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Cardiac inflammation and microvascular procoagulant changes are decreased in second wave compared to first wave deceased COVID-19 patients

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\textbf{A B S T R A C T}

\textbf{Background:} Compelling evidence has shown cardiac involvement in COVID-19 patients. However, the overall majority of these studies use data obtained during the first wave of the pandemic, while recently differences have been reported in disease course and mortality between first- and second wave COVID-19 patients. The aim of this study was to analyze and compare cardiac pathology between first- and second wave COVID-19 patients.

\textbf{Methods:} Autopsied hearts from first- (n = 15) and second wave (n = 10) COVID-19 patients and from 18 non-COVID-19 control patients were (immuno)histochemically analyzed. CD45+ leukocyte, CD68+ macrophage and CD3+ T lymphocyte infiltration, cardiomyocyte necrosis and microvascular thrombosis were quantified. In addition, the procoagulant factors Tissue Factor (TF), Factor VII (FVII), Factor XII (FXII), the anticoagulant protein Dipeptidyl Peptidase 4 (DPP4) and the advanced glycation end-product N(\(\epsilon\))-Carboxymethyllysine (CML), as markers of microvascular thrombogenicity and dysfunction, were quantified.

\textbf{Results:} Cardiac inflammation was significantly decreased in second wave compared to first wave COVID-19 patients, predominantly related to a decrease in infiltrated lymphocytes and the occurrence of lymphocytic myocarditis. This was accompanied by significant decreases in cardiomyocyte injury and microvascular thrombosis. Moreover, microvascular deposits of FVII and CML were significantly lower in second wave compared to first wave COVID-19 patients.

\textbf{Conclusions:} These results show that in our cohort of fatal COVID-19 cases cardiac inflammation, cardiomyocyte injury and microvascular thrombogenicity were markedly decreased in second wave compared to first wave COVID-19 patients.
1. Introduction

Compelling evidence has been reported of cardiac involvement in coronavirus disease 2019 (COVID-19) patients. Elevated blood levels of cardiac Troponins and Creatine Kinase MB, indicative for acute myocardial injury, were found in 5% to 38% of hospitalized COVID-19 patients [2] and appear to associate with a fatal outcome [5]. In addition, cardiac magnetic resonance imaging studies have revealed myocardial abnormalities, including scar formation and myocardial edema, in patients with ongoing [6,7] and who recently recovered from COVID-19 [8]. Histopathological studies have shown increased cardiac inflammation consisting of infiltrating lymphocytes, macrophages and neutrophils, either or not coinciding with focal cardiomyocyte injury, in autopsied hearts of deceased COVID-19 patients [3,4,9,10] and in endomyocardial biopsies (EMB) of living COVID-19 patients [11,12], although some controversy exists about the incidence of myocarditis in COVID-19 patients [13–15]. In addition, evidence points to COVID-19-associated microvascular dysfunction and increased thrombogenicity in the heart. For instance, microvascular thrombosis has been observed in autopsied hearts of COVID-19 patients [3,10,16,17], which may predispose towards focal myocardial ischemia and myocardial injury.

During 2020 in many countries around the world, including Western Europe, the pandemic has surged in two distinct waves: the first between February/March and the end of May/June, and the second from September until the end of the year. The overall majority of studies on cardiac involvement in COVID-19 use data obtained during the first wave of the pandemic. Recently however, differences in patient demographics, disease course and mortality were reported between first and second wave COVID-19 [18–23]. These include a decrease in the proportion of hospitalized patients requiring ICU treatment or mechanical ventilation [18,19]. An increase in younger patients that require hospitalization [19] and a decrease in case fatality rates [18,20] during the second wave. Whether and how COVID-19-related cardiac pathology compares between patients from the first and second wave of the pandemic is unknown.

We therefore analyzed and compared cellular inflammation, cardiomyocyte injury, microvascular thrombosis and markers of increased microvascular thrombogenicity and dysfunction in the hearts of COVID-19 patients who died during the first and second wave.

