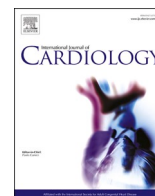




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## SARS-CoV-2 infects human cardiomyocytes promoted by inflammation and oxidative stress

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### ABSTRACT

**Introduction:** The respiratory illness triggered by severe acute respiratory syndrome virus-2 (SARS-CoV-2) is often particularly serious or fatal amongst patients with pre-existing heart conditions. Although the mechanisms underlying SARS-CoV-2-related cardiac damage remain elusive, inflammation (i.e. ‘cytokine storm’) and oxidative stress are likely involved.

**Methods and results:** Here we sought to determine: 1) if cardiomyocytes are targeted by SARS-CoV-2 and 2) how inflammation and oxidative stress promote the viral entry into cardiac cells. We analysed pro-inflammatory and oxidative stress and its impact on virus entry and virus-associated cardiac damage from SARS-CoV-2 infected patients and compared it to left ventricular myocardial tissues obtained from non-infected transplanted hearts either from end stage heart failure or non-failing hearts (donor group). We found that neuropilin-1 potentiates SARS-CoV-2 entry into human cardiomyocytes, a phenomenon driven by inflammatory and oxidant signals. These changes accounted for increased proteases activity and apoptotic markers thus leading to cell damage and apoptosis.

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<sup>2</sup> All authors take responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

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**Conclusion:** This study provides new insights into the mechanisms of SARS-CoV-2 entry into the heart and defines promising targets for antiviral interventions for COVID-19 patients with pre-existing heart conditions or patients with co-morbidities.

## 1. Introduction

Although cardiovascular co-morbidities play a prominent role in SARS-CoV-2 infection and existing cardiovascular diseases are associated with poor outcomes, the relationship between SARS-CoV-2 and the cardiovascular system is still poorly understood [1–4]. A study of 273 COVID-19 patients for markers of cardiac injury at hospital admission found myoglobin elevation in 10.6%, high-sensitivity troponin I in 9.9% and NT-proBNP elevation in 12.5% of patients. Death occurred in 24 patients (8.8%), and correlated significantly with the presence of cardiac injury at admission [2]. Furthermore, a substantial percentage of patients infected with COVID-19 (approx. 7–20%) develop additional cardiac injury [1,3,5].

SARS-CoV-2 is thought to enter a cell via interaction of the viral spike (S) protein with the ACE2 cell surface receptor, initiating membrane fusion followed by uncoating and viral replication [6,7]. Intriguingly, despite the relatively low level of ACE2 expression in the heart [8], cardiovascular complications are frequent and often dramatic in COVID-19 patients. Post-mortem studies have found SARS-CoV-2 in myocardial tissues, demonstrating entry of the virus despite low ACE2 levels [9]. Therefore and based on the low ACE2 expression in the heart, we believe other mechanisms are involved. The mechanisms of cardiac damage due to SARS-CoV-2 infection remain speculative, but ongoing inflammation, a ‘cytokine storm’ and oxidative stress are possible contributory factors. Inflammatory signals attract leukocytes to a site of injury or infection and leukocytes (neutrophils and macrophages) respond by producing numerous reactive oxygen species (ROS), including hydrogen peroxide, superoxide anion and others. Advanced age, cardiovascular risk factors and heart diseases predispose to heart failure (HF) and share a common environment of pro-inflammatory signals and oxidative stress within cardiac cells [10–12]. Epidemiological studies from China show that approximately 20% of COVID-19 patients have developed cardiovascular diseases and are more likely life threatening complications in the course of infection [13]. Even more, some patients have developed myocarditis, a cardiac disorder characterized by inflammatory cell infiltration of the heart and greater risk of deterioration of cardiac function [14], indicating SARS-CoV-2-associated myocarditis or “myocarditis-like syndromes”. Neutrophils express and release cytokines, which in turn amplify inflammatory reactions by several other cell types. In addition to employing and activating other cells of the immune system, neutrophils play an important role in the defence against invading pathogens. Human neutrophils express Toll-like receptor (TLR2) and (TLR4) protein on the cell surface and both receptors regulate neutrophil activation and their life span [15]. In addition, neuropilin-1 (NPR-1) functions as an endogenous negative modulator of the TLR4-NFκB pathway and facilitates together with the receptor for advanced glycation end products (RAGE) SARS-CoV-2 cell entry and infectivity [16,17].

We hypothesize that oxidative stress and a pro-inflammatory environment exacerbates SARS-CoV-2-associated damage via an yet unknown mechanism.

We therefore investigated 1) whether SARS-CoV-2 is found in the heart and if cardiomyocytes are targeted by SARS-CoV-2 and 2) how inflammation and oxidative stress promote the viral entry into cardiac cells. Furthermore, using cardiac tissue samples from SARS-CoV-2 infected patients, we analysed pro-inflammatory and oxidative stress [10–12] and its impact on virus entry and virus-associated cardiac damage.

## 2. Online methods

A detailed description of methods is provided in the online supplementary methods.

### 2.1. Human studies

All details related to the patient’s characteristics are included either in the extended supplementary section or in the results section.

### 2.2. RNA isolation and reverse transcription

Whole transcriptome sequencing was performed for each sample. For details, see supplementary methods section.

### 2.3. Immunofluorescence imaging

Frozen LV slides were fixed, blocked and either single- or dual-stained with various antibodies, followed by appropriate secondary antibodies. Immunostained samples were analysed using confocal laser scanning microscopy [10,12,18].

### 2.4. Quantification of enzyme activities, tissue oxidative stress, inflammatory response, inflammation, and inflammasome components

Myocardial enzyme activities, oxidative stress and inflammatory markers were assessed using enzyme-linked immunosorbent assay (ELISA) and colorimetric assay kits [10,12,18].

### 2.5. Protein phosphorylation analysis by Western blot

Homogenized myocardial samples were analysed using either 1.8% (for titin isoform switching and phosphorylation) or 10 and 15% SDS-PAGE, followed by Western blotting to analyse the expression and post-translational modifications of small proteins [18].

### 2.6. RNA-sequencing

Whole transcriptome sequencing was performed for each sample. For details, see supplementary methods section.

