Yin-Yang Balance of ACE/ACE2 Pathways: The Rational for Administration of ACE2 Pathway Inhibitors in Patients Infected by SARS-CoV-2

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Abstract

The article describes the rational for inhibition of the angiotensin-converting enzyme 2 (ACE2) pathways as specific targets in patients infected by SARS-CoV-2 in order to prevent the establishment of positive feedback loops triggered by COVID-19 in some predisposed subjects. Making use of a large quantity of published reports in which human/rodent ACE2 pathway inhibitors were administered *in vivo*, it is hypothesized a possible therapeutic pharmacological intervention through an inhibition strategy of the zinc metalloprotease ACE2 and its downstream pathway for SARS-CoV-2 patients. Of even more interest, metal (zinc) chelators and renin inhibitors (both FDA approved drugs) may also work alone or in combination in inhibiting the positive feedback loops, initially triggered by COVID-19 and subsequently sustained by hypoxia independently on viral trigger, when both arms of renin-angiotensin system (ACE2 and ACE) are upregulated, leading to critical, advanced and untreatable stages of the disease.

Keywords: Severe Acute Respiratory Syndrome Coronavirus-2; (soluble) ACE2, eosinophil, asthma, IL-10, Lung fibrosis, hypercapnic acidosis, hypoxia, infarction, hypertension, cardiac dysfunction, respiratory distress, coagulopathy, Angiotensin, renin, Ang (1-7), Ang (1-9), Mas receptor, AT2 receptor.

Introduction

The clinical characteristics and allergy status of 140 patients infected by COVID-19 have been recently described [1]. Infection is known to produce either non-severe or severe symptoms and, in the latter case, it may lead to severe acute respiratory syndrome (SARS) and even death [1][2][3][4][5]. Elder age and different comorbidities were associated with critical patients. Lymphopaenia and eosinopaenia were observed in most patients, and the critical ones have extremely low values of eosinophils, suggesting that eosinopaenia may be a potential marker for diagnosis [1]. Moreover, cardiac dysfunction/injury, respiratory distress/illness, coagulopathy, hypoproteinaemia, acidosis, hypoxia and a cytokine storm mainly involving IL-6, IL-8, IL-1β and IL-10 upregulation were associated with most serious SARS cases [1][2][3][4][5]. Of note, hypertension, an age-related disease, was the most common comorbidity (15-31% up to 24-58% in severe disease), while asthma and other allergic diseases were not reported by any of the 140 infected patients [1], nor by any patient in other reports [2][3][4][5], considering that the overall prevalence of asthma in

China is estimated to be around 4% [6]. It is generally known that viral infections may increase the risk of allergic disease exacerbation; however, the reported data seem to suggest that, in this case, the opposite is true. Indeed, in the examined cohorts (more than 1000 patients), the reduced number of asthmatic patients suggests that they might be protected from virus induced SARS, whereas pre-existing hypertensive disease and/or pre-existing antihypertensive treatments may represent a risk factor for virus infection, considering that the overall prevalence of hypertension in Chinese adult population ≥18 years of age is estimated to be around 23% [7]. Therefore, according to the clinical picture of infected patients, Th2-mediated allergic diseases (usually with high eosinophil counts) may play a protective role against this severe acute respiratory syndrome (usually with low eosinophil counts), while still obscure mechanisms related to hypertensive conditions may exacerbate symptoms.

ACE2 mediated SARS-CoV-1/2 infections

It is known that SARS-CoV-2 virus shares about 80% sequencing identity with the original SARS-CoV virus [8]. Of note, angiotensin-converting enzyme 2 (ACE2) was identified as a receptor for the spike (S) protein of SARS-CoV after its priming by transmembrane protease serine 2 (TMPRSS2), finally facilitating viral entry into target cells [8]. ACE2 is abundantly expressed in airway epithelial cells and it is believed to play a crucial role in the control of acute lung injury induced by SARS-Cov [9]. Spike-Fc protein treatment (3h) resulted in a downregulation of ACE2 protein expression either in an *in vitro* system (cell lines) or *in vivo* in lung cells of mice [9][10], suggesting that ACE2 pathway may be down-modulated during infection. However, ACE2 is constitutively expressed and released from the apical cell surface of human airway epithelia into airway surface liquid [11] and its surface down-modulation upon spike protein challenge has been shown to be due to ACE2 shedding mediated by activation of extracellular ADAM17/TACE metalloprotease, which concomitantly induces shedding/production of TNFα [12][13]. Interestingly, ACE2 shedding is enhanced not only by binding with spike protein [12][13], but also by IL-1β and TNFα inflammatory cytokines [11], cytokines that are secreted at relatively high concentration in COVID-19 patients [2]. Moreover, soluble (s)ACE2 (induced or not by virus binding) released from human airway epithelia has been demonstrated to retain both its enzymatic activity and its binding ability for spike viral protein, finally reducing spike protein-mediated viral entry into target cells [11][12]. Therefore, the engagement of ACE2 by spike protein of SARS-CoV induces a cellular “protective” ACE2 shedding feedback response that initially limits viral entry. Nevertheless, ADAM17/TACE-mediated ACE2 shedding or ACE2 enzymatic activity have been shown to intriguingly correlate positively with viral infection and disease complications [12][14][15]. In particular, HNL63-CoV binds to ACE2, infects ACE2-bearing cells but induces neither ACE2 shedding nor SARS [12] and catalytically inactive forms of sACE2 can potently inhibit SARS-CoV infection [14][16], suggesting that events downstream of ACE2 shedding and/or its enzymatic