2. Methods

2.1. Patients

Heart tissue was obtained from 43 deceased patients: patients who died of clinical PCR-confirmed COVID-19 (n = 25) and control patients who died without any form of heart disease nor had inflammation of the heart (n = 18). Fifteen of the included COVID-19 patients died during the first wave of the pandemic (March or April 2020), while ten COVID-19 patients died during the second wave (between October and the end of December 2020). The controls all died >1 year before the COVID-19 outbreak. The general histopathological and immunohistological findings of the included first wave COVID-19 patients were previously published [3]. All autopsies were performed within 24 h after death. From each patient transmural sections of the posterior, lateral and anterior walls of the left ventricle (LV) and the septum were examined. These samples were formalin-fixed and paraffin embedded for (immuno)histochemical analyses. The diagnosis of LM was made in case the inflammatory infiltrate in the heart consisted of clusters of predominantly adherent T lymphocytes and to a lesser degree macrophages in the myocardium, that in all cases reached ≧14 leucocytes/mm² including up to 4 macrophages/mm² with the presence of CD3+ T-lymphocytes ≥7 cells/mm², accompanied by cardiomyocyte necrosis of non-ischemic origin, conform the European Society of Cardiology (ESC) criteria [24]. From eight first wave-, six second wave COVID-19 patients and five control patients, additional LV samples were taken and snap frozen in liquid N₂.

This study followed the guidelines of the ethics committee of the Amsterdam UMC (Amsterdam, the Netherlands), and conforms to the Declaration of Helsinki. The use of autopsy material for research after completion of the diagnostic process was consented in all cases.

2.2. Immunohistochemistry

Deparaffinized slide-mounted tissue-sections (4 µm) were used. Endogenous peroxidases were blocked with 0.3% H₂O₂ in methanol for 30 min. Antigen retrieval was performed either by heat inactivation in Citrate buffer (pH = 6.0; CD68, CD3, C3d, FVII, Tris-EDTA buffer (pH = 9.0; CD31, TF) or enzymatically in 0.1% pepsin (37 °C for 30 min; FXII, CML). No antigen retrieval was performed for CD45 stainings. DPP4 (CD26) was analyzed on acetone-fixed frozen heart sections (5 µm). Primary antibodies were added for 1 h at room temperature (RT): mouse-anti-human CD45 (1:100, Dako Santa Clara, USA; M0701), rabbit-anti-human CD68 (1:400, Dako; M0814), rabbit-anti-human CD3 (1:100, Dako; A0452), rabbit-anti-human C3d (1:1000, Dako; A0063), mouse-anti-human CD31 (1:50, Dako; M0823), mouse-anti-human TF (1:250, Biirbyt Cambridge, UK, ORB100189), mouse-anti-human FXII (1:100, Sanquin Research, Amsterdam, The Netherlands), mouse-anti-human FXII (1:25, Sanquin), mouse-anti-human CML (1:500 [25]) or mouse-anti-human CD26 (1:100, Bio-Rad, Lunteren, The Netherlands, MCA1317T). After a wash in PBS, the slides were incubated with goat-anti-rabbit/mouse Envision secondary antibodies (undiluted, Dako; K5007) for 30 min at RT. The stainings were visualized using 3,3'-diaminobenzidine (DAB)(0.1 mg/mL) and counterstained with hematoxylin. For each staining, slides incubated without a primary antibody were included as a negative control and these slides were found to be negative (data not shown).

2.3. Immunopathological and immunohistochemical analyses

All slides were analyzed using light microscopy and during immunoscopying the researchers were blinded to the group allocation. Increases in extravasated CD45+ leukocytes, CD3+ T lymphocytes and CD68+ macrophages in the myocardium were semi-quantitatively determined to be either ‘no’ (no increase), ‘focal’ (small increases in certain areas), ‘mild’, ‘moderate’ or ‘strong’ (respectively mild, moderate or strong diffuse increases throughout the myocardium). In addition, the number of extravascular CD3+ cells in combination with up to 4 macrophages per mm² was determined in the LV endocardium in accordance with the ESC criteria [24,26]. Cardiomyocyte death was identified on CD3-stained slides. Microvascular fibrin platelet thrombi were identified CD31-stained slides. The number of intramyocardial blood vessels wherein endothelial cells stained positive for TF, FXII, FXI or DPP4 was counted. CML was quantified using intensity scoring as described previously [25]. For all markers the numbers of positive blood vessels were divided by the surface areas (in cm²) of the analyzed tissues. Immunoscopying was performed by 3 independent researchers (L.W., B.U. and P.A.I.K.) and the inter-observation variation was below 10%.

2.4. Statistical analysis

All statistical analyses were performed with SPSS (version 22.0, Armonk, NY, USA). All graphs were designed using GraphPad Prism
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software version 8.2.1 (San Diego, CA, USA). Differences between two
groups were evaluated by independent Student t-test or Mann-Whitney
U test for normal or non-normal distributed data respectively. Com-
parisons between multiple groups (more than two) were evaluated by
either a one-way ANOVA or Kruskal-Wallis test with post-hoc Dunn’s
multiple comparisons for respectively normal or non-normal distributed
data. Differences in semi-quantitatively determined myocardial
inflammation as well as frequency distributions of non-parametric var-
ables between patient groups were analyzed with Pearson Chi-square
tests. Spearman rank correlation coefficients were used for correlation
between groups with a non-normal distribution. P-values < 0.05 were
considered statistically significant.