### 2.7. Force measurements on isolated cardiomyocytes

Cardiomyocytes were skinned and single isolated cells were attached between a force transducer and motor [12]. Total tension was recorded, and the sarcomere length (SL) 2.2 μm and  $F_{\text{passive}}$  were recorded over an SL range of 1.8 to 2.3 μm.

### 2.8. Statistical analysis

Data are given as mean values ±SEM. Student’s *t*-test was used for statistical analysis of parametric data for two groups, while the Mann-Whitney test was used for non-parametric data. One-way ANOVA was used in the analysis of parametric data comparing more than two groups. *P*-values were corrected for multiple comparisons using the Tukey method. Fisher’s exact test was used for analysis of proportions. All analyses were performed using GraphPad Prism 8. *P*-values are two-sided and considered statistically significant if *P* < 0.05.

### 3. Results

#### 3.1. Study population

Left ventricle (LV) myocardial tissues were obtained from patients with end-stage HF (NYHA III-IV) ( $n = 14$ ; 8 males, 6 females; mean age  $62 \pm 2$  years) characterized by LV dilation, LV systolic dysfunction and with ejection fractions of  $25 \pm 2.8\%$  suffering from hypertension and/or diabetes mellitus. Healthy LV myocardium ( $n = 14$ ) was obtained from healthy donor hearts that were not transplanted due to technical reasons ( $n = 14$ ; 8 males, 6 females mean age  $58 \pm 6$  years). Furthermore, we included 59 deceased patients with SARS-CoV-2 infection confirmed by qRT-PCR. All SARS-CoV-2 patients selected in this study were sorted according to their virus load within the myocardium and we have selected patients with a viral load above 1000 copies per  $\mu\text{g}$  RNA and the average virus load per  $\mu\text{g}$  RNA was  $26,530 \pm 12,432$ . These patients had high cardiovascular risk or established cardiovascular disease and were subsequently referred to as “SARS-CoV-2 patients”. The majority of patients died as a result of pneumonia/acute respiratory distress syndrome or multi-organ failure. We also employed other autopsy hearts without SARS-CoV-2 infection to confirm that the alterations found in SARS-CoV-2 infected patients are due to the virus infection rather than the storage conditions of the tissues. Our findings suggested that the alteration maps in autopsy hearts without SARS-CoV-2 infection are similar to the alterations found in tissues from HF and donors, but different from SARS-CoV-2 infected patients, indicating that our molecular and functional findings in SARS-CoV-2 infected patients are mainly a consequence of the viral infection (Fig.S1).

#### 3.2. Functional alterations of single skinned cardiomyocytes from patients with SARS-CoV-2 infection

We first assessed the effect of SARS-CoV-2 infection on cardiomyocyte function. Representative images (Fig. 1.A-C) show skinned cardiomyocytes and accompanying original recordings of force responses to stepwise cell stretching (sarcomere length (SL) 1.8–2.3  $\mu\text{m}$ ) in relaxing buffer and activating buffer.  $\text{Ca}^{2+}$ -activated tension generated by a single cardiomyocyte was severely reduced in SARS-CoV-2 patients compared to HF patients and the healthy donor group. Remarkably, only around 10% of all SARS-CoV-2 cardiomyocytes showed any force development and those that did showed an almost 75% reduction compared to HF patients and the donor group (Fig. 1.B). Accordingly, the rate constant of force redevelopment ( $k_{tr}$ ) was significantly lower in the remaining functional SARS-CoV-2 patient cardiomyocytes ( $0.10 \pm 0.0012 \text{ s}^{-1}$ ) compared to HF patients ( $0.56 \pm 0.002 \text{ s}^{-1}$ ) and the donor group cardiomyocytes ( $0.59 \pm 0.002 \text{ s}^{-1}$ ). Considerable degradation of cardiac myosin binding protein C (cMyBPC) and cardiac troponin I (cTnI) was in fact observed (Fig. 1.F-G). In addition, the passive sarcomere length-tension relationship in isolated skinned cardiomyocytes from SARS-CoV-2 patients was higher compared to HF patients and the donor group (Fig. 1.C). This may relate to high titin degradation [10,12,18–25], as demonstrated by Coomassie blue staining and Western blotting with specific anti-titin antibodies (Fig. 1.D-E). While these events may partly explain the severe impairment of cardiac function in of SARS-CoV-2 patients, it is not clear how SARS-CoV-2 enters cardiomyocytes and how infection impacts cardiomyocyte function.

#### 3.3. Increased proteolytic activity and apoptosis of cardiomyocytes in SARS-CoV-2 patients

Oxidative stress can activate a range of transcription factors, which lead to the differential expression of genes involved in inflammatory pathways. The inflammation triggered by oxidative stress is the cause of many chronic diseases [11,12,25]. Proteases are known to be central in the inflammation process. One potential effect of SARS-CoV-2 infection on cardiomyocytes is the activation of proteolytic enzymes (Fig. 2.A).