activity may indirectly and subsequently favor viral infection and/or disease complications. To this regard, sACE was also associated with myocardial pathological conditions [17] and cardiovascular complications including hypotension (known to enhance both renin and angiotensin I, the substrate of ACE/ACE2) and tachycardia were common in SARS-CoV patients [18]. Since spike protein has been shown to not inhibit ACE2 enzymatic activity that is retained by sACE2-spike protein complex [11][12][19] and, in general, sACE2 maintains its enzymatic activity, we cannot consider its higher circulating expression a mere disease biomarker. Indeed, ACE2 shedding might repress its local function but it certainly enhances its circulating/systemic activity. Interestingly, a recent integrative bioinformatics analysis shows that the expression of ACE2 in human bronchial cells infected with SARS-CoV is dramatically increased 24h after infection and remained at a high level for at least 2 days, suggesting that ACE2 may be involved in a positive feed-back loop post-infection [20]. In the same report, it has been shown that ACE2 expression level in bronchial epithelium obtained by brushing from asthmatic and normal subjects was similar, suggesting that respiratory epithelial cells of healthy subjects and asthmatic patients have similar ability to bind to SARS-CoV-2 through ACE2. Of note, ACE2 was also identified as the receptor for the novel (TMPRSS2 primed) spike protein of SARS-CoV-2 [8]. Although the role of ACE2 in the pathogenesis of SARS-CoV-2 and in inducing lung injury is still unknown, ACE2 behaves similarly to original SARS-CoV [8].

Pathological effects of (s)ACE2/Ang (1-7)/Mas receptor pathway upregulation and feedback mechanisms of pathway regulation

ACE2 and ACE are key enzymes of the renin-angiotensin system (RAS). ACE2 processes angiotensin (Ang) I and II into Ang (1-9) and Ang (1-7), respectively, and it also has other known peptide targets, such as des-Arg(9)-bradykinin, which mediates B1 receptor activation. Ang (1-7) and Ang (1-9) peptides, opposing the effects of ACE/Ang II/Ang II type 1 receptor (AT1R) pathway, are known to mediate vasodilatative (hypotension), antiproliferative and apoptotic effects through Mas and AT2 receptors, respectively, both involving downstream nitric oxide synthase (NOS) pathway activation [21][22][23][24][25][26].

Most of the experiments show that increased ACE2 activity leads to beneficial effects; however, they were performed using models in which its “antagonist“ (ACE) pathway was upregulated or ACE2 itself was downregulated, therefore balancing an unbalanced situation. What does it happen in models in which the opposite occurs?

(s)ACE2 or Ang (1-7) upregulation have been associated to some pathological conditions such as inflammation of the gastrointestinal tract, infarction, human cirrhosis and lung injury/fibrosis. For example, in the plasma of patients with inflammatory bowel disease, (s)ACE2 activity and Ang (1-7) concentrations were higher compared to healthy subjects [27]. Moreover, elevated plasma sACE2 activity was associated both with greater severity of myocardial dysfunction and with an independent prediction of adverse clinical events

