A lesson from an old friend: high molecular weight kininogen (HMWK) impact in COVID-19.

Chiara Colarusso[1], Michela Terlizzi[1], Aldo Pinto[1], and Rosalinda Sorrentino[1]

[1]University of Salerno

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) is a newly identified coronavirus which has spread from China to the rest of the world causing the pandemic coronavirus disease 19 (COVID-19). It has fatality rate that floats from 5 to 15% and the symtoms are fever, cough, myalgia and/or fatigue up to dyspnea, responsible for hospitalization and in most of the cases of artificial oxygenation. In the attempt to understand how the virus spreads and how to pharmacologically abolish it, it was highlighted that SARS-CoV2 infects human cells by means of angiotensin converting enzyme 2 (ACE2), transmembrane protease serine 2 (TMPRSS2) and 3-chymotrypsin-like protease (3CLpro), also known as Mpro. Once bound to its receptor ACE2, the other two proteases, in concert with the receptor-mediated signaling, allow virus replication and spread throughout the body. Our attention has been focused on the role of ACE2 in that its blockade by the virus increases Bradykinin and its metabolites, well known to facilitate inflammation in the lung (responsible for cough and fever), facilitate both the coagulation and complement system, three mechanisms that are typical of angioedema, cardiovascular dysfunction and sepsis, pathologies which symptoms occur in COVID-19 patients. Thus, we propose to pharmacologically block the kallicrein-kinin system upstream bradykinin and the ensuing inflammation, coagulation and complement activation by means of lanadelumab, which is a clinically approved drug for hereditary angioedema.

Abbreviations:

2'-O-MTase: 2'-O-Ribose Methyltransferase

3CLpro: 3-chymotrypsin-like protease

AAK1: AP2-associated protein kinase 1

ALT: alanine aminotransferase

ARDS: acute respiratory distress syndrome

BKB1R: bradykinin receptor B1

Bradykinin: BK

CFR: case fatality rate

cGAS: cyclic guanosine monophosphate–adenosine monophosphate

cGAMP: cyclic guanosine monophosphate–adenosine monophosphate

CoV: coronavirus

COVID-19: coronavirus disease 19

CT: computed tomography

CXCL1: C-X-C motif chemokine 1

CXCL5: C-X-C motif chemokine 5

DABK: des-Arg9bradykinin

DMARDS: disease-modifying antirheumatic drugs

EMA: European medical agency

eNOS: endothelial NOS

HIV: Human Immuno-deficiency Virus

HMWK: high molecular weight kininogen

hrsACE2: human recombinant soluble ACE2

ICU: intensive care unit

iNOS: inducible NOS

INSTI: integrase strand transfer inhibitors

KC: keratinocyte-derived chemokine

KKS: kallikrein-kinin system

LMWK: low-molecular-weight kininogen

Lys-BK: lysyl-bradykinin

mAbs: monoclonal antibodies

MERS: Middle East Respiratory Syndrome

MERS-CoV: Middle East Respiratory Syndrome-CoV

MHC: major histocompatibility complex

MIP2: macrophage inflammatory protein-2

NLRP3: Nod-like receptor protein 3

PAI-1: plasminogen activator inhibitor 1

PAR: protease-activated receptors

PD: peptidase domain

PGI2: prostaglandin I2

RAS: renin-angiotensin system

RDB: receptor binding domain

RdRp: RNA-dependent RNA polymerase

S: Spike

SARS: Severe Acute Respiratory Syndrome

SARS-CoV: Severe Acute Respiratory Syndrome-CoV

SARS-CoV2: Severe acute respiratory syndrome coronavirus 2

scRNA-seq: single-cell RNA sequencing

STING: stimulator of interferon genes

TF: tissue factor

TLR: Toll-like receptor

TMA: thrombotic microangiopathy

TMPRSS2: transmembrane protease serine 2

u-PA: urokinase-type plasminogen activator

WHO: World Health Organization

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV2) is a newly identified coronavirus which emerged for the first time in the city of Wuhan and rapidly spread through China to cause a disease known as coronavirus disease 19 (COVID-19) (http://www.who.int/csr/don/12-january-2020-novel-coronavirus-china/en/). Because the outbreak of COVID-19 has rapidly spread worldwide, affecting millions of people, the World Health Organization (WHO) has declared SARS-CoV2 as a global pandemic (https://www.who.int/dg/speeches/detail/who-director-general-s-opening-remarks-at-the-media-briefing-on-covid-19—11-march-2020).

SARS-CoV2 is a new *beta-coronavirus* belonging to the same sub-group as Severe Acute Respiratory Syndrome-CoV (SARS-CoV) and the Middle East Respiratory Syndrome-CoV (MERS-CoV) which caused SARS and MERS outbreak in 2002 and 2012, respectively (Chen, Liu and Guo, 2020). Several studies have identified a sequence homology of 79.5% between SARS-CoV2 and SARS-CoV (Zhou *et al* . 2020b; Wu *et al* . 2020). Therefore, SARS-CoV2 genome sequencing was rapidly performed, leading to the rapid availability of real-time PCR diagnostic test which is actually used to identify infected subjects allowing the epidemiologic tracking (Corman *et al* . 2020). SARS-CoV2 is a single-stranded RNA virus characterized by an envelope-anchored Spike glycoprotein which drives virus entry into target cells by binding membrane receptors of sensitive cells and leading to viral replication (Xu *et al* . 2020b).