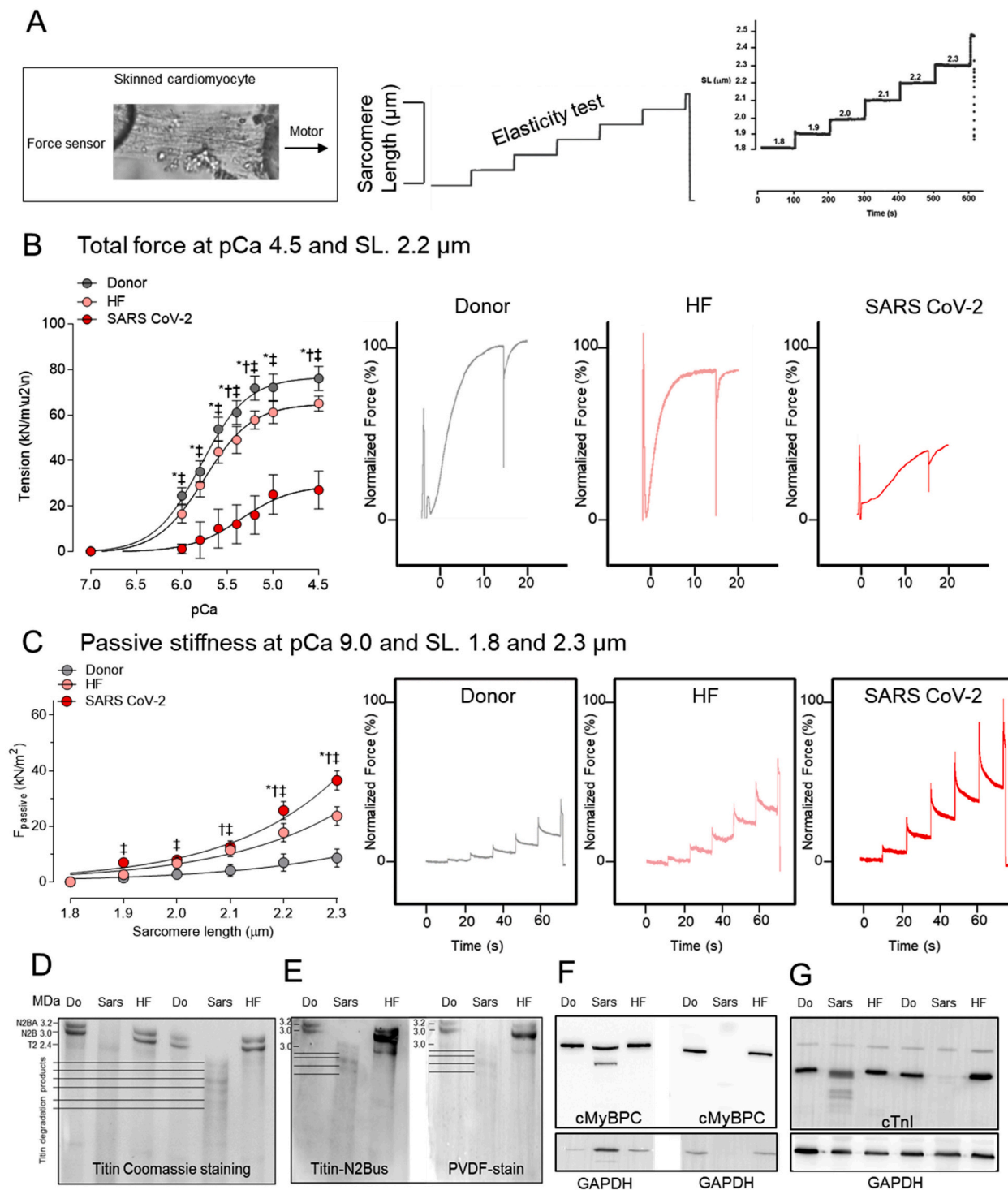
Indeed, we found significant upregulation of matrix metalloproteinase (MMP)-2 (Fig. 2.B), MMP-9 (Fig. 2.C) and cathepsin (Fig. 2.D) in SARS-CoV-2 compared to the HF patient and donor group. Cathepsin is known to play an important role in SARS-CoV-2 viral entry by activating the virus spike protein in the endosome/lysosome and inhibiting cathepsin blocks or decreases viral entry [26,27]. Many viruses, including SARS-CoV-2, enter host cells via cleavage and activation of the S-protein by host proteases [28,29], and membrane fusion is dependent on proteolysis of the S-protein by host cathepsin L, as shown for SARS-CoV-1 [30,31]. We therefore assessed the possible colocalization of spike protein with cathepsin in the heart using Duolink staining, which is a proximity ligation assay (PLA) that monitors protein-protein interaction for ranges up to 40 nm. Duolink staining showed that cathepsin and spike protein do indeed interact (Fig. 2.E), thus suggesting that cathepsin inhibition may be a viable target in the treatment of SARS-CoV-2. The transmembrane serine protease 2 (TMPRSS2) gene level was higher in SARS-CoV-2 patients compared to non-failing hearts (Fig. S3-D).

Proteolytic enzymes and proteins that cause apoptosis include Bcl2-associated X (BAX) (an indicator of mitochondrial damage) and nuclear factor of activated T-cells (NFAT). We found increased expression and activity of both these proteins (Fig. 2.G,I,F,H). Increased NFAT activity may indicate inhibition of the innate immune system by SARS-CoV-2 in HF patients. Apoptotic proteins such as caspase 3 (Fig. 2.J) and 9 (Fig. 2.L) also showed increased activity but dramatically reduced expression levels, indicating protein cleavage and activation of apoptosis (Fig. 2.K, M). Some other apoptotic signaling genes were altered, but others unchanged in SARS-CoV-2 patients (Fig.S3). In addition, we found increased calpain and calcineurin activity (Fig. 2.N,P), but no change in their expression levels (Fig. 2.O,Q). Calpain inhibitors have been suggested as a potential therapy for SARS [32]. These findings indicate that, together with oxidative stress, apoptosis may contribute to contractile deterioration.