[17][28]. Transgenic mice with increased cardiac ACE2 expression suffer from lethal ventricular arrhythmia (Heart block, ventricular tachycardia and terminal ventricular fibrillation) consequent upon downregulation of connexins involved in gap junction formation [29]. Interestingly, in a rat model of myocardial infarction following coronary artery ligation, there is evidence that C16/MLN-4760 (a specific ACE2 inhibitor, see later) administration inhibits fibrosis and hypertrophy of non-infarcted myocardium and increases diastolic relaxation, raising the possibility that ACE2 activity may have some adverse effects on post-myocardial infarction [30]. The risk factors for cardiovascular disease are often accompanied by alterations in platelet function and coagulation with an increased risk of thrombosis. Ang II and hypercholesterolemia are known to participate in microvascular thrombosis and enhanced thrombus formation in the microvasculature may contribute to microinfarctions. To this regard, in a rat model in which AT1R activation produce baseline thrombosis by platelet aggregation, Ang (1–9), known to bind AT2R [25], has been shown to enhance the thrombotic process [31]. Moreover, in a mouse model, AT2R activation (inhibited by AT2R antagonist, PD12319) mediated the onset of arteriolar microvascular thrombosis following chronic Ang II infusion [32], indicating both the recruitment of the AT2R pathway downstream of AT1R activation and its involvement in arteriolar thrombosis. Of interest, disseminated intravascular coagulation is associated with hypoproteinaemia and deficit of coagulation and anticoagulation proteins, which can originate by their renal loss (and consequent proteinuria) or by their reduced hepatic synthesis. To this regard, in healthy livers, ACE2 is limited to perivenular hepatocytes and endothelial cells; instead, in human cirrhosis, ACE2 protein expression is widespread in the hepatic parenchyma. Notably, human hepatocytes cultured in hypoxic conditions upregulated ACE2 protein expression [33] and ACE2 mRNA, protein and activity were increased in response to hypoxia and by IL-1β in human hepatocellular carcinoma-derived cells [34]. In line with these observations, in peripheral blood human CD34+ cells, Mas receptor (MasR) expression and ACE2 expression, activity and shedding (of sACE2 catalytically active forms) were increased under hypoxia [35]. Moreover, under hypoxic conditions human pulmonary artery smooth muscle cells upregulated both (arms of the RAS) ACE and ACE2 mRNA and protein expression, and ACE2 was subsequently downregulated by (ACE-derived) Ang II through an AT1R-mediated process [36]. In line with these observations, a strong correlation between the gene expression of ACE and that of ACE2 was observed in human renal cortical biopsy specimen, suggesting a link between the two gene transcription, not exclusively related to hypoxia [37]. A link that tends to maintain the balance of ACE/ACE2 ratio but that may be disrupted by ACE inhibitors (ACEIs) or by Ang II type 1 receptor blockers (ARBs) which both have been shown to upregulate ACE2 mRNA expression in left ventricle of Lewis rats, possibly through two different mechanisms involving upregulation of Ang (1-7) or Ang II, respectively [38]. To this regard, in rat aortic vascular smooth muscle cells, Ang (1-7) prevented the Ang II-mediated reduction in ACE2 mRNA, an effect blocked by a selective MasR antagonist, D-

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Ala7-Ang-(1-7), also known as A779 [39]. Therefore, Ang (1-7) (as well as ACEIs and ARBs), preventing Ang II-mediated ACE2 downregulation, shift the angiotensin peptide balance in favour of Ang II metabolisation by ACE2, which, in turn, leads to further production of Ang (1–7), finally sustaining ACE2 transcription, membrane protein expression and eventually shedding that enhance ACE2 circulating activity. Altogether these observations indicate a complex interplay of regulation between the two arms of the RAS in which feedback mechanisms of reciprocal (ACE/ACE2) pathway inhibition are involved at different levels: Ang II/AT1 receptor mediates a negative-feedback signal on the ACE2 enzymatic expression/activity and Ang (1-7)/MasR mediated a negative feedback signal on the AT1 receptor activity (see **Fig.1**). These negative feedback signals in some cases (e.g. hypoxia/COVID-19) can give rise to positive feedback loops that markedly shift the balance between Ang II/AT1R and the antagonist Ang (1-7)/MasR (see **Fig.1**). For example, under hypoxic conditions both arms of the RAS are upregulated and the presence of COVID-19 can affect Ang (1-7)/Ang II balance (which might be further influenced by ACEI/ARBs) by shifting it in favour of an increased ACE2 systemic activity and Ang (1-7) production. This, in turn, through the activation of MasR, can lead to further ACE2 cell membrane expression (increasing the probability of viral entry) and eventually shedding by binding to spike-SARS-CoV-2, finally establishing a positive feedback loop.