3. Results

3.1. Patient characteristics

The characteristics of the control (n = 18), first wave COVID-19
(Wave 1; n = 15) and second wave COVID-19 (Wave 2; n = 10) pa-
tients are presented in Supplementary Table 1. There was no significant
difference in gender distribution between the three groups, with a ma-
jority of males in all groups (control n = 14 (78%); wave 1 COVID-19 n = 12 (80%); wave 2 COVID-19 n = 6 (60%)). The average age of the
wave 1 and wave 2 COVID-19 patients (mean 68 and 67 years respec-
tively) was significantly higher than the controls (mean 53 years; p =
0.0053 (wave 1) and p = 0.0141 (wave 2)). All COVID-19 patients were
hospitalized. Prior to admittance, significantly more wave 1 (n = 8;
53%) than wave 2 COVID-19 patients (n = 1; 10%) were hypertensive (p

Fig. 1. Examples of inflammation, myocytolysis and microvascular thrombosis in the hearts of COVID-19 patients.
Shown are examples of increased diffuse presence of CD68+ macrophages (A) and CD45+ leukocytes (B) in a wave 1 COVID-19 patient with diffuse cardiac
inflammation (DCI), as well as immunohistochemical examples of clusters of adherent CD45+ leukocytes (C; arrow) and CD3+ T lymphocytes (D; arrows) in a wave 1
COVID-19 patient with lymphocytic myocarditis (LM). In addition an example of myocytolysis, detected as complement factor C3d+ cardiomyocytes (E; arrow), and
an example of intravascular aggregated CD31+ platelets and fibrin, indicative of a microvascular thrombus (F; arrow) in wave 1 COVID-19 patients.
= 0.027), whereas the other comorbidities, or cardiac symptoms did not differ between the groups. Thrombotic events, including deep-venous thrombosis and pulmonary embolism, were observed in 5 (33%) and 4 (27%) wave 1 and in 1 (10%) and 7 (70%) wave 2 patients respectively, indicative of increased systemic thrombogenicity. Of note, diffuse alveolar damage (DAD) was found to be equally severe in wave 1 and wave 2 patients.

3.2. Less cardiac inflammation in wave 2 than in wave 1 COVID-19 patients

All COVID-19 patients showed signs of increased cardiac inflammation, as confirmed immunohistochemically by increases in extravasated CD45+ leukocytes, CD3+ T lymphocytes and CD68+ macrophages in the myocardium (Fig. 1). In most patients this inflammatory infiltrate was diffusely present throughout the myocardium (Fig. 1A+B). Semi-quantitative analysis showed that wave 1 patients scored significantly higher amounts of CD45+ and of CD3+ cells in the myocardium than wave 2 patients (p = 0.018 and p = 0.019 respectively), while CD68+ macrophage scores were similar (Fig. 2A). In 7 out of 15 wave 1 patients the infiltrate consisted predominantly of clusters of adherent T lymphocytes and to a lesser degree macrophages in the myocardium, consistent with lymphocytic myocarditis (LM) [24]. No LM was observed in wave 2 patients. The other 8 wave 1 patients and all wave 2 patients showed a more dispersed mixed infiltration of lymphocytes and macrophages that we refer to here as diffuse cardiac inflammation (DCI) [27]. In the LM patients of wave 1, the scores for CD45+ and CD3+ cells were significantly higher than in DCI patients of wave 1 (p = 0.006 and p = 0.03 respectively) and of wave 2 (p = 0.018 and p = 0.002 respectively) (Fig. 2B). The scores between wave 1 and wave 2 DCI patients did not differ significantly, although the difference in scores for CD45+ cells was borderline significant (p = 0.05). Lastly, the number of extravascular CD3+ cells in combination with up to 4 macrophages per mm² in the ventricular endocardium of LM patients (29, SD = 4 cells/mm²) was significantly higher compared to wave 1 and wave 2 DCI patients (p = 0.0087 and p = 0.0015 respectively; Fig. 2C).

Injury in dispersed small cardiomyocyte clusters or individual cells, objectified by complement factor C3d immunostaining [3] (Fig. 1E) was observed in all wave 1 patients, but only in 4 out of 10 wave 2 patients (p = 0.001; Fig. 2D). Furthermore, intravascular thrombi, consisting of aggregated CD31+ platelets and fibrin were observed in the myocardium of 47% of wave 1 COVID-19 patients (Fig. 1F) [3], both in case of LM and DCI, while no intravascular thrombi were found in wave 2 patients (p = 0.011; Fig. 2D). No cardiac inflammation, nor intravascular thrombi were found in the control group.