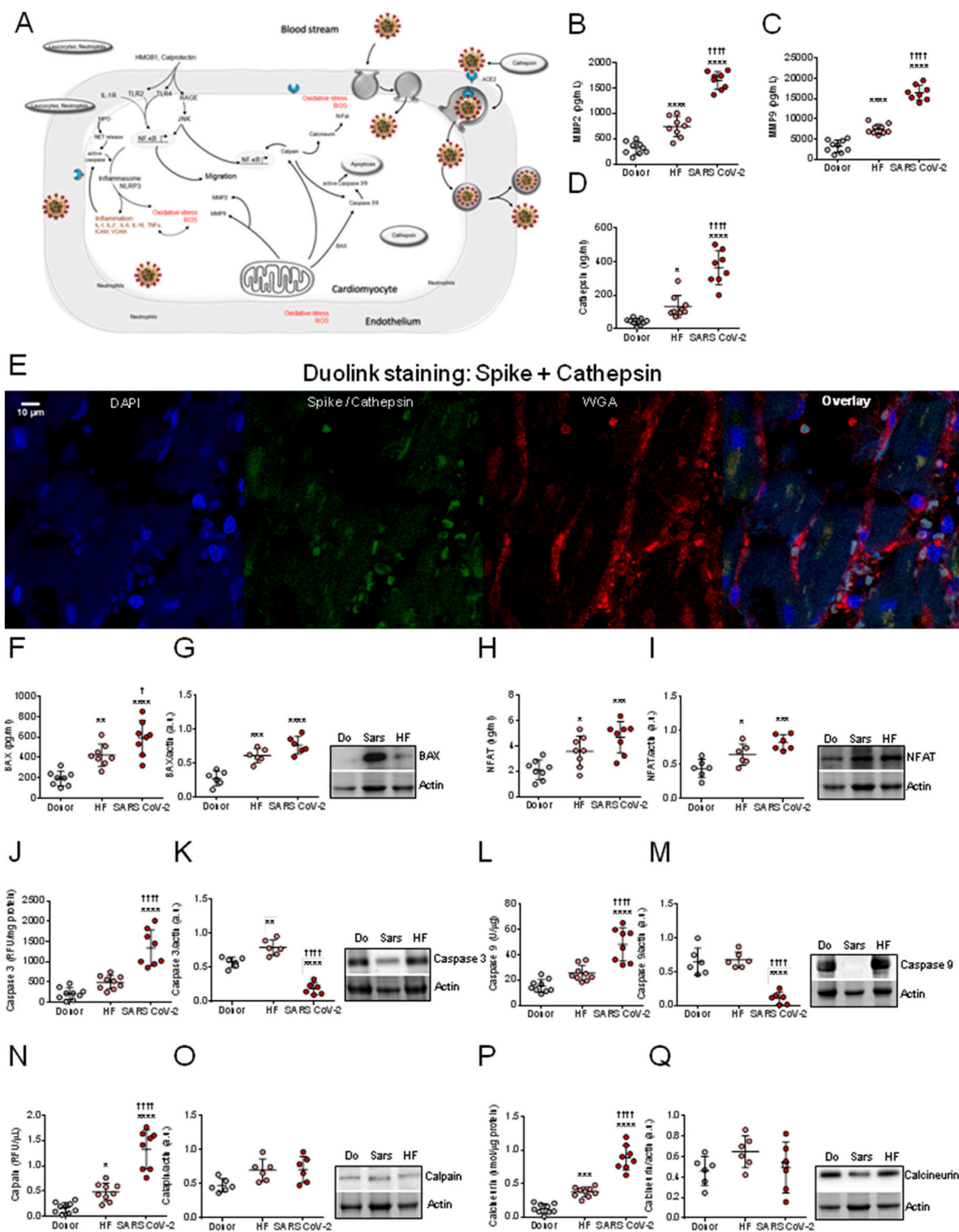
#### 3.4. Enhanced inflammasome and oxidized cardiomyocytes in SARS-CoV-2 infected hearts

The activity and composition of cardiac inflammasomes in SARS-CoV-2 infected patients is still poorly understood. Fig. 3.A proposes an inflammasome-inflammation pathway. The proinflammatory mediators high mobility group box 1 (HMGB-1) protein (Fig. 3.B; Fig.S2.A) and calprotectin (Fig. 3.C) were significantly elevated in SARS-CoV-2 as compared to donors and HF patients. Both mediators can induce activation of intracellular signaling pathways via interactions with at least three pattern recognition receptors, including toll-like receptor-2 and -4 (TRL-2, -4) or the receptor for advanced glycation end products (RAGE). Compared to HF patients and the donors, SARS-CoV-2 patients showed enhanced mRNA expression and activities of these receptors (Fig. 3.D-F; Fig.S2-B-C). The SARS-CoV-2 spike protein has been shown to interact with TLR-4 [33], and SARS-CoV-2 patients showed increased receptor activity that enhanced downstream intracellular factors such as c-Jun N-terminal kinase (JNK) (Fig. 3.G) and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B, Fig. 3.H; Fig.S2-D). These transcription factors are associated with major upregulation of inflammatory signaling and gene expression (also elicited by SARS-CoV-2 infection [34]).

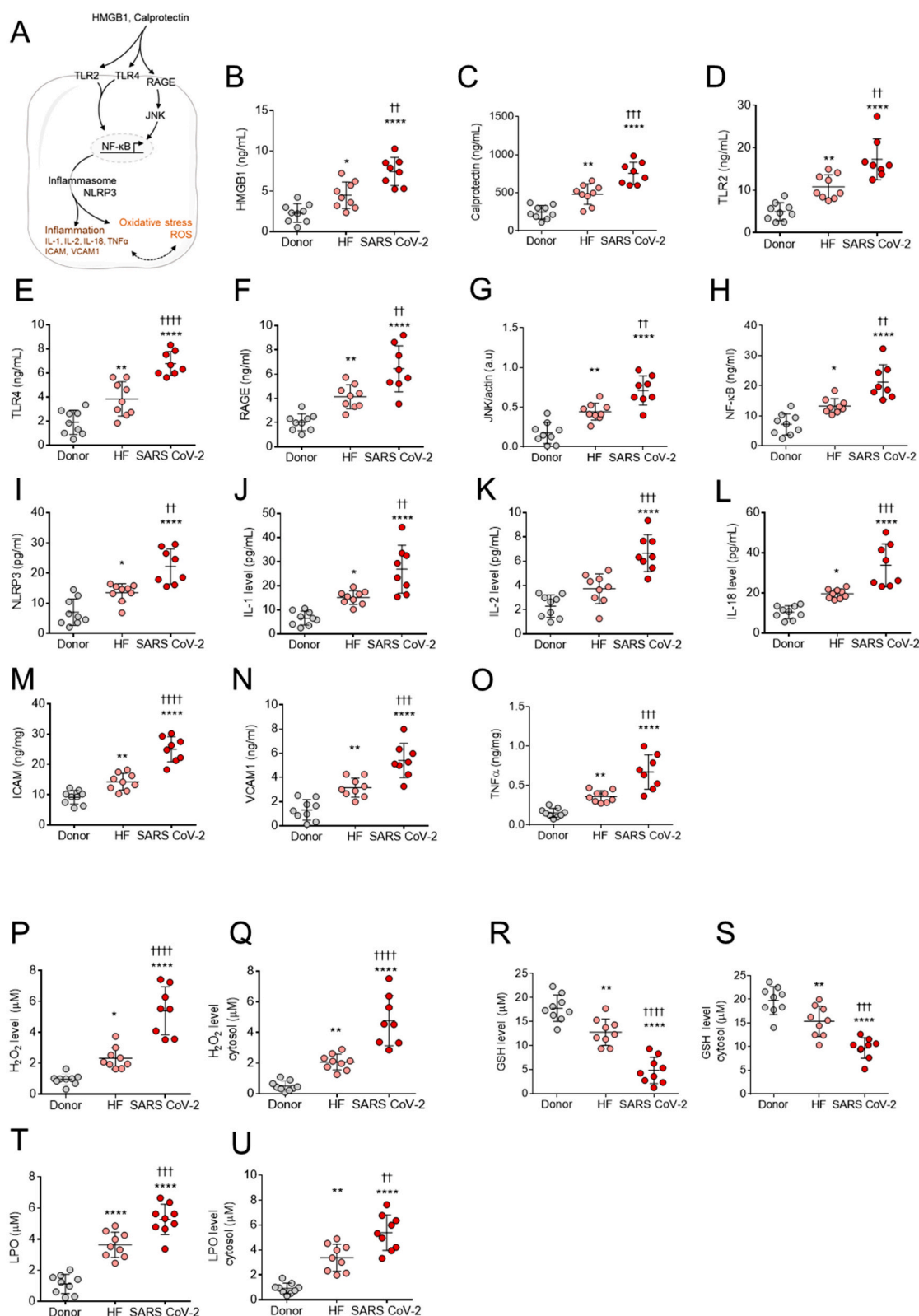
NLR family pyrin domain-containing protein 3 (NLRP3) acts as an intracellular sensor of endogenous danger signals and environmental irritants, resulting in the formation and activation of the NLRP3 inflammasome, caspase-dependent release of the pro-inflammatory cytokines IL-1 $\beta$  and IL-18 and subsequent pyroptotic cell death [35]. We detected increased expression of NLRP3 (Fig. 3.I; Fig.S2.E) in response to SARS-CoV-2 infection. Our data further indicated the upregulation of a range of pro-inflammatory cytokines and chemokines, including interleukin-1 (IL-1) (Fig. 3.J; Fig.S2.F), IL-2 (Fig. 3.K), IL-18 (Fig. 3.L), intracellular adhesion molecule-1 (ICAM1) (Fig. 3.M), and vascular cell