Epidemiological data indicate that SARS-CoV2 infection progresses through human-to-human contact, which is predominantly realized via droplet transmission (Ong *et al* . 2020). As reported by WHO, the incubation period for SARS-CoV2 is 2-14 days, although a longer period may be at the basis of asymptomatic and subclinical infection (https://www.who.int/docs/default-source/coronaviruse/who-china-joint-mission-on-covid-19-final-report.pdf), whereas illness establishment occurs mainly in 10 days (Guan *et al* . 2020). Although the estimated case fatality rate (CFR) of COVID-19 floats from 5 to 15%, the number of deaths is very high. Indeed, as of May 4th 2020, the virus has infected over 3.4 million individuals in 215 countries, and 238198 people have lost their lives (https://www.who.int/emergencies/diseases/novel-coronavirus-2019).

Several reports have summarized the clinical and epidemiological features of patients affected by COVID-19. In the first published cohort of 41 laboratory-confirmed cases infected with SARS-CoV-2 (Huang *et al* . 2020), it was reported that infected patients had a median age of 49.0 years and 73% of them were men. The common symptoms were fever (98%), cough (76%), myalgia and/or fatigue (44%), and dyspnea occurred within 8 days from the establishment of the pathology in 55% of patients. Very few COVID-19 patients had gastrointestinal symptoms and prominent upper respiratory tract signs and symptoms, indicating that the target cells might be located in the upper and lower airways. All hospitalized patients showed abnormalities in chest computed tomography (CT) images, which were characterized by grinding glass-like and consolidation areas in 98% of the cases reporting bilateral lungs impairment at the basis of bilateral interstitial pneumonia. Because of complications, 32% of patients were admitted to an intensive care unit (ICU), among which 15% worsened in a short period of time. Most of them died of respiratory failure, but it is not excluded that death was due to organ failure, coagulation alteration with ensuing thrombosis and embolism as a consequence of blood clotting due to septic shock and/or cardiovascular complications (Huang *et al* . 2020). An exacerbation

of SARS-CoV2-induced acute respiratory distress syndrome (ARDS) is characterized by thrombosis and ischemic events so that the check for coagulation parameters are daily needed.

It is well-known that in the early stages of ARDS, fluid from the pulmonary capillaries start to leak into the lungs, making oxygenation very difficult (https://www.lung.org/lung-health-diseases/lung-disease-lookup/ards/learn-about-ards). CT images from COVID-19 patients showed the presence of widespread ground-glass opacities in the lung, which represent the most common evidence of pulmonary edema (Hewitt *et al* . 2014) associated to bilateral diffuse alveolar damage due to high levels of pro-inflammatory concentrates typical of ARDS, as revealed by biopsy lung specimens from COVID-19 affected patients (Xu *et al* . 2020d).

2. Biological targets for SARS-CoV2

One key discovery in understanding the secrets of SARS-CoV2 infection involves virus structure, especially viral spike (S) protein, which facilitates viral entry into target cells by binding host-cell receptor and then by fusing viral and host membranes (Li, 2016). SARS-CoV2 specifically recognizes Angiotensin-Converting Enzyme 2 (ACE2) as the receptor binding domain (RDB) for its S protein to mediate viral entry and infection (Zhou *et al* . 2020b). Based on the fact that SARS-CoV2 engages the same receptor used by SARS-CoV to mediate infection, and that both virus share sequence similarities of 80% between their S proteins (Zhou *et al* . 2020b), it was suggested that they could act in a similar way. It has to be pointed out that the viral attachment to host cell membrane via ACE2 is the first of a multi-step process involved in coronavirus infection; indeed, after the ligation to ACE2, indispensable for infection, the next step is the priming of S protein by cellular proteases, which consists of S protein cleavage at the S1/S2 and the S2 site, which allows fusion of viral and cellular membranes proteases on the host cell (Letko *et al* . 2020; Chen *et al* . 2020). As in the case of SARS-CoV (Li *et al* . 2005a), the S1 subunit, which contains the RBD, directly interacts with the peptidase domain (PD) of ACE2 providing for tight and higher affinity binding between virus and the host cell. Based on the fact that RBD of SARS-CoV2 is the critical determinant of viral tropism and infectivity, it was demonstrated that its mutations could alter the affinity to the binding receptor, leading to increased viral load (Ou*et al* . 2020). In particular, three mutants of SARS-CoV2 RBD (V367F, W436R, and D364Y) are correlated to higher human ACE2 affinity, ensuing higher infectivity. This discovery provides insights into SARS-CoV2 evolution and highlights how an increased affinity for human ACE2 due to RDB mutations could further favor COVID-19 diffusion. Because ACE2 is the receptor that SARS-CoV2 uses to anchor host cell, it is obvious to speculate that its expression could be correlated to viral infection susceptibility. Therefore, scientific efforts are focused on the study of ACE2 localization, in order to identify the possible route of viral infection, spread and replication throughout the body. ACE2 expression in the lung is concentrated in a small population of type II pneumocytes, which also express other genes positively correlated to SARS-CoV2 reproduction and transmission (Zhao *et al* . 2020), suggesting that alveolar pneumocytes could be a potential site of entrance of this virus, and prove a possible explanation for rapid lung viral expansion and pulmonary manifestations typical of COVID-19 patients. If on one side, ACE2-expressing lung cells may be the main target cells for coronavirus infection, on the other, Hamming *et al* . 2004 have already reported that other organs express ACE2, maybe explaining why some COVID-19 patients also exhibit non-respiratory symptoms. According to the single-cell RNA sequencing (scRNA-seq) and protein datasets, apart from lung and type II alveolar cells, heart, esophagus, kidney, bladder, ileum, oral cavity and testes are the organs at risk due to higher ACE2 expression (Zou *et al* . 2020; Xu*et al* . 2020a). To date, in the attempt to find a potential drug against COVID-19, human recombinant soluble ACE2 (hrsACE2), which has already been tested in phase 1 and phase 2 clinical trials for ARDS and COVID-19 (Haschke *et al* . 2013; Khan *et al* . 2017; https://clinicaltrials.gov/ct2/show/NCT00886353), can reduce viral growth in Vero E6 cells, most probably by acting as a decoy receptor and preventing viral binding to the natural membrane-bound ACE2 (Monteil*et al* . 2020).