3.3. Increased presence of coagulation factors in the cardiac microvasculature of COVID-19 patients

Procoagulant TF, FVII and FXII were all present on the endothelium of intramyocardial blood vessels of COVID-19 patients in both waves (Figs. 3A-C). The positive blood vessels were diffusely distributed throughout the myocardium. High levels of TF were also present in neutrophils (not shown). The numbers of TF+ blood vessels/cm² in wave 1 and wave 2 patients were significantly higher than in controls (p
< 0.0001 and \( p = 0.0005 \) respectively; Fig. 4A), but were similar in both waves. In contrast, the number of FVII+ blood vessels/cm\(^2\) in wave 1 patients were significantly higher than in controls (\( p = 0.0001 \)), while in wave 2 patients these were similar to controls and significantly lower than in wave 1 patients (\( p = 0.0362 \); Fig. 4B). The number of FXII+ blood vessels/cm\(^2\) again was significantly higher than controls in both waves (\( p = 0.0017 \) and \( p = 0.0007 \) respectively; Fig. 4C).

3.4. CML is increased in the cardiac microvasculature of wave 1 but not of wave 2 COVID-19 patients

Cardiac microvascular dysfunction can coincide with increased levels of N(ε)-Carboxymethyllysine (CML; an advanced glycation endproduct) [25,30].

CML was found in the endothelium and smooth muscle cells of both in wave 1 and wave 2 COVID-19 hearts we observed a significant decrease of DPP4+ blood vessels (Fig. 3E) compared with controls (\( p = 0.0242 \) and \( p = 0.0044 \) respectively; Fig. 4D).
intramyocardial blood vessels in COVID-19 patients, again diffusely distributed throughout the myocardium (Fig. 4E). The CML immuno-
histochemical (IH)-score/cm² in wave 1 patients was significantly higher than in controls (p = 0.0021), while in wave 2 patients these were similar to controls and significantly lower than in wave 1 patients (p < 0.0001; Fig. 4E). The increased CML IH-score in wave 1 COVID-19 hearts was mainly due to increased numbers of moderate and strong positive vessels (staining intensity 2 (p = 0.0003) and 3 (p = 0.0002) respectively), while in wave 2 patients the number of CML+ blood vessels of all staining intensities were significantly lower than wave 1 patients (p < 0.0001 for intensities 1 and 2, and p = 0.0003 for intensity 3) and in case of weak positive vessels even lower than in controls (p = 0.0017; Fig. 4F).

3.5. Coagulation and microvascular dysfunction factors are comparable between LM and DCI COVID-19 patients

The microvascular coagulation factor- and CML levels between wave 1 LM, wave 1 DCI and wave 2 DCI patients were then compared (Fig. 5A). The numbers of TF++ (Fig. 5A), FVII+ (Fig. 5B), FXII+ (Fig. 5C) blood vessels/cm² as well as the CML IH-score/cm² (Fig. 5D) and CML intensity scores (Fig. 5E) did not differ significantly between wave 1 LM and wave 1 DCI patients. In wave 2 DCI patients, the number of FVII++-blood vessels/cm² was significantly lower than wave 1 DCI patients (p = 0.0183; Fig. 5B). Also, the CML IH-scores/cm² in wave 2 DCI patients were significantly lower than in wave 1 DCI (p = 0.0072) and wave 1 LM patients (p = 0.0048). This was reflected in significantly lower CML intensity 1, 2 and 3 scores/cm² compared to wave 1 DCI (p = 0.0022, p = 0.0027, p = 0.0069 respectively) and wave 1 LM (p = 0.001, p = 0.0017, p = 0.0018 respectively) patients.

4. Discussion

The aim of this study was to compare COVID-19-related cardiac pathology between patients from the first and second wave of the SARS-
CoV-2 pandemic. The extent of cardiac inflammation in second wave patients was significantly decreased compared to first wave patients, that appeared predominantly related to a decrease in infiltrated lymphocytes and occurrence of LM. This was accompanied by a decrease in cardiomyocyte injury and microvascular thrombosis in second wave patients, that coincided with a decreased presence of procoagulant factors in the cardiac microvasculature in second wave COVID-19 patients. These results highlight a markedly decreased cardiac pathology in deceased second wave COVID-19 patients.
wave patients corresponded with a decreased occurrence of cardiomyocyte injury. This suggests that cardiomyocyte injury is related more to infiltrated lymphocytes than macrophages, the levels of which remained high also in second wave patients.