**Fig. 1.** Cardiomyocyte force production and passive stiffness ( $F_{passive}$ ). (A) Representative image of skinned cardiomyocyte and elasticity test protocol with original recordings of force development at sarcomere length (SL) 2.2  $\mu\text{m}$  (B) in activating buffer and response to stepwise cell stretching (SL 1.8–2.3  $\mu\text{m}$ ) (C) in relaxing buffer. In (B) at a constant sarcomere length of 2.2  $\mu\text{m}$  calcium dependent force development is presented from donor, HF, and SARS-CoV-2 cardiomyocytes. (C) Passive stiffness (pCa 9.0) is measured between sarcomere lengths of 1.8 and 2.3  $\mu\text{m}$  donor, HF, and SARS-CoV-2 cardiomyocytes. Fit curves are 2-order polynomials to the means. (D) is titin Coomassie blue, (E) Western blot with titin specific antibody which recognizes the elastic region of titin the N2B unique sequence (N2Bus). (F,G) Western blot with cardiac myosin binding protein C (cMyBPC) and cardiac troponin I (cTnI) specific antibodies which recognizes both proteins. Data are shown as mean  $\pm$  SEM; ( $n = 16\text{--}20/4\text{--}5$ ) cardiomyocytes/hearts.  $\ddagger P < 0.05$  SARS-CoV-2 vs. donor,  $\uparrow P < 0.05$  HF vs. donor,  $*P < 0.05$  SARS-CoV-2 vs. HF by Student *t*-test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** SARS-CoV-2 infection of the heart and proteolytic enzymes. In (A) schematically pathway and effects of SARS-CoV-2 infection on cardiomyocytes are presented with integrated inflammatory, proteolytic and oxidative stress pathways. The enzymatic activities of MMP-2 (B), MMP-9 (C) and cathepsin (D) in donor, HF patients and SARS-CoV-2 infected patients. In cardiac tissue a viral spike/cathepsin interaction is shown using immunofluorescence and duolink (E) DAPI staining and WGA (anti-wheat agglutinine 555 conjugate, red) staining are used for nucleic acids and membranes, respectively. Bax (Bcl-2-associated X protein) (F,G) and NFAT (nuclear factor of activated T-cells) (H,I) activity and expression. In addition, activities of apoptotic enzymes caspase 3 (J) and caspase 9 (L) and their expression level (K) and (M) respectively. Intracellular calpain activity (N) and expression (O). Calcineurin activity (P) and expression (Q). Inserts show characteristic Western blot section. Data are shown as mean ± SEM; n = 8–9 patient/group. \*P < 0.05/\*\*P < 0.01/\*\*P < 0.001/\*\*\*\*P < 0.0001 HF and SARS-CoV-2 vs. donor, ††††P < 0.0001 SARS-CoV-2 vs. HF by one-way ANOVA. P-values were corrected for multiple comparisons by the Tukey method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



**Fig. 3.** Inflammasome and oxidative stress markers in SARS-CoV-2 infected cardiac tissue. (A) Represents the whole inflammatory pathway investigated in this section, enzymatic activities of (B) HMGB1 (high mobility group box 1), (C) calprotectin, (D,E) TLR (toll-like receptor) 2 and 4, (F) RAGE (receptor for advanced glycation endproducts), (G) expression of JNK (C-jun-N-terminal kinase), enzymatic activities of (H) NF-κB (nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells), (I) NLRP3 (NLR family pyrin domain containing 3), (J) IL-1, (K) IL-2, (L) IL-18 (interleukin), (M) ICAM-1 (intracellular adhesion molecule-1) and (N) VCAM1 (vascular cell adhesion molecule-1) and (O) TNFα (tumor necrosis factor α). Further enzymatic activities of (P) H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide), (Q) H<sub>2</sub>O<sub>2</sub> level in cytosol, (R) GSH (reduced glutathione), (S) GSH level in cytosol (T) LPO (lipid peroxidase), (U) LPO level in cytosol in cardiac tissue from healthy donors, heart failure (HF) patients and severely infected SARS-CoV-2 patients. Data are shown as mean ± SEM; n = 8–9 patient/group. \*P < 0.05/\*\*P < 0.01/\*\*\*P < 0.0001 HF and SARS-

CoV-2 vs. donor,  $\uparrow\uparrow P < 0.01 / \uparrow\uparrow\uparrow P < 0.001 / \uparrow\uparrow\uparrow\uparrow P < 0.0001$  SARS-CoV-2 vs. HF by one-way ANOVA. P-values were corrected for multiple comparisons by the Tukey method.

adhesion protein-1 (VCAM1) (Fig. 3.N), likely indicating an antiviral response. In addition, tumor necrosis factor alpha (TNF $\alpha$ ) (Fig. 3.O; Fig. S2.L) was also increased in SARS-CoV-2 patients compared to HF patients and the donor group. Interestingly, certain genes remained unchanged in SARS-CoV-2 patients (Fig.S2.G,J,K).