Beyond ACE2, it was recently found that SARS-CoV2 also uses the cellular transmembrane protease serine 2 (TMPRSS2) for S protein priming, another key event for virus entrance into host cell (Hoffmann et al., 2020). TMPRSS2 is a cell surface protein of the serine protease transmembrane family type II that is

broadly expressed by epithelial cells (Zou*et al* . 2020; Xu *et al* . 2020a) and is involved in the cleavage of the SARS-CoV and influenza virus hemagglutinin protein (Böttcher *et al* . 2006). As other member of its family, TMPRSS2 favors the entry of the virus into the lungs leading to respiratory infections (Shulla *et al* . 2011). This protease was already described by Gierer *et al* . (2013) and Matsuyama *et al* . (2010) as the enzyme responsible for SARS-CoV infection. Hoffman et al., (2020) found that, SARS-CoV2 uses both TMPRSS2 and endosomal cysteine proteases cathepsin B and L (CatB/L) to enter host cells. The inhibition of TMPRSS2 by means of Camostat mesylate, an TMPRSS2 inhibitor, partially blocked SARS-CoV2 entry, suggesting CatB/L involvement (Kawase*et al.* 2012). Moreover, the same authors found that co-treatment with Camostat mesylate and E-64d, an inhibitor of CatB/L, completely abrogated virus entry in the same cells, indicating that the virus can use both CatB/L as well as TMPRSS2 for S protein priming in these cell lines. In contrast, the sole Camostat mesylate was not able to block SARS-CoV-2 entry into the TMPRSS2 knock-down 293T cells, confirming that the S protein of SARS-CoV-2 could employ TMPRSS2 for its priming.

Other lines of research are focusing their attention on the coronavirus 3-chymotrypsin-like protease (3CL-pro), also known as Mpro, a cysteine protease present in the Coronavirus replicase polyprotein (Zhou *et al* . 2019). This protease plays a critical role both in the immune regulation and in viral replication in that it regulates the proteolytic cleavage of some polyprotein. 3CLpro drives the cleavage of polyproteins pp1a and pp1ab, which in turn are responsible for the generation of functional proteins such as RNA polymerase, endoribonuclease and exoribonuclease (Khan *et al* . 2020). For this reason, it was speculated that 3CL-pro could represent an attractive target for COVID-19 treatment. In this context, two different molecular docking and molecular dynamic simulation studies reveled 4 drugs that could act against 3CLpro: the antibacterial drug talampicillin, the antipsychotic drug lurasidone (Elmezayen *et al.* 2020), and the antiviral drug raltegravir and paritaprevir, which were already used in the antiretroviral therapy against the Human Immuno-deficiency Virus (HIV) infections as integrase strand transfer inhibitors (INSTI) (Khan*et al* . 2020). 3CLpro also cleaves the 2'-O-Ribose Methyltransferase (2'-O-MTase), a protein that catalyzes the methylation of 5'-terminal cap structure of viral mRNAs (Chen *et al* . 2011). Because this reaction is crucial for viral replication and expression in host cells (Menachery *et al* . 2014), 2'-OMTase was suggested as another possible druggable target for COVID-19 treatment (Khan *et al* . 2020), although it is still unclear whether 2'-O-MTase, as well as 3CLpro, contributes to SARS-CoV2 infection.

3. ACE2 and Bradykinin

ACE2 is a membrane-associated aminopeptidase and belongs to the angiotensin-converting enzyme family of dipeptidyl carboxydipeptidases and has high homology to human angiotensin 1 converting enzyme (ACE1) (Tipnis *et al* . 2000). A region of the extracellular portion of ACE2 that includes the first α-helix and lysine 353 and proximal residues of the N terminus of β-sheet 5 interacts with high affinity to the receptor binding domain of the SARS-CoV S glycoprotein (Li *et al* . 2005b). The secreted ACE2 catalyzes the cleavage of angiotensin I into angiotensin 1-9, and angiotensin II into the vasodilator angiotensin 1-7, explaining the negative regulation activity exerted on angiotensin II-induced increase of blood pressure (Patel *et al* . 2016). Beyond its role in the cardiovascular system, it plays a role in the regulation of renal function and fertility (Koitka *et al* . 2008; Pan *et al* . 2013). Of recent outbreak, it was demonstrated that ACE2 plays as a functional receptor for the S glycoprotein of the human coronavirus SARS-CoV2 (COVID-19 virus) (Imai *et al* . 2005; Zhou *et al* . 2020b). Once the virus binds with its glycoprotein moiety of the extracellular S protein to ACE2, it is endocytosed into the host cell allowing its reproduction, leading to ARDS- and SARS-induced pulmonary edema. The ligation of ACE2 by the virus provides ACE2 blockade which systemically translates into higher hypertensive activity of Angiotensin II, which is not catabolized into angiotensin 1-7 or angiotensin 1-9, promoting not only the well-known hypertensive and hypertrophic activity on the cardiovascular system, but also the leakage of pulmonary blood vessels, as demonstrated in an *in vivo*model (Imai *et al* . 2005) (Figure 1). This likely leads to what we are actually assisting in terms of high blood pressure in COVID-19 patients and pulmonary edema up to angioedema, which underlies the fact that ACE2 cleaves a single-terminal residue of several bioactive peptides, such as neurotensin, dynorphin A (1-13), apelin-13, and des-Arg^{9}bradykinin (DABK) (Vickers *et al* . 2002; Donoghue *et al* .