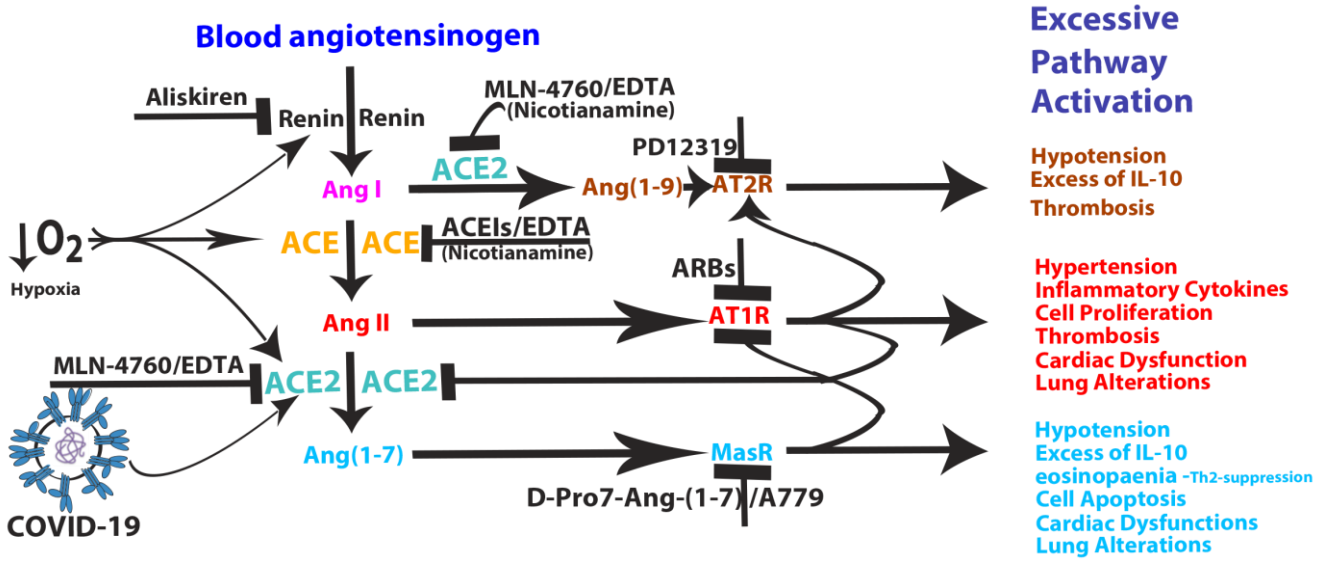

Fig.1 Interplay of regulation between the two arms of the renin-angiotensin system. Reciprocal (ACE/ACE2) pathway inhibition, RAS inhibitor sites of action and influence of hypoxia/COVID-19 on the RAS are indicated. (for reference, see the text)

Of interest, chronic hypoxia induced activation of ACE2/Ang (1-7)/MasR axis and suppression of ACE/Ang II/AT1 receptor axis in lungs of pulmonary hypertensive Ren-2 transgenic rats (constructed by inserting the mouse *Ren*-2 renin gene) but not in normotensive transgene-negative control rats [40], suggesting that the baseline renin activity in hypertensive rats may be crucial to determine the differential response to hypoxia and that renin inhibition might be useful to inhibit the ACE to ACE2 pathway shift under hypoxic conditions. Of interest, hypoxia alone or combined with hypercapnia has been shown to significantly increase both plasma renin expression or activity and plasma angiotensin II expression [41][42]. Moreover, hypercapnic acidosis (pH 6.8/6.9, a condition that could occur in SARS) induced in isolated rat lungs has been shown to induce a (compensatory) venular dilatation mediated by cyclooxygenase activation (inhibited by indomethacin) [43], an enzyme that has been shown to be induced downstream of ACE2/Ang (1-7)/MasR pathway in isolated rat hearts (again inhibited by indomethacin) [44], suggesting the involvement of ACE2/Ang (1-7) pathway in mediating CO_2-dependent lung venular vasodilation**.** Altogether these observations indicates that there is a high probability that hypoxia/hypercapnia, a condition that occurs in SARS patients, might upregulate the activity of both arms of the RAS by suppling high amounts of renin product, Ang I, to ACE which subsequently provides high amounts of Ang II to ACE2, finally producing high amounts of Ang (1-7). Interestingly, Ang (1-7) alone can either activate the bax/caspase–dependent apoptotic pathway or upregulate NF-kB signaling in lung fibroblast cultures [45]. Moreover, in vivo administration of Ang (1-7) alone (in Wistar rats) showed to promote morphological lung alterations, extracellular matrix accumulation and inflammatory cytokine production (including TNF-α and IL-6), characteristics of lung inflammation in pulmonary fibrosis [45]. A condition that, deteriorating lung function, could lead to hypoxia/hypercapnia. This, in turn, will activate both arms of the RAS which further increase MasR pathway, finally generating a positive feedback loop that might sustain SARS independently on viral infection. Of note, in lung aspirates of acid- and/or spike-treated mice, Ang II and ACE2 are synergistically upregulated and cell surface downregulated (shed), respectively, suggesting their involvement in the increased lung microvascular permeability and pulmonary oedema [9][10]; however, this condition might subsequently favour the diffusion, in neighbouring lung tissues, of both (s)ACE2 and Ang (1-7), the Ang II-derived product of (s)ACE2 processing. As already proposed, enhanced ACE2 shedding may locally reduce ACE2 activity in lung, however, it likely increases ACE2 systemic activity and subsequent production of circulating Ang (1-7). Interestingly, Ang (1–7) has been also reported to promote eosinophil apoptosis in lungs and in bronco-alveolar lavage fluid (BALF) [23]. Moreover, Ang (1-7)/MasR axis inhibits allergic airway inflammation and eosinophil cell counts in the BALF of a murine model of asthma, indicating both that an impairment of ACE2 pathway may favour asthma and that ACE2 pathway activation can reduces asthma symptoms [46]. Moreover, a compound that mimics the Ang (1–7) actions has been shown to induce IL-10 upregulation via a MasR-dependent