### 3.5. Oxidative stress triggers inflammatory cytokines: the mechanisms and impact of SARS-CoV-2 entry

Inflammation-associated oxidative stress or vice versa leads to a deterioration of cardiomyocyte function that is partly due to a modification of contractile proteins [24,36]. In cardiac tissue, an overall increase of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) (Fig. 3.P) was noted, along with increased H<sub>2</sub>O<sub>2</sub> levels in the cytosol (Fig. 3.Q). Oxidative stress-induced defence mechanisms were reciprocally affected, as levels of reducing glutathione (GSH) decreased (Fig. 3.R,S), while lipid peroxidase (LPO) production increased (Fig. 3.T,U), indicating that cardiac ROS production is elevated upon SARS-CoV-2 infection. Of note, a strong association between ROS and pro-inflammatory signals has previously been reported in various lung diseases, including SARS-CoV-2 infection [37].

### 3.6. Upregulation of neuropilin-1 (NRP-1) by IL-6 in neutrophils enhances SARS-CoV-2 entry and infection

Neutrophils are one of the primary cell types releasing proteolytic enzymes, including neutrophil elastase and play an essential role during an inflammatory response. They are rapidly mobilized from the circulation into damaged tissues. As proteolytic enzymes are increased, we wanted to define the receptor-mediated signaling events responsible for IL-6-driven neutrophil trafficking, we investigated the entire cascade in the heart. In SARS-CoV-2 patients, IL-6 activity was significantly increased compared to HF patients and the donor group (Fig. 4.A), while its relative expression was unchanged (Fig. 4.B). Myeloperoxidase, a peroxidase enzyme abundantly present in neutrophil granulocytes, showed a significant increase in both enzyme activity (Fig. 4.C) and expression (Fig. 4.D). Neuropilin-1 (NRP-1), a receptor found in the vasculature of the heart, functions as an alternative SARS-CoV-2 receptor [16]. NRP-1 activity was increased in SARS-CoV-2 compared to HF and donor group hearts (Fig. 4.E). Using Western blot, several bands of different molecular weights were detected, in addition to a cleavage product (bands at 75 kDa and 63 kDa) (Fig. 4.I), a band at 100 kDa (Fig. 4.F,I) and a band at 135 kDa (Fig. 4.G,I). The NPR-1100 kDa and 135 kDa proteins were significantly reduced in SARS-CoV-2 hearts (Fig. 4.F,G,I), while the cleaved products were entirely confined to SARS-CoV-2 patients (Fig. 4.I). These findings accord with the study which showed that NRP1 potentiates SARS-CoV-2 infectivity in human embryonic kidney 293 T cells in vitro [16]. Neutrophil elastase activity was increased in SARS-CoV-2 (Fig. 4.H), potentiating infection in the presence of other host elements. Using confocal microscopy, we detected staining of the SARS-CoV-2 spike protein with IL-6 in cardiomyocytes (Fig. 4.J). Importantly, the spike protein co-localized with NRP-1 in cardiomyocytes (Fig. 4.K).

### 3.7. NETosis and the release of histones by neutrophils

Histones are important pro-inflammatory agents and act as the major pro-inflammatory component of neutrophils, in addition to other well-known nuclear functions [38], potentiating signaling by recruiting TLR4 to histone-containing endosomes. Neutrophil extracellular traps (NETs), which comprise a DNA backbone coiled around histones accompanied by enzymes found in neutrophil cytoplasmic granules, are released during cell damage (NETosis). We therefore investigated

histones and related mechanisms of cell damage following SARS-CoV-2 infection.

As NET formation is altered by specific properties of histone beads, we investigated the expression of HDAC4 and the posttranslational modification of histones. Various molecular weights were detected using Western blot (Fig. 4.P), and the expression levels of HDAC4 245 kDa and 75 kDa proteins were significantly reduced in SARS-CoV-2 compared to HF patients and the donor group (Fig. 4.L,O), while the expression levels of the 135 kDa and 100 kDa proteins were increased (Fig. 4.M,N). Histones can be altered through various chemical modifications including acetylation, methylation, phosphorylation, ubiquitination and acylation of free N-terminal tails or globular domains that physically interact with DNA. While histone 3 showed an overall increased expression in SARS-CoV-2 (Fig. 4.Q), the acetylation (Fig. 4.R), dimethylation (Fig. 4.S) and phosphorylation (Fig. 4.T) of histone 3 were all significantly reduced.