2003). Herein, once ACE2 is blocked by SARS-CoV2, besides the perturbation of the pulmonary renin-angiotensin system (RAS) (Imai *et al* . 2005; Kuba *et al* . 2005), increasing inflammation and vascular permeability occur, due to the activity of DABK that binding to bradykinin receptor B1 (BKB1R) can lead to acute lung inflammation (Sodhi *et al* . 2018) (Figure 1). In support, in a mouse model of endotoxin inhalation, the absence of ACE2 led to the activation of the DABK/BKB1R axis, release of pro-inflammatory chemokines such as C-X-C motif chemokine 5 (CXCL5), macrophage inflammatory protein-2 (MIP2), C-X-C motif chemokine 1 (CXCL1), also known as keratinocyte-derived chemokine (KC), and TNF-α from airway epithelia, increasing neutrophilia and inflammation with an ensuing lung injury. To date, the same authors showed that endotoxin administration in mice induced ACE2 attenuation in the lung partly due to NF-ϰB signaling, which is constantly activated during an acute inflammatory process, especially by IL-1β, as well as IL-6, and other pro-inflammatory cytokines, exacerbating lung inflammation/edema up to organ dysfunction (Sodhi *et al* . 2018).

Therefore, if we consider the clinical outcome of COVID-19 patients, we could speculate that the blockade or under-expression of ACE2 by SARS-CoV2 and the ensuing pro-inflammatory mediators (i.e. IL-1β and IL-6) contribute to the pathogenesis of lung inflammation because of an impaired catabolism of DABK, leading to an enhanced BKB1R signaling, resulting in what is actually called "cytokine storm" during the early onset of COVID-19. Bradykinin receptors are B1, an induced receptor during inflammatory conditions, and B2, a constitutive and ubiquitous receptor (Marceau, Hess, and Bachvarov, 1998). B2 receptor mediates the action of BK and lysyl-bradykinin (Lys-BK), the first set of bioactive kinins formed in response to injury from kininogen precursors through the action of plasma and tissue kallikreins; whereas B1 receptor mediates the action of DABK and Lys-DABK, the second set of bioactive kinins formed through the action of carboxypeptidases on BK and Lys-BK, respectively (Couture *et al* . 2001). The B2 receptor is ubiquitous and constitutively expressed, whereas the B1 receptor is expressed at very low levels in healthy tissues but it is induced following injury by various pro-inflammatory cytokines such as IL-1β (Marceau , Hess, and Bachvarov, 1998). Both receptors act through $G\alpha_q$ to stimulate phospholipase Cβ followed by phosphoinositide hydrolysis and intracellular free Ca^{2+} mobilization, and through $G\alpha_i$ to inhibit adenylate cyclase and stimulate the MAPK pathways (Leeb-Lundberg, 2004) (Figure 2). Although little is known about the cross-talk between B1 and B2 receptors, it is well established that B2 signalling can mediate B1 upregulation via MAPK- and NF-kB-dependent pathways, and that the expression of both receptors can be induced by pro-inflammatory cytokines (i.e. TNFα and IL-1β), creating a "*catch-22 loop* " (Brechter *et al* . 2008). This molecular mechanism/s translated into clinical outcomes underlie vascular permeability and dilation, bronchoconstriction (cough) and pain (hyperalgesia, muscular pain) with ensuing fever due to the cytokine storm, all symptoms of COVID-19. (Figure 1).

An important issue is that BK is produced by the kallikrein-kinin system (KKS). The KKS consists of prekallikrein in complex with high molecular weight kininogen (HMWK) (Hooley, McEwan, and Emsley, 2007) (Figure 2). HMWK is a multifunctional single-chain plasma glycoprotein primarily expressed by the liver and secreted into the bloodstream. HMWK consists of 6 different proteic domains (Shariat-Madar and Schmaier, 1999) and binds to prekallikrein by means of a sequence in domain 6. The detachment of the domain 4 liberates BK (Griffin and Cochrane, 1979). Kallikreins are serine proteases responsible for the release of kinins, vasoactive peptides that cause vascular smooth muscle relaxation and an increase of vascular permeability (Bhoola, Figueroa, and Worthy, 1992). It has been found that kallikrein exists in two different forms: plasma kallikrein, which cleaves HMWK into BK, which in turn interacts with the constitutive B2 receptor, and tissue kallikrein which processes low-molecular-weight kininogen (LMWK) into Lys-BK. The interaction of BK or Lys-BK onto B1 and B2 receptors will increase the activation of both endothelial NOS (eNOS) and inducible NOS (iNOS), with an ensuing release of nitric oxide, potent vasodilator, of prostaglandin I2 (PGI2) and pro-inflammatory cytokines and chemokines responsible for acute inflammation that is accompanied by vasodilation, pain, cell proliferation and fibrosis (Kuhr *et al* . 2010; Tsai *et al* . 2015), symptoms typical of COVID-19 (Figure 1; Figure 2).