pathway in BALF [47] and IL-10 is thought to mediate anti-inflammatory effects of MasR pathway activation [48]. IL-10 production was also induced by an AT2 receptor agonist in mouse plasma and renal cortex via nitric oxide signalling [49] and IL-10 is known to suppress antigen-specific Th2-mediated immune responses including eosinophil expansion in allergic inflammation [50]. Indeed, increased IL-10 production (e.g. by T regulatory cells) is often associated with immune tolerance [50]. Of note, IL-10 is one of the cytokines downstream ACE2 pathway [20] and is significantly upregulated in the most severe forms of SARS-Cov-2 [2], indicating an important correlation between ACE2/Ang (1-7) axis activation and IL-10 upregulation which might lead to eosinopaenia/lymphopaenia in SARS-CoV-2 patients.

COVID-19 can induce RAS-mediated positive feedback loops at different levels: possible targets of intervention

Intriguingly, the binding affinity of ACE2 to SARS-CoV-2 binding domain has been reported to be either equal to or 10- to 20-fold higher than ACE2 binding to SARS-CoV [51][52]. The affinity of spike protein for ACE2 has been shown to correlate with the severity of disease [19]. However, although SARS-CoV produces more severe respiratory symptoms than NL63-CoV does, the 2 viral receptor binding domains bind to ACE2 with similar affinity [53], indicating that SARS development is not related to the strength of binding affinity and depends on other mechanisms. To this regard, in contrast to SARS-Cov, NL63-Cov did not induce ADAM17/TACE-mediated both ACE2 shedding and TNF-α secretion [12], suggesting that increased cleavability of both S1-S2 boundary and ACE2 receptor may be crucial for disease severity. Interestingly, the spike glycoprotein of SARS-Cov-2 but not of SARS-CoV, contains a furin-like cleavage site at the S1-S2 boundary which indicates an increased cleavability [8]. Of note, TNF-α and IL-1β were shown to both be upregulated in SARS-CoV-2 [2] and induce viral-independent ACE2 shedding from epithelial airway cells [11]. Moreover, viral-independent ACE2 surface release from epithelial cells was not only inducible by cytokines (e.g. TNF-α and IL-1β) but also constitutively and spontaneously produced when ACE2 surface expression was upregulated [11], for example upon IL-1β stimulation [34], suggesting a possible increase of sACE2 mediated by systemic release of proinflammatory cytokines such as TNF-α and IL-1β. Of note, activation of ADAM17/TACE metalloprotease was induced by SARS-CoV and necessary for efficient infection (and TNF-α secretion) [12]. Intriguingly, both viral infection and TNF-α secretion were significantly attenuated by knock-down of ADAM17/TACE expression by siRNA or deletion of the ACE2 cytoplasmic tail which has been shown to mediate ADAM17/TACE activation induced by the spike protein of SARS-CoV [12]. Altogether these observations suggest the possibility that SARS-CoV may induce a positive feedback loop leading to surface expression and shedding of both ACE2 and TNF-α. Indeed, upon spike protein binding to ACE2, downstream pathway activation can sustain positive feedback loops at different levels (see **Fig.2**):

1) SARS-Cov can induce IL-1β and TNF-α systemic secretion that can mediate viral-independent surface membrane ACE2 upregulation and shedding. This, on one hand, protects from viral infection but, on the other hand, increases circulating/systemic active sACE2 leading to its downstream pathway activation.

2) hypoxia in combination or not with hypercapnia can upregulate the activity of both arms of the renin–angiotensin system by inducing renin, ACE and ACE2 synthesis, which can increase expression of Ang I, Ang II, Ang (1-7), Ang (1-9) and the inactive metabolite bradykinin (1-7), but also membrane bound ACE2, finally giving more chances to COVID-19 viral entry.

3) ACE2 can induce vasodilatative hypotensive effects by Ang II catabolism. Hypotension can induce again renin and ACE upregulation finally providing further Ang II, as a ACE2 substrate for further Ang (1-7) production.

4) Ang (1-7) antiproliferative and apoptotic effects, possibly in part through IL-10, may mediate eosinopaenia and lymphopaenia that, on one hand, reduce inflammatory responses but, on the other hand, impair immune system ability to counter virus infection, finally predisposing the organism for further infections. Ang (1-7) immunosuppressive activity, mediated or not by IL-10, may also support the reduced ability to generate an effective immunization to SARS-CoV-2 infection.

5) Ang (1-7)/Mar receptor pathway can sustain ACE2 synthesis even in the presence of elevated concentrations of Ang II, such as in hypoxia, which are known to down-modulate ACE2 synthesis.

6) Ang (1-7)/Mar receptor pathway can produce cardiac dysfunctions and lung alteration leading to systemic hypoxia, which, in turn, upregulates the activity of both arms of the RAS.