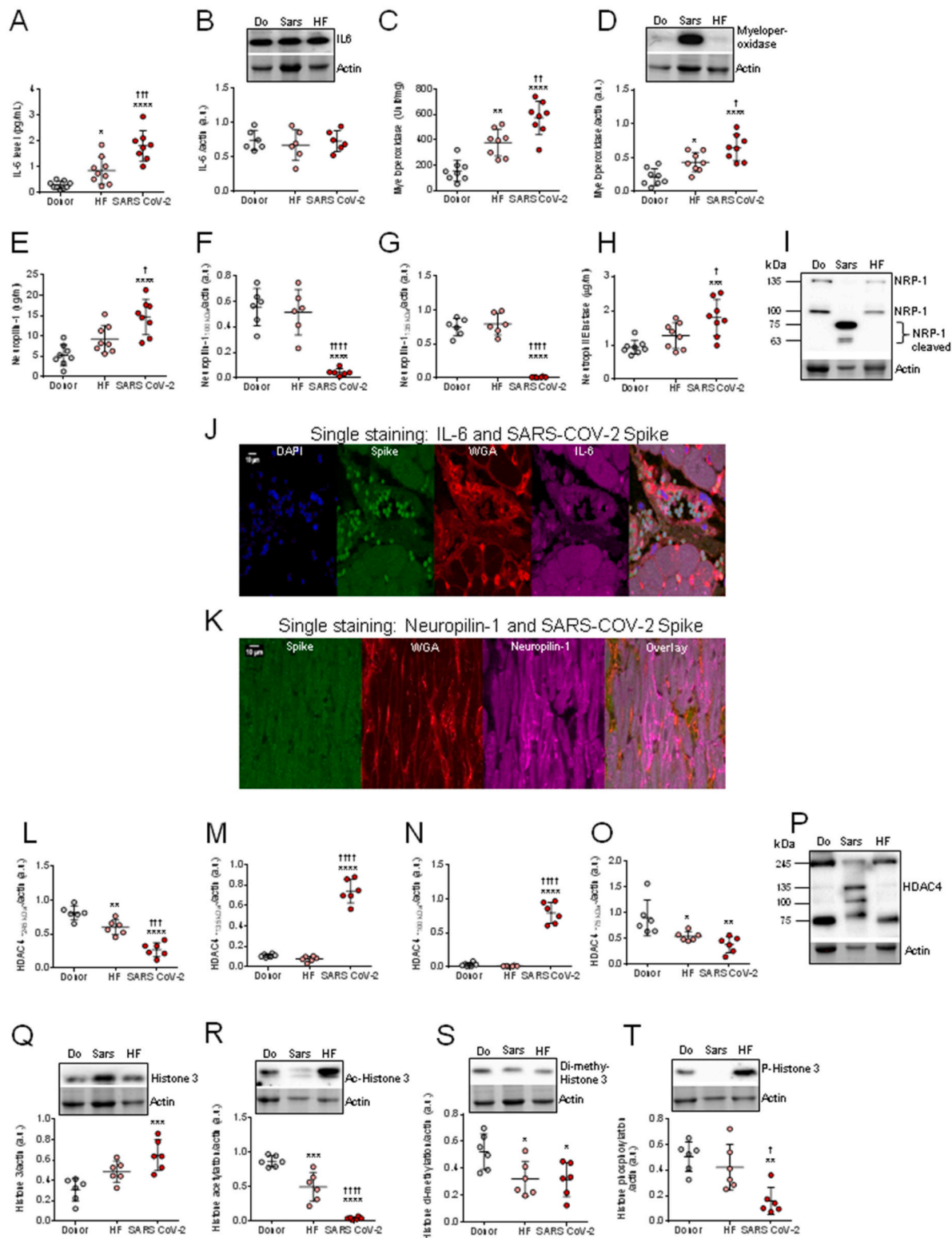
## 4. Discussion and conclusions

Taken together, our data suggest that SARS-CoV-2 infection and the resultant oxidized microenvironment cause alterations of protein localization and expression, enzyme activity, inflammation, oxidative stress, which together lead to severe cardiomyocyte damage and subsequent cell death.

A recent study demonstrated that human iPSC cardiomyocytes are susceptible to SARS-CoV-2, with cardiomyocyte infection resulting in viral replication, cytopathy and an induction of apoptosis that was followed by a cessation of beating 72 h after infection [39]. Viruses display considerable redundancy and flexibility so that they can exploit weak multivalent interactions to enhance affinity. Recent studies of SARS-CoV-2 entry have focused almost entirely on the ACE2 receptor, which in many organs, as well as respiratory and olfactory epithelial cells, actually shows low protein levels [40]. By contrast, our data shows that in the failing human heart an environment with increased protease activity is required to facilitate SARS-CoV-2-host cell interactions in cardiomyocytes.

Our paradigm emphasizes the role of inflammation and oxidative stress to promote SARS-CoV-2 entry to cardiomyocytes. We defined the receptor-mediated signaling events responsible for IL6-driven neutrophil trafficking. Remarkably, the inflammatory signaling pathways were highly regulated in cardiomyocytes, hence suggesting a key role for the organelle in COVID-19 and its associated inflammatory pathologies. Virus infection seems to excessively activate monocytes and macrophages leading to the development of a cytokine storm and subsequently to the appearance of acute respiratory distress syndrome [41]. Our findings are consistent with SARS and Middle East Respiratory Syndrome as the presence of “cytokine storm” may have a key role in the pathogenesis of SARS-CoV-2 [42,43]. Infected SARS-CoV-2 cells are reprogrammed for virus particle production and die after their lytic release, potentially due to the cytosolic components release by removing cells from the system that induce the massive inflammatory reaction leading to an “over-reaction” of the immune system „cytokine storm“ [44]. A number of neutrophils has been reported to be increased during inflammation, but were also shown to have the capacity to produce cytokines such as TNF- $\alpha$ , IL-6, and IL-8, all of which are involved in the regulation of the immune response and inflammation. Some of the ILs initiate degranulation and the production of ROS which induce oxidative stress, which plays a key role in the pathogenesis of HF development [11,12,25,45]. Oxidative stress and ROS modify lipids, proteins, carbohydrates, nucleic acids, and induce the mitochondrial permeability transition leading to autophagy, apoptosis, and necrosis. The HMGB1 protein, a chromatin-binding nuclear protein and damage-associated molecular pattern molecule, is integral to oxidative stress and





**Fig. 4.** SARS-CoV-2 and Neuropilin-1 cascade. IL-6 activity and expression in SARS-CoV-2 patients compared to donors and HF patients (A,B), myeloperoxidase activity (C) and expression levels (D). Neuropilin-1 (E) and expression (F,G,I) in SARS-CoV-2 infected patients. (H) Neutrophil elastase activity. (J,K) immunofluorescence showing the interaction/colocalization of SARS-CoV-2 spike with IL-6 (J) or Neuropilin-1 (K) single staining. DAPI staining (blue) and WGA (anti-wheat agglutinin 555 conjugate, red) staining are used for nucleic acids and membranes. (L-P) HDAC4 expression level. Histone expression and modifications (Q-T), histone 3 expression level (Q), acetylated (R), Di-methylated (S), phosphorylated (T) in SARS-CoV-2 and donors and compared to HF patients. Data are shown as mean ± SEM; n = 8–9 patient/group. \*P < 0.05/\*\*P < 0.01/\*\*\*\*P < 0.0001 HF and SARS-CoV-2 vs. donor, †P < 0.05/††P < 0.01/†††P < 0.001/††††P < 0.0001 SARS-CoV-2 vs. HF by one-way ANOVA. P-values were corrected for multiple comparisons by the Tukey method. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

downstream of apoptosis or survival. Accumulation of HMGB1 at sites of oxidative DNA damage can lead to DNA repair, suggesting a DNA damage in SARS-CoV-2 infected patients. A potential mechanism linking ROS and myocardial inflammation, in particular in conditions of hyperglycaemia, is the activation of NF- $\kappa$ B signaling, since NF- $\kappa$ B activation induces various pro-inflammatory genes which, in turn, lead to ROS generation. These imply the pivotal role of ROS and inflammation in activating NF- $\kappa$ B signaling in hyperglycemia conditions [46] and potentially in SARS-CoV-2 infection. Increased H<sub>2</sub>O<sub>2</sub> and 3-nitrotyrosine, as shown previously [11,12,25] could have resulted from the uncoupling of eNOS, thereby switching the eNOS dimer to a superoxide anion-generating monomer, hypothetically the same is true for SARS-CoV-2 infected patients.

