digital tissue specimens were examined using NDPview 2.0 slide viewer program (Hamamatsu, Japan).

**Immunohistochemical staining**

The immunohistochemical staining was performed automatically by using BOND-MAX (Leica Biosystem, Germany) and Ventana (Medical Systems/Roche, USA). The instrument requires diluted antibodies (Supplementary Table 1). After immunohistochemical staining and dehydration, the slides were mounted with coverslips (Tissue-Tek Film®, Coverslipper, Japan).

| Heart and body characteristics of the patients. |
|-----------------------------------------------|
| Age (year) | Median | Min | Max |
| Body mass index | 27 | 19 | 51 |
| Body weight (kg) | 84 | 55 | 150 |
| Total heart weight (g) | 507 | 290 | 906 |
| Male heart weight (g) | 520 | 303 | 906 |
| Female heart weight (g) | 435 | 290 | 703 |

**RNA in situ hybridization (RNAscope®)**

RNAscope® was performed against SARS-CoV-2 by producing special human-designed Z probes (Bond III Leica Systems, USA). Three manufactured probes have been used including SARS-CoV-2 spike (COVSPIKE, 21,631 – 23,303 bp, ACD # 848568), one negative control probe against the bacterial hydroxy-tetrahydrodipicolinate reductase (414-862 bp), and one positive control probe Ubiquitin C. (342 – 1503 bp, Advanced Cell Diagnostics, USA).

**Results**

**Comparison of heart weight to the body weight**

The patients’ median body weight in Covid-19 was 84 kg while the median body mass index (BMI) of the Covid-19 patient was 27 kg m² (Table 1).

The mean heart weight in Covid-19 patients (525 g for males and 455 g for females) (Table 1) in comparison to the normal heart weight (353 g for males and 303 g for females) of the same median body-weight group (84 kg) of healthy individuals [19] showed ~1.5-fold increase for both sex groups.

**Macroscopic examination**

Three Covid-19 patients suffered from myocardial infarction; two cases had occluded right coronary artery with remnant after old infact and minimal histological changes in the posterior wall. In one case, an occluded left coronary artery showed a massive fresh infarct in the anterior wall. The macroscopic findings of the Covid-19 victims’ (C16) hearts show the signs of myocardial infarction with blood clots in the left anterior descending and the left circumflex coronary arteries blocking the blood flow. The lack of oxygen caused damage to the myocardium (Supplementary Figure 1). Detailed information about occlusion grading and hypertrophy in all patients is provided in Supplementary Table 2. All patients summary is provided in Supplementary Table 3.

**Histopathological changes**

Covid-19 victims with AMI showed specific histological changes such as waviness of myocytes, eosinophilia, karyopyknosis, vacuolization, and contract band necrosis. The waviness of myocytes occurs when the isolated contractility of myocytes is lost but the neighboring fibers are still contracting. The waviness of myocytes is demonstrated...
by myofibrils narrowing and becoming wavy. Over 90% of the cases showed the presence of wavy cardiocytes in at least one of the histological samples. Fibrosis was seen as a clear sign of old infarction where the dead heart muscle cells are replaced by collagen-rich connective tissue. Remnants of old infarct in form of fibrosis were detected only in the three cases with AMI due to coronary occlusion (Figure 1(A)).

Contraction band necrosis often occurs as a myocardial injury at a site of reperfusion when oxygen-rich blood returns to the ischemic area, typically at the edge of the infarcted area. Almost half of the Covid-19 hearts showed contract band necrosis even in the absence of coronary occlusion (Figure 1(B)).

Vacuolization, the sign of decreased ion pump activity, caused by hypoxia-induced ATP depletion, occurs in the cardiomyocyte sarcoplasm forming cavities (Figure 1(C)).