In addition, we observed microvascular thrombosis in 47% of first wave cases, but in none of the second wave cases. As microvascular thrombosis can cause focal ischemia in the heart, it may contribute to the observed cardiomyocyte injury. The coinciding decreases in microvascular thrombosis and cardiomyocyte injury in second wave patients support this. Cardiac microvascular thrombosis in COVID-19 patients may be the result of the systemic hypercoagulability that often accompanies COVID-19 [31]. Indeed, deep vein thrombosis and pulmonary emboli were prevalent findings in our COVID-19 patients. However, the increased levels of TF, CML and decreased levels of DPP4, together with the increased deposits of the clotting factors FVII and FXII in the cardiac microvasculature of first wave COVID-19 patients, point to a procoagulant and pro-inflammatory phenotype of the microvascular endothelium that may locally facilitate the formation of thrombi. We previously showed a similar TF increase and DPP4 decrease in the cardiac microvascular endothelium of MI patients [29]. We also showed that inhibition of DPP4 enzymatic activity augmented TF expression and platelet adherence on HUVECs [29], emphasizing their importance in coagulation regulation on endothelial cells. The deposition of FVII and FXII indicates possible involvement of both extrinsic and intrinsic coagulation pathways. FVII can bind TF, whereas FXII is activated on negatively charged surfaces, including cell-free DNA. Both injured microvascular endothelium, that can release genomic material, and neutrophil extracellular traps (NETs), have been shown in the cardiac microvasculature of first wave COVID-19 patients [3], and may explain the presence of FXII. The decrease in microvascular thrombosis in second wave patients coincided with a decreased presence of FVII, arguing for an important role for the extrinsic pathway in COVID-19 related cardiac thrombosis.

CML is an indicator of microvascular inflammation and dysfunction and we previously showed increased microvascular CML levels in the hearts of patients with diastolic heart failure, MI and myocarditis [25,30]. The significantly lower microvascular CML levels in second wave COVID-19 patients are in line with the decreased cardiac inflammation and microvascular thrombogenicity that we see in those patients.

The exact mechanisms underlying COVID-19 associated cardiac pathology so far remain to be elucidated. The observed LM suggests a viral etiology, which is supported by the detection of SARS-CoV-2 RNA in autopsied hearts and in EMB from living COVID-19 patients [11,32], although only in infiltrated macrophages, rather than in cardiomyocytes or endothelial cells [33]. The absence of LM in second wave COVID-19 patients would then imply a lower cardiac prevalence of SARS-CoV-2 during the second wave, although this remains to be established. Alternatively, high levels of circulating pro-inflammatory cytokines often accompany COVID-19 [34] and may also contribute to cardiac inflammation, injury and microvascular dysfunction as was shown previously in patients with long term sepsis [35]. Other factors may include increased cardiac stress due to impaired pulmonary perfusion [27], anxiety, and mechanical ventilation, that was shown to induce cardiac inflammation in rats [36].

Recent studies have reported a generally less severe disease course and decreased mortality in second wave COVID-19 compared to the first wave [18–23]. This may f.i. relate to shorter times between disease onset and admission and an increase in the proportion of younger patients that require hospitalization and advances in treatment. Younger patients tend to have less underlying cardiovascular co-morbidities and may therefore have a better outcome than older patients. However, in our case series, the ages of the first and second wave patients were similar and age is therefore an unlikely contributor to the decreased cardiac pathology. Unfortunately no data are available regarding the SARS-CoV-2 virus variants that infected our cohort. The prevalent variants in Europe and the Netherlands differed between the first and second wave. The most prevalent variants in the Netherlands in the first wave were
and to decrease cardiac microvascular CML levels in mice with myocarditis has been shown before to decrease cardiac inflammation in myocarditis patients [41]. None of the earlier studies, however, compared the pathologic involvement in a patient with coronavirus disease 2019 (COVID-19), JAMA Cardiol. 5 (2020) 802–810, https://doi.org/10.1001/jamacardio.2020.1096.

In conclusion, in this pathology study we show that cardiac inflammation, cardiomyocyte injury and microvascular thrombogenicity in our cohort of deceased COVID-19 patients were markedly decreased in second wave compared to first wave patients.

5. Study limitations

A limitation of this study is the relatively small number of patients in which these observations were made. In addition, this study has been performed in patients who died as a result of very severe COVID-19. Whether, and if so to what extent, the results we describe here also occur in patients with less severe COVID-19 remains to be studied.

Data sharing statement

The data that support the findings of this study (in deidentified form) are available from the corresponding author upon reasonable request.

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Disclosures

No conflicts of interest exist.

Declaration of Competing Interest

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jjcard.2021.11.079.

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