7) Although the ACE2 catalytic efficiency is 400-fold lower with Ang I than with angiotensin II [54], high concentration of circulating ACE2 may be able to produce significant increase of Ang (1-9) that, binding AT2 receptors, can enhance the thrombotic process finally generating local hypoxic conditions and local upregulation both arms of RAS.

Although ACE2 hypertranscription and consequent increase of membrane (m)ACE2 exposure induced by hypoxia/hypotension may facilitate COVID-19 entry and its lifecycle into mACE2 expressing cells, the release of ACE2 from the cell membranes and its subsequent activity in the bloodstream and in local (lung/cardiac) extracellular fluids are likely critical steps in contributing to systemic disease pathogenesis, which involves several organs in relation to subject's predisposition. Nevertheless, the involvement of B1 receptor pathway suppression through the metabolisation of des-Arg-bradykinin to inactive bradykinin (1-7) or other ACE2-downstream pathways cannot be rule out.

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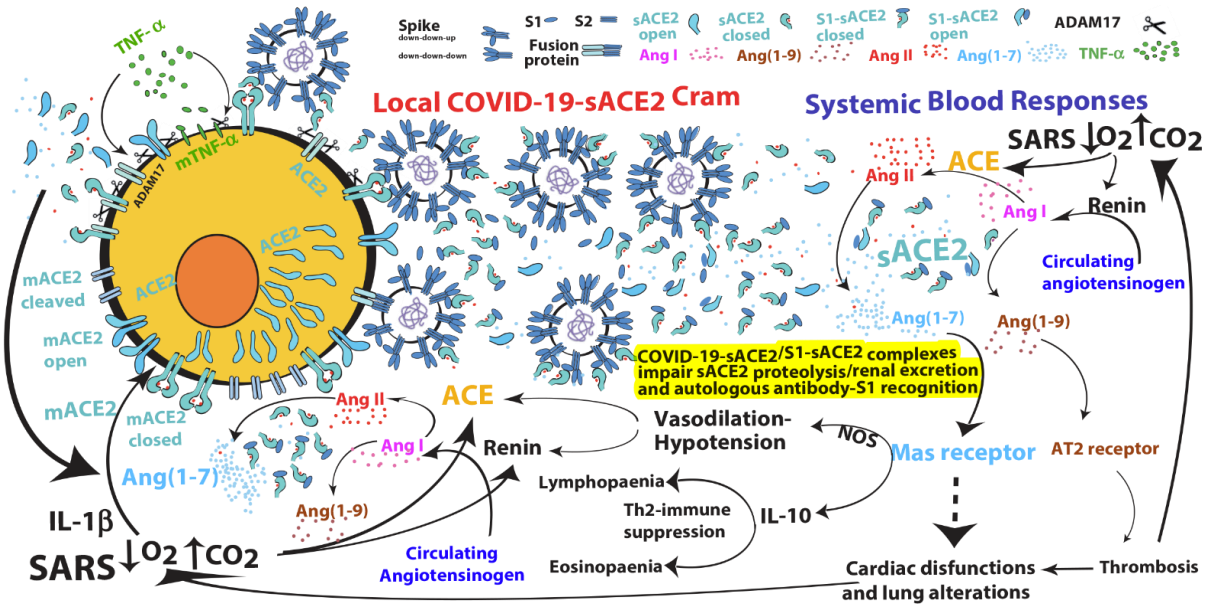

Fig.2 COVID-19 induced positive feedback loops mediated by RAS activation and physio-pathological consequences of the systemic excess of circulating ACE2 enzyme. (for reference, see the text)

Based on these observations, in order to block the positive feedback loops, different pharmacological targets can be hypothesized and pursued alone or in combination. Among them, pharmacological inhibition of ADAM17, renin and/or ACE2 enzymatic activity or its pathway are expected to stop positive feedback loops and their detrimental consequences. Indeed, inhibition of ADAM17 enzymatic activity has been already proposed about ten years ago by Haga and colleagues [15], although inhibition of ADAM17 is expected to increase membrane ACE2 expression and the probability of viral entry. Nevertheless, in the early phases of the disease in absence of systemic ACE2 pathway upregulation and, in particular, of ACE2-mediated immune suppression, it is expectable that maintenance/recovery of correct organismal immune responses in concert with cellular adaptive immune responses mediated by APOBEC systems [55] may anyway work to induce both an effective “immunization” and the viral eradication. However, of more interest are the inhibitor of ACE2 and renin. Regarding renin inhibitors, Aliskiren is the unique compound in the class of these drugs that was approved by the US Food and Drug Administration in March 2007 and commercialized for the management of hypertension [56] and it may be used alone or in combination. Among the inhibitors of ACE2 pathway, different strategy can be pursued involving either ACE2 enzymatic activity or its downstream MasR pathway. Inhibition of ACE2 enzymatic activity and its involvement in SARS-CoV-1/2 will be extensively discussed in the next sections, instead a brief description of MasR inhibition will be presented in Box1.