Plasma as well as tissue kallikrein are initially secreted as inactive, but both of them are activated by serine protease activity (Bhoola, Figueroa, and Worthy, 1992). The reciprocal activation of Factor XIIa (Hageman

Factor) and plasma prekallikrein promotes the activation of the kallikrein, which, besides the catabolism of HMWK into BK, initiate the intrinsic pathway of coagulation, influencing fibrinolysis (Figure 2). At the same time, tissue pre-kallikrein cleaves low molecular weight kininogen (LMWK) in des-Arg-kallidin and des-Arg^9-BK which interact with B1 receptors further enhancing inflammation. The intrinsic pathway of coagulation is then correlated to the extrinsic pathway of the coagulation in that Factor XIIa activates Factor XI, which leads to the activation of factor IX which subsequently leads into the common pathway by the activation of Factor X and then thrombin, with fibrin aggregates generation, hence the need to detect D-dimer in COVID-19 patients (Figure 2). In this context, studies looking at rat models that express both BK receptors show, *in vitro* , that BK acting through the B2 receptor on the surface of endothelial cells promotes the expression of procoagulant and antifibrinolytic proteins, such as tissue factor (TF) and plasminogen activator inhibitor 1 (PAI-1) (Kimura *et al* . 2002). On the other hand, plasma kallikrein can align pro-urokinase plasminogen activator (u-PA) in such close proximity as to drive plasminogen activation into plasmin which degrades fibrin aggregates (Selvarajan *et al* . 2001), effects that are widely observed in sepsis, another co-morbidity of COVID-19. However, it has been shown that the complex HMWK and Factor XIIa can also bind to another of the three endothelial cell-binding sites, the 33-kDa cell surface receptor for the first component of complement C1q (gC1qR/p33) which has high affinity for HMWK (Ghebrehiwet *et al* . 2006). Therefore, the activation of the classical pathway of the complement together with the activation of the plasmin on the conversion of C3 into C3a and C3b induce the activation of both lecithin and extrinsic pathways of the complement with the ensuing activation of the humoral immunity, exacerbating the inflammatory process (Figure 2).

These events may happen in COVID-19 patients from the early onset up to the severe step of the pathology. To date the above pathological conditions are typical of angioedema, cardiovascular dysfunction and sepsis, pathologies which symptoms occur in COVID-19 patients. But it is obvious to ask the correlation between these symptoms and the viral infection. Why would this happen? Our hypothesis is that the infection by SARS-CoV2 that "uses" ACE2 to enter the host, blocks the activity of the degradation of angiotensin II, but at the same time the degradation of BK is altered and impaired in that to trigger and enhance all the above described clinical events.

4. Approaching therapies for COVID-19 patients.

In the attempt to identify the effective anti-SARS-CoV2 therapy some clinical trials are still ongoing. In particular, the therapeutic approach can be classified in two big branches: the antiviral, which aims to diminish virus replication, and the anti-inflammatory agents to hijack the cytokine storm that the virus is able to induce. In particular, the antiviral drug that is currently proving of efficacy in COVID-19 is remdesivir, which tightly binds and inhibits the virus RNA-dependent RNA polymerase (RdRp) (Elfiky, 2020). In a cohort of patients hospitalized for severe COVID-19 who were treated with compassionate-use, remdesivir proved of clinical improvement in 36 out of 53 patients (68%) (Grein *et al.* 2020). Instead, lopinavir and ritonavir, anti-HIV drugs, showed disappointing results beyond standard care in that the viral load and the mortality were not altered (Cao*et al.* 2020).

On the other hand, disease-modifying antirheumatic drugs (DMARDS), such as chloroquine and hydroxychloroquine, as well as immunotherapeutic agents, such as monoclonal antibodies (mAbs), are being used. In particular, chloroquine as well as hydroxychloroquine, antimalarial drugs, can interfere with lysosomal activity and autophagy, interact with membrane stability and alter signalling pathways and transcriptional activity, which can result in inhibition of cytokine production and modulation of immune co-stimulatory molecules (Schrezenmeier and Dörner, 2020). Thus, they can inhibit lysosomal activity, preventing major histocompatibility complex (MHC) class II-mediated antigen presentation. Moreover, they can accumulate in endosomes and bind to double-stranded DNA, inhibiting both Toll-like receptor (TLR) signaling (i.e. TLR7 and TLR9) (Kuznik *et al.*2011) and the nucleic acid sensor cyclic guanosine monophosphate–adenosine monophosphate (cGMP-AMP or cGAMP) synthase (cGAS) (Zhang *et al.* 2014). By preventing TLR signalling and cGAS–stimulator of interferon genes (STING) signalling, hydroxychloroquine can reduce the production of pro-inflammatory cytokines (van den Borne *et al.* 1997). However, the adverse effects need to be taken

into consideration, especially in regards to the alteration of heart rhythm which cautiously limits their use.

Importantly, immunotherapeutic agents, such as tocilizumab or sarilumab, which are mAbs against IL-6 signalling, highly released during the interstitial pneumonia, have proved an effective treatment in severe patients of COVID-19 to calm the inflammatory storm and reduce mortality (Xu *et al.* 2020c; http://www.news.sanofi.us/2020-03-16-Sanofi-and-Regeneron-begin-global-Kevzara-R-sarilumab-clinical-trial-program-in-patients-with-severe-COVID-19). This encouraging clinical trial indicates that neutralizing mAbs against other pro-inflammatory cytokines may also be of use, with potential targets including IL-1, IL-17 and their respective receptors. However, tocilizumab, as well as sarilumab, can induce hepatotoxicity, neutropenia, tumorigenesis, hypersensitivity, opportunistic infections (*https://www.accessdata.fda.gov/drugsatfda_docs/label/2016/125276s107_-125472s018lbl.pdf*;*https://www.accessdata.fda.gov/drugsatfda_docs/label/2018/761037s001lbl.pdf*); therefore, mAbs targeting IL-6 signalling cannot be administered to all patients, because of co-morbidities to COVID-19 need carefully to be taken into consideration. Nevertheless, they represent the promise for blocking cytokine storm-related immunopathology of moderate to severe COVID-19.