Neutrophil infiltration is associated with myocyte necrosis and is a sign of the late phase of myocardial infarction. Disintegrating cardiomyocytes release mitochondria and mitochondrial DNA that is interpreted as a PAMP by the innate immune system leading to the recruitment of the neutrophil granulocytes as shown in control cases with the AMI (Figure 1(D)). However, none of the Covid-19 victims showed signs of neutrophil infiltration in the heart indicating that only a short time has elapsed between the development of severe hypoxia and the death.

**Immunohistochemical results**

Representative cases, with short postmortem time showing severe forms of the described histological changes, were selected for immunohistochemical staining for CD31, IgM, WT-1, and CD61 markers.

CD31 stains the endocardial endothelium of the myocardium marker allowing the detection of endothelial activation. Activated endothelial cells became fibroblast-like with protruding filopodia and large activated euchromatic nuclei showing various degrees of cytoplasmic vacuolization and migrating from the vessel wall to form new capillary structures (Figure 2).

Production of IgM antibodies is the first immune response in the blood during viral infection or recovery after a viral infection/vaccination. The IgM staining was used to detect the presence of IgM antibodies in the bloodstream or if it occurs outside the bloodstream indicating endothelium damage [20]. IgM stained the entire myocardium tissue in 6 out of 8 representative Covid-19 cases (Figure 3).

A section from the Covid-19 victim’s heart (C8) (left) and control (right).

Transcription factor Wilms’ tumor-1 (WT-1) is a HIF1 target gene induced by hypoxia which is stable even after a longer postmortem time; therefore, a reliable marker to demonstrate ischemic damage (Figure 4).
CD61 staining was used to detect individual or aggregated thrombocytes in the bloodstream; 6 out of 8 cases showed abnormal thrombocyte aggregation (Figure 5). In addition, CD3/CD20 and CD8/CD4 stains did not detect any increase in lymphocytes.

Detection of Sars-Cov-2 using RNA in situ hybridization

To detect the presence of the Sars-Cov-2 virus in the heart of Covid-19 patients, RNAscope® was conducted. However, replicating the SARS-CoV-2 virus was not detected in the heart of Covid-19 patients (Figure 6). In contrast, the presence of replicating the Sars-CoV-2 virus was readily detected in the lung tissue in the same individuals (Figure 7).

Discussion

This study aimed to characterize myocardial involvement in Covid-19 critically ill patients by using a panel of antibodies to detect hypoxic and inflammatory changes and also the presence of SARS-CoV-2 proteins using RNA Scope. In addition, the integrity of the walls of small vessels was analyzed. SARS-CoV-2 causes irreversible changes in several organs like the heart; which is reflected by the increased cardiac
risk in COVID-19 patients who survived [10]. Several studies claim that Sars-CoV-2 infects the heart causing inflammation and leading to the secretion of inflammatory factors such as chemokines and cytokines, e.g. interleukin 1b, 6, 8, 10, MCP-1, MIP-1A, and NFα [5,6]. During the autopsy examination of this study, 3 out of 37 patients showed to have suffered from the AMI due to occluded coronary arteries. The inflammatory secretions were not identified but the heart weight of Covid-19 victims was ~1.5-fold more compared to normal heart weight in the same body-weight range.

It has been shown that Sars-CoV-2 virus replicates in pulmonary epithelial cells [21]. Bulfamante, Perrucci [22] also claimed the presence of SARS-CoV-2 in the heart of Covid-19 victims. In our study, HTX staining showed varying degrees of histopathological damages that are associated with heart ischemia and hypoxic damage of the myocardium including the waviness and vacuolization (>90 of the cases) of myocytes and contract band necrosis (~50% of cases). However, RNA in situ hybridization could not detect virus replication in the heart of Covid-19 victims. The negative RNA in situ hybridization coupled with the positive localization in the lung does favor the hypothesis of a systemic mechanism for the cardiac changes [23].