Box1. Mas receptor pathway inhibition and side effects of using MasR inhibitors

A779 also known as D-Ala7-Ang-(1-7)] and D-Pro7-Ang-(1-7) are two distinct Mas receptor antagonists able to prevent Ang-(1-7)-mediated downstream activation in human cells. The existence of several Mas receptor subtypes has been suggested based on the differential capacity of the two Mas receptor blockers to fully inhibit some biological actions of Ang-(1-7) [48]; therefore, differently from ACE2 enzymatic inhibitors, MasR antagonists have to be administered in combinations in order to inhibit an excess of ACE2 activity. In human aortic smooth muscle cells, they have been shown to restore NADPH oxidase/NF-kB /iNOS inflammatory pathway induced by Ang II when it is inhibited by Ang (1-7) co-administration [57]. Regarding *in vivo* experiments, acute studies in anesthetized mice revealed that a Mas receptor blocker (A779) administered alone was not associated with systolic blood pressure alterations, and the hypotensive effect produced by rACE2 in co-infusion with Ang II was unaffected by A779 co-administration, indicating that the hypotensive activity of rACE2 mainly depended on Ang II degradation rather than on increase of Ang (1-7) and MasR activation [58]. In another report, spontaneously hypertensive rats (SHRs) that received A-779 alone for a total of two weeks did not significantly alter basal blood pressure and alter urinary protein excretion [59]. Moreover, in SHRs treated with A-779 in combination with Ang II, renal injury sores and interstitial infiltration of macrophages and T cells were surprisingly reduced as compared with SHRs treated with Ang II alone, suggesting a safe use of A-779 drug in *in vivo* infusions [59]. Another report showed that infusion of A-779 alone for 7 days did not produce a significant effect neither on blood pressure nor on heart rate in SHRs [60]. In a rat model of cardiac arrhythmia, administration of A-779 alone did not cause any significant alteration in the number of arrhythmic events, confirming that A-779 can be safely delivered to rodents *in vivo*. [61]. Although Mas receptor antagonists has been shown to be safe in acute and chronic *in vivo* studies either with mice or rats, the existence of different Mas receptor subtypes in the vasculature require combinations of MasR antagonists to inhibit an excess of ACE2 activity as for example may occur in COVID-19 patients.

Spike-ACE2 complexes generated by COVID-19-ACE2 interplay may impair ACE2 inactivation/excretion and autologous anti-S1-RBD antibody recognition

ACE2 serves as an entry receptor for SARS-CoV-1/2, however during the viral infection some important conformational changes, cleavages and fusion of both spike viral and ACE2 proteins occur. The SARS coronavirus surface spike glycoproteins consist of trimers formed by two spike, capsid-proximal S1 and –distal S2, protein regions, located on the outer envelope of the virion. Spike proteins are clove-shaped trimers with three S1 heads and a trimeric S2 stalk that can bind the cellular ACE2 receptor when one S1 (possessing the receptor-binding domain) in the trimer adopts an “up” conformation (**see Fig.2**). The subsequent binding of ACE2 receptor to the spike glycoprotein triggers both the ADAM17-mediated ACE2 shedding and dissociation of S1 fragments by exogenous (furin-related or other) proteases from the spike trimer, which leads on one hand to one S1-ACE2 complex and two S1 free fragments, and on the other hand to fusion of S2 viral trimers and cellular membrane structures, finally producing cell infection. The molecular mechanism model for

SARS-CoV recognition and infection was well described by Song and collaborators [62] and a similar molecular interphase between SARS-CoV-2 and ACE2 has been recently hypothesized [52] [63]. To complete the picture, a recent structural work reveals that the full-length human ACE2 is assembled as a dimer associated or not with amino acid transporter B^0AT1, which sandwich ACE2 [63]. Binding of the spike protein trimer onto the ACE2 dimer suggests simultaneous binding of two spike protein trimers to an ACE2 dimer [63]. Each monomer of the ACE2 homodimer is composed of a membrane-proximal collectrin-like domain mediating homodimerization and necessary to position the molecule to the cell membrane, and of a membrane-distal ACE2 peptidase domain responsible for ACE2 substrate cleavage. Collectrin-like domain consists of an extracellular ferredoxin-like fold domain, also called neck domain (residues 616 to 726), a single transmembrane helix and an intracellular segment. The ACE2 cleavage site mediated by ADAM17/TACE has been predicted between amino acids 716 and 741 [11]), which corresponds to neck-transmembrane boundary of ACE2 collectrin-like domain. It is tempting to speculate that, after ACE2 shedding, S2 viral trimers may fuse with the residual ACE2 collectrin-like fragments (complexed or not with B^0AT1 transporters) which are eventually exposed on the cell surface membranes. Then, when the virus is inside the cell, a second proteolytic cleavage mediated by endosomal proteases (such as TMPRSS2) in the S2 region of the spike-ACE2 fusion protein might be necessary for intracellular virus “release” and active infection.