Another clinical trial for stable COVID-19 patients is on the activity of colchicine, an anti-gout drug, which blocks the mitotic cells in metaphase, but is also able to block Nod-like receptor protein 3 (NLRP3) inflammasome inhibiting the release of IL-1-like cytokines (*https://clinicaltrials.gov/ct2/show/NCT04322565*), such as IL-1β, that was in the attempt to be blocked by means of anakinra in another clinical trial (https://clinicaltrials.gov/ct2/show/NCT04366232). Additionally, emapalumab, a monoclonal antibody against IFN-γ, associated to anakinra, has been proposed (https://clinicaltrials.gov/ct2/show/NCT04324021). However, the inhibition of such an important anti-viral cytokine could be on one side important to block the cytokine storm, but on the other can be hijacked by the virus due to the absence of one of the most important army against viral infections, creating further opportunistic pathologies.

Baricitinib has been identified as a molecule potentially useful in COVID-19 because of a double action to down-modulate the inflammatory storm and reduce the entry of the virus into type II pneumocytes due to the blockade of the AP2-associated protein kinase 1 (AAK1), a regulator of the endocytosis of the virus (Richardson *et al.* 2020). Moreover, baricitinib also binds to the cyclin G-associated kinase, another regulator of endocytosis. Thus, baricitinib may be useful for both reducing inflammatory response and viral endocytosis.

It has to be pointed out that all the above ongoing clinical trials include monitoring of coagulation parameters, such as D-dimer, which is a metabolite of fibrin aggregates. Although there are no published case series reporting abnormal coagulation parameters in hospitalized severe COVID-19 patients, in a multicenter retrospective cohort study in China, elevated D-dimer levels ($>$ 1 g/L) were strongly associated with in-hospital deaths, therefore to severe COVID-19 (Zhou *et al.* 2020a). To date, low molecular weight heparin (LMWH), enoxaparin, has been proposed for these patients either to avoid thromboembolism events (Tang *et al.* 2020a) or to inhibit the cytokine storm (Shi*et al.* 2020), due to non-anticoagulant fraction of enoxaparin suppresses *in vitro* IL-6 and IL-8 release from human pulmonary epithelial cells (Shastri *et al.* , 2015). Moreover both *in vitro* and *in vivo* experimental studies have shown that human coronaviruses utilize heparin sulfate proteoglycans for attachment to target cells (Milewska *et al.* 2014). Indeed, interaction between the SARS-CoV2 Spike S1 protein receptor binding domain (SARS-CoV-2 S1 RBD) and heparin has been recently showed, suggesting a role for heparin in the therapeutic armamentarium against COVID-19 (Mycroft-West *et al.* 2020).

Another immunotherapeutic agent that was suggested is eculizumab, a mAb against C5 complement. Diffuse microvascular thrombi in multiple organs in COVID-19 non-survivors have been announced and even more important, thrombotic microangiopathy (TMA) can occur in many different clinical scenarios including pathogenic complement activation (Campbell and Kahwash, 2020). Altered complement system occurs in a number of pathologic settings, leading to diffuse thrombotic microangiopathy (TMA), microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure up to organ dysfunction. If given early,

eculizumab therapy can reverse both renal and cardiac dysfunction (Campbell and Kahwash, 2020). Campbell and Kahwash (2020) suggest that complement inhibition could be a promising treatment for severe COVID19 by reducing the innate immune-mediated consequences of severe coronavirus infection, and it would pair well with direct anti-viral therapy.

Another suggested approach includes the off-label use of Camostat or Nafamostat mesylate, inhibitors of the host cell protease TMPRSS2, that could arrest coronavirus infections by controlling viral entry into the human cells. It has to be noted that, if on one side TMPRSS2 inhibitors could prevent SARS-CoV2 replication by blocking the fusion of the virus envelope with host cell surface membranes, they could also be effective in controlling pathological conditions correlated to COVID-19, such as coagulation and inflammation, based on their pharmacological properties. As it is well-known, Nafamostat mesylate has been used as a short-acting anticoagulant in patients with disseminative blood vessel coagulation, hemorrhagic lesions, and hemorrhagic tendencies (Maruyama *et al.*2011; Choi *et al.* 2015) due to its ability to competitively inhibit various enzyme systems, such as coagulation and fibrinolytic systems (thrombin, Xa, and XIIa), the KKS, the complement system, pancreatic proteases and activation of protease-activated receptors (PARs) (Kim *et al.* 2016). Similarly, Gabexate mesylate, binds and inhibits kallikrein, plasmin and thrombin (Tamura *et al.* 1977). Therefore, it was suggested the use of these drugs to prevent thrombosis and disseminated intravascular coagulation typical of COVID-19 patients (Tang *et al.* 2020b; Cui *et al.* 2020). Beyond their anticoagulant propriety, both Gabexate and Nafamostat mesylate show anti-inflammatory effects, which could be useful in COVID-19 uncontrolled inflammation (Tay *et al.* 2020). In particular, Gabexate mesylate decreases the production of inflammatory cytokines, such as TNF-α by attenuating NF-ϰB and JNK pathway activity, most probably through the proteolytic destruction of IϰB (Yuksel *et al.* 2003); Nafamostat mesylate shows an anti-inflammatory effect*in vitro* , where it mediates the inhibition of lipopolysaccharide-induced nitric oxide production, apoptosis, IL-6 and IL-8 production in cell cultures (Kang *et al.* 2015; Choi *et al.* 2016). In this context, Camostat mesylate could be also useful to reduce the production of inflammatory cytokines due to SARS-CoV2 infection. Indeed, it was already found that Camostat mesylate reduces the release of IL-6 and TNF-α into cell supernatants infected with influenza virus (Yamaya *et al.* 2016).