Furthermore, any sign of virus-induced cytopathic effects or any antiviral lymphocytic reaction typical for viral
myocarditis was not detected in any cases. Also, signs of antiviral inflammation were not observed. Some studies claim there is a sign of lymphocyte infiltration in the Covid-19 heart [24]. For example, multifocal lymphocytic myocarditis was observed in a small fraction of the cases in a multicenter COVID-19 pathological study [25]. Furthermore, quantitative analysis of inflammatory infiltrates in COVID-19 hearts showed a higher number of CD68⁺ cells proposing that COVID-19 may cause a different type of myocarditis than conventional viral myocarditis, one that is associated with diffusely infiltrative monocyte/macrophage cells [26]. However, we didn’t detect any lymphocyte or

Figure 6. Absence of Sars-CoV-2 virus RNA in the heart of Covid-19 patients. Covid-19 heart (C9) does not show virus replication. Ubiquitin C positive control (Left), dap B negative control (middle), and COVSPIKE (right).

Figure 7. Presence of Sars-CoV-2 virus RNA in the lung. Covid-19 lung (C9) showed virus replication in the desquamated lung epithelial cells. Ubiquitin C positive control (Left), COVSPIKE (middle), and dap B negative control (right).
granulocytic infiltration in the Covid-19 cohort as a hallmark of myocarditis.

The myocardial hypoxia marker WT-1 was shown to be induced in 3 out of the 8 cases. Acute hypoxia due to rapidly deteriorating lung function rapidly killed most Covid-19 critically ill patients. Ventilated patients with overpressure oxygen in the breathing air likely suffered from the slower decline of systemic oxygen and had time to develop systemic prolonged multiorgan hypoxia including diffuse prolonged hypoxia of the heart [17].

Thrombocyte aggregation was often present in the small vessels of the Covid-19 hearts cases indicating vessel wall damage. IgM leakage into the extravascular space also indicated endothelial damage. One possible explanation for increased vascular permeability can be the overexpression of VEGF [27]. For example, it has been shown that the SARS-CoV-2 infection causes endothelial exocytosis, which activates two parallel pathways, microvascular thrombosis, and microvascular inflammation, eventually leading to hyperinflammation and diffuse thrombosis seen in severe COVID-19 cases [28].

Endothelial activation was detected in all cases independently if local prolonged ischemia (shown as WT-1 induction) was evident. A possible explanation for this phenomenon is that the endothelium activating agent was not originating from the local hypoxic tissue but from other sites in the body, e.g. from the consolidated hypoxic areas of the Covid-19 lungs. The most likely explanation for the endothelial activation is the presence of circulating VEGF that is known to be produced in the Covid-19 lungs in large quantities [27]. In addition, immunohistochemical staining (CD31 and CD146) showed signs of endothelial activation due to the enlarged fibroblast-like endothelial cells with activated euchromatic nuclei. Our study showed the activation of the endothelium in the form of aggregation of fibroblastoid cells with vacuolated cytoplasm leading to further capillary formation [29].

**Conclusion**

Histological examination of Covid-19 heart victims (37 cases) revealed subtle to severe signs of acute myocardial hypoxia in all cases. Three patients had AMI due to Covid-19 independent coronary thrombosis. All patients showed signs of endothelial activation and half of them showed prolonged signs of severe myocardial hypoxia. The presence of SARS-CoV-2 virus, virus-induced tissue damage, or virus-induced inflammatory response was not detected in any of the heart tissues. Particularly, lymphocyte infiltration, as a hallmark of myocarditis, was not seen in any cases of this study. Thus, any sign of histological myocarditis was not proved in our Covid-19 victims’ cohort of the study. These findings are mostly congruent with the hypothesis that most cardiac damage is due to generalized hypoxia and endothelial leakage is likely due to the presence of circulating endothelium activating factor, e.g. VEGF, originating outside of the heart, most probable from the hypoxic part of the Covid-19 lungs.

**Ethical approval**

The regional ethical authority in Stockholm has approved the project by registration numbers (DNR 2020-02446, DNR 2020-04339, and DNR 202100-2973).

**Disclosure statement**

No potential conflict of interest was reported by the author.

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