A significant invasion of neutrophils occurred in the affected organs during first stage of infection [47]. Neutrophils combat pathogens either by release of their granular content leading to production of ROS, by phagocytosis, or by neutrophil extracellular traps (NETs). NETs are web-like structures formed from decondensed chromatin coated with histones and oxygenases like myeloperoxidase [48]. NETs were shown to capture and inactivate invading pathogens like bacteria and viruses [49,50]. The NET producing neutrophils may survive or undergo cell death (NETosis). Their persistence leads to adverse effects as reported in different pathological situations like sepsis, pulmonary diseases, and cystic fibrosis [51]. Induction of microvascular thrombosis by persistent NETs has been shown [52, 53]. Accumulation of neutrophils within inflamed infection areas and massive occlusion of microvessels by thrombotic fibrin clots has been reported within affected organs [51]. The SARS-CoV-2 infected cardiomyocytes could therefore be responsible for enhanced NETs formation supporting the onset of COVID-19 coagulopathy and microvascular thrombosis. This suggests that the prevention of NET formation and/or their rapid degradation might offer additional therapeutic strategies.

Inflammation and oxidative stress cause protein oxidation, which may act as a tagging system for the degradation of an irreparably damaged protein by the proteasomal system [54, 55] leading to dysregulation of cellular redox states [56] inducing apoptosis [57]. In our previous study, increased inflammation and oxidative stress in HF patients has predicted to be the most likely underlying cause of the abnormal cardiomyocyte resulting to myofilament contractile dysfunction. During cardiac stunning, ROS depress contractile performance and alter force production and stiffness either directly due to protein oxidation or indirectly due to altered signaling pathways. Respiratory viral infections are most of the time associated with cytokine production, inflammation, cell death, and other pathophysiological processes, which could be linked with a redox imbalance or oxidative stress. It is well known that ROS overproduction and antioxidant mechanisms deprivation are crucial for viral replication and the subsequent virus-associated disease [58]. Some studies suggested that the severe lung injury onset in SARS-CoV-2 infected patients depends on activation of oxidative stress that is coupled with overreactive innate immunity activating different transcription factors resulting in an aggravated proinflammatory host response [59].

Hence, our study indicates that cardiomyocyte dysfunction could be explained most likely encompass events associated with 1) Increased inflammation and oxidative stress; 2) protein oxidation/modification; 3) catecholamine surge [60]; 4) enzymatic dysfunction or degradation of cardiomyocyte proteins by activated proteases. and thereby 5) apoptosis. Proteases are involved in inflammatory processes [61]. Inflammatory caspases are activated via the inflammasome [62]. The

inflammasome generally responds to inducers of inflammation as well as infectious agents such as viruses, bacteria and fungi. Thereby, some of the caspases will promote the secretion of some inflammatory cytokines as the case in SARS-CoV-2 infected patients.

## 5. Conclusion

Our data provide new insights into the mechanisms of SARS-CoV-2 entry into the heart and define promising targets of antiviral interventions for COVID-19 patients suffering from pre-existing heart condition, heart failure patients and/or patients with co-morbidities.

## 6. Therapeutic approaches

We could observe a reversed altered force production and stiffness of SARS-CoV-2 cardiomyocytes upon treatment with Mito-TEMPO, an antioxidant that targets mitochondria (Fig.S4). Remarkably, in SARS-CoV-2 infected patients Mito-TEMPO treatment significantly reduced inflammatory molecules IL-6 and 18 (Fig.S4-D,E) and oxidative stress levels, as assessed by H<sub>2</sub>O<sub>2</sub> (Fig.S4-F) and increased anti-oxidant GSH (Fig.S4.G). This approach may be appropriate in high risk groups with pre-existing heart conditions or patients with co-morbidities characterized by increased oxidative stress and inflammation. Additionally, and based on our study and other studies, calpain inhibitors could also be used as a treatment option for SARS infected patients and have already been suggested as a potential therapy for SARS [32]. Moreover, cathepsin is elevated in SARS-CoV-2 infection and positively correlated with disease course and severity. Cathepsin is also known to cleave the SARS-CoV-2 spike protein and enhanced virus entry. It is therefore suggested that cathepsin inhibition could prevent SARS-CoV-2 infection. Indeed, this suggestion has been experimentally *in vivo* employed showing a significant inhibition of SARS-CoV-2 in Huh7 cells treated with a cathepsin inhibitor [63]. Finally, since increased inflammation and oxidative stress is a hallmark of SARS-CoV-2, we believe that a one-component therapy will not alone be sufficient in these patients. We also propose drugs or biological modulators that inhibit viral spreading and replication in recipient cells, combined with enhancer defence mechanisms that reduce oxidative stress and inflammation, which will be more effective than a single agent. An anti-viral drug could be combined with an immune system booster and anti-oxidants, aiming for redox balance at the cellular level via enhanced antioxidant metabolites, thus protecting enzyme function and preventing mitochondrial destruction. This approach may be appropriate in high risk groups with pre-existing heart conditions or patients with co-morbidities characterized by increased oxidative stress and inflammation. Finally, regarding cytokine storms sometimes reported in SARS-CoV-2 HF patients, we suggest a multi-drug cocktail could be combined with an IL-6 inhibitor. Gathering together mechanistic insights on SARS-CoV-2 cellular entry and replication, several potential targets of future antiviral therapeutics emerge for infected HF patients and patients with co-morbidities

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## Appendix A. Supplementary data

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