5. Further therapeutic hypotheses.

So far, the published clinical observations of biochemical markers in COVID-19 patients include elevated LDH, D-dimer, bilirubin, high levels of pro-inflammatory cytokines that accompany interstitial pneumonia, renal and cardiac injury due to thromboembolic events, which also underlie septic shock that occurs in severe COVID-19 patients. Therefore, based on what described above and cross-linking biochemical with clinical outcomes, in this review we propose another therapeutic approach based on the inhibition of both BK receptors and HMWK. Icatibant is an antagonist of B2 receptor blocking the activity of the BK avoiding both the pro-inflammatory cytokine storm and cell proliferation; it is a drug approved by the European medical agency (EMA) for the treatment of angioedema in both children and adults (https://www.ema.europa.eu/en/documents/assessment-report/firazyr-epar-public-assessment-report_en.pdf). No specific adverse events have been reported, unless urticaria, nausea and headache, though, specific attention to be paid in patients with compromised cardiovascular system (i.e. ischemia and angina pectoris). However, preclinical studies did not show any genotoxic activity, alteration of the cardiac conduction and ischemia events or hemodynamic parameters. Nevertheless, it has been demonstrated that icatibant highly binds to B2 receptor, while the affinity to the analogous B1 receptor is at least 100 times lower. It has to be pointed out, though, that little is known about B1 receptor, which is the inducible receptor during inflammatory conditions, although several pre-clinical and phase I/II trials have been performed to evaluate possible use of agents targeting B1 receptor for inflammation-related diseases (Qadri and Bader, 2018).

In addition, another drug to point the attention on could be lanadelumab, which is a monoclonal antibody against the plasmatic kallikrein, which is important for the cleavage of HMWK into BK, and is involved in the coagulation as well as in the induction of the complement system (Figure 2). Actually, lanadelumab is used for the treatment of angioedema and has not reported adverse, severe events, other than hypersensitivity, myalgia and hepatic alteration of

alanine aminotransferase (ALT) (https://www.ema.europa.eu/en/documents/assessment-report/takhzyro-epar-public-assessment-report_en.pdf). Differently from icatibant, lanadelumab could block upstream the activity of BK, avoiding the inflammatory and coagulation storm besides the complement system in SARS-CoV2 infected patients, likely preventing the exacerbation of COVID-19, in parallel with antiviral therapy.

In conclusion, we believe that the blockade of ACE2 increases not only the activity of angiotensin II on the cardiovascular system, but also the levels of DABK derived by HMWK. Therefore, the hypothesis to block the production of DABK upstream by blocking the metabolism of HMWK could be another option to face this tremendous pandemic event that affected whole world life style obliging to social limitations and stay-at-home politics.

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No conflict of interest to disclose.

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Figures and Figure Legends

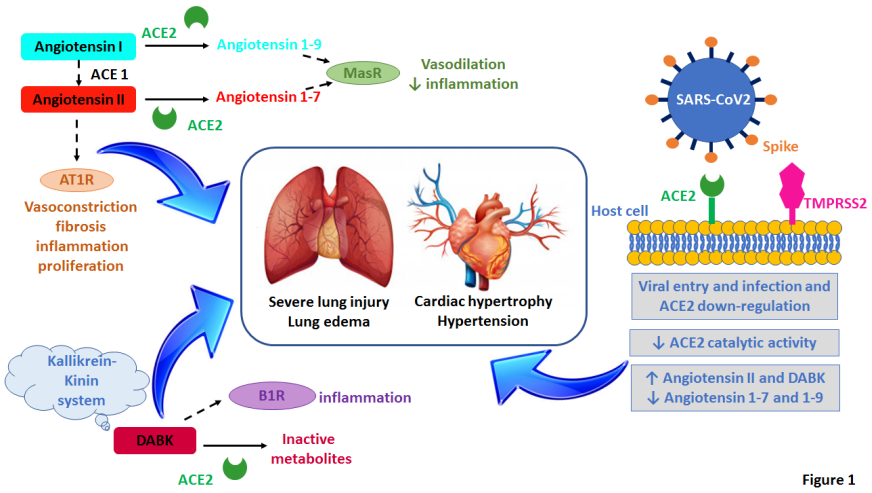

Figure 1. ACE2 function and its regulation in SARS-CoV2 infection.

ACE2 is a carboxypeptidases which catalyzes and inactivates Angiotensin I and Angiotensin II, respectively, into the vasodilator peptides Angiotensin 1–9 and Angiotensin 1–7, which bind Mas receptor (MasR) leading to reduced inflammation and vasodilation. ACE2 also cleaves des-Arg^{9}bradykinin (DABK), a bioactive kinin derived from kininogen pathway, into inactive metabolites. ACE2 is the cell entry receptor for SARS-CoV2; the binding of viral spike glycoprotein with ACE2 and the priming of the spike through the transmembrane protease serine 2 (TMPRSS2), leads to SARS-CoV2 infection. The binding of SARS-CoV2 downregulates ACE2 expression, leading to a reduction of its enzymatic activity and the ensuing increase of Angiotensin II and DABK levels. Angiotensin II takes its deleterious effect by binding the Angiotensin II type 1 receptor (AT1R), whereas DABK concurs to inflammation by binding BK receptor B1 (B1R), resulting in severe lung injury, pulmonary inflammation and edema, increased coagulation, hypertension and cardiac hypertrophy, which are all features of COVID-19 patients.

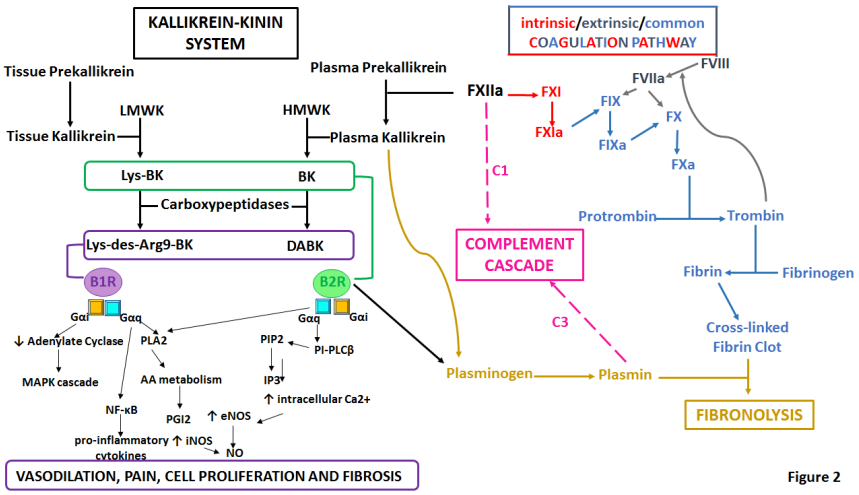

Figure 2

Figure 2. Crosstalk between Kallikrein-Kinin System (KKS), coagulation, fibrinolysis and complement cascade.

Kallikrein-Kinin system (KKS) (black box and arrows) consists of tissue and plasma kallikrein which act on high molecular weight kininogen (HMWK) and low molecular weight kininogen (LMWK) to generate bradykinin (BK) and kallidin (Lys-BK). BK and Lys-BK, and their metabolites (Lys-des-Arg^9-BK and DABK) act via two G-coupled receptors, B1R and B2R, resulting in increased vascular permeability, vasodilation, edema formation and ultimately hypotension. Plasma kallikrein, which is induced by the reciprocal activation of the Factor XIIa (FXIIa) and plasma prekallikrein, also influences the fibrinolytic pathway by activating plasminogen into plasmin and leading to fibrin degradation and D-dimer generation (yellow box and arrows). Beyond its role in KKS, FXIIa starts the intrinsic coagulation pathway (red arrows). Blood coagulation consists of an intrinsic and extrinsic (grey arrows) pathways, both resulting in activation of Factor X (FX), which subsequently leads to thrombin and fibrin generation (common pathway; blue arrows). The coagulation cascade is also a starting point for the complement system (pink box and arrows). FXIIa binds C1q component of the complement triggering the classic pathway; moreover, plasmin activation, which is also promoted via B2 signalling, triggers C3 cleavage inducing the activation of both lecithin and extrinsic pathways of the complement.

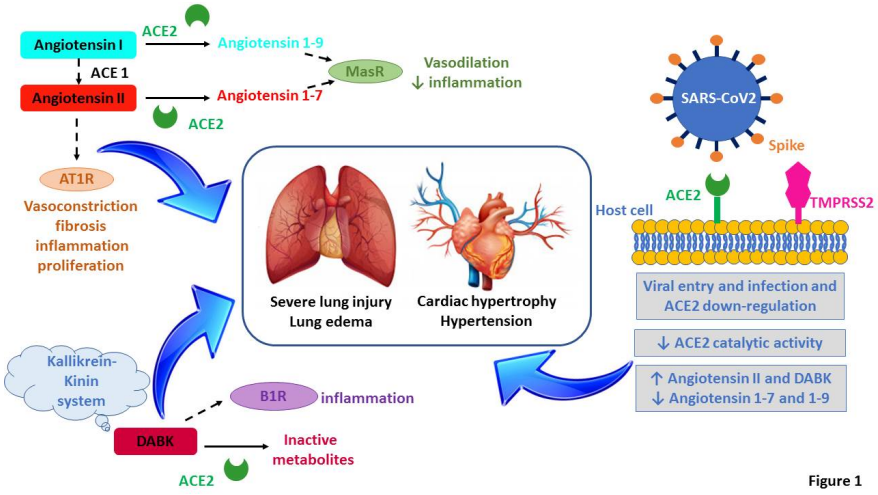

Figure 1

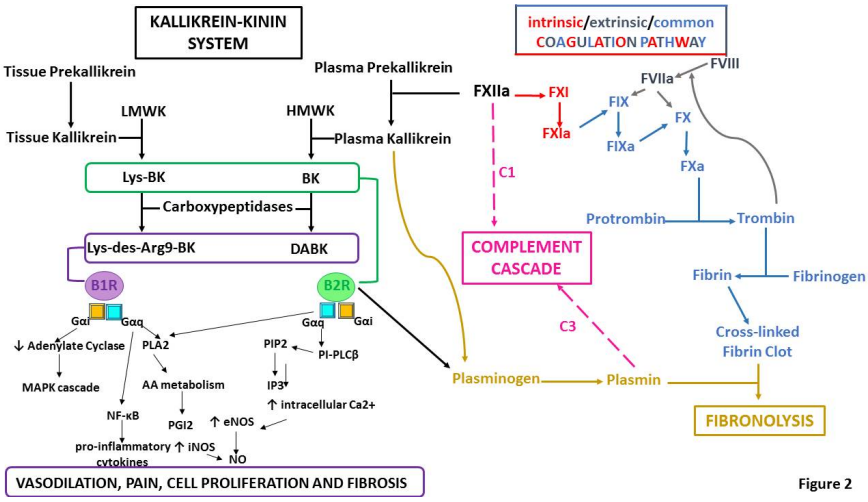

Figure 2