Several S1-sACE2 complexes, free S1 fragments or sACE2 are likely released in bloodstream of COVID-19 infected patients by spike and ACE2 receptor cleavages. As already mentioned, circulating sACE2 protein shedding independent on virus contact has been also described either spontaneously when ACE2 transcription is upregulated or upon cytokine activation; it is therefore likely that in the bloodstreams ACE2 proteins are available to bind to both free S1 fragments and COVID-19 viral particles, finally leading to more and more S1-sACE2 and COVID-19-sACE2 complexes bearing enzymatic active sACE2 (see **Fig.2**). Of note, COVID-19-sACE2 complexes, sequestrating sACE2, can drive part of the sACE2 activity and concentrate it locally where the organs possess cells expressing ACE2 on their surface membrane, i.e. following the viral tropism. Indeed, although the viral load in sputum of adult SARS-Cov patients was usually higher than 10^4 copies/mL, reports indicate that plasma viral RNA concentrations are low, usually lower than 190 copies/mL and lymphocytes have much higher concentrations of SARS-CoV RNA than plasma [64]. Similarly, a very low RNA concentration with no difference between patients with mild or severe symptoms were detected in plasma from COVID-19 patients [64]. Of interest, SARS-Cov infected pediatric patients have more than double the amount of plasma RNA copies/mL when compared to adult patients [64], suggesting a different viral tropism between adults and children. Notably, free S1 fragments may have a decoy function for autologous/exogenous anti-S1-receptor binding domain (RBD) antibodies, however sACE2-S1 masking activity might also impair either generation of

autologous high affinity antibodies against S1-RBD or recognition/inactivation of S1-RBD on COVID-19 by autologous/exogenous antibodies and antibodies that do not compete with ACE2 for the binding to S1-RBD might be more effective in neutralizing SARS-Cov-2 [51]. Moreover, ACE2-loaded COVID-19 virions and S1-ACE2 complexes may impair both sACE2 proteolytic degradation by blood proteases and its renal excretion, finally blunting the removal of its systemic enzymatic activity and indicating that circulating viral particles are dangerous even when they are not able to entry into the cells. Knowing that the number of spike proteins per virion is estimated to be around 70 and that every spike protein bears three S1 receptor binding domains, which are masked to autologous antibody recognition when in a "down" conformation, it makes clear how the coronaviruses are sophisticated and lethal weapons of infection able to bypass immune responses induced or not by vaccination.

Spike-ACE2 complexes are still sensitive to ACE2 inhibitors: mechanism of action and potential risk of using ACE2 inhibitors in SARS-CoV-2

ACE2 is a zinc metalloprotease that is inhibited by zinc chelating EDTA and activated by high concentrations of chloride or fluoride [54]. It is known that the catalytic cleft of ACE2 consists of two peptidase subdomains: one membrane-distal and the other one membrane-proximal. Their weak interactions are consistent with the ability to transition from open to the closed ACE2 conformation [63][65]. Indeed, the two subdomains undergo a large hinge-bending motion in which membrane-proximal subdomain remains almost unchanged, while membrane-distal subdomain moves to close the distance between the two subdomains, mimicking the opening/closing movement of a clam shell [65]. ACE2 open conformation likely reflects free state of the enzyme available to catch substrates (or inhibitors), then, when the ACE2 receptor binds to a substrate, the membrane-distal subdomain closes around the substrate (or the inhibitor), finally performing the enzymatic activity [19] [65]. Interestingly, only the closed state of ACE2 was observed in the spike-ACE2 ternary complex [63], suggesting that two spike protein trimers preferentially bind to the substrate-bound conformer of membrane ACE2 homodimer. The spike binding sites on ACE2 homodimer are localized above the membrane-distal peptidase subdomain of each ACE2 monomer, nevertheless neither ACE2 shedding nor ACE2 binding to spike proteins have been shown to inhibit ACE2 enzymatic activity [11][12][19]. On the other hand, the S-protein-binding region of membrane-distal ACE2 subdomain is not significantly perturbed by the receptor conformational changes and maintains the ability to associate with soluble spike proteins independently on open/closed conformations [19]. Interestingly, an ACE2 specific inhibitor (MLN-4760) has been shown to induce the closed (inhibitor-bound) ACE2 structure [65] and to retain its inhibitory effects on soluble ACE2 bound to spike proteins [19].

MLN4760 is a potent and selective human ACE2 inhibitor (IC50 = 0.44 nM against soluble human ACE2) whose synthesis produces a racemic mixture of two diastereomers that

showed 75:25 ratio for Isomer A: Isomer B [66][67] and the purified isomer B is the commercially available isomer by Merck Millipore [67]. Testing MLN-4760 racemic mixture and its isomers, it was observed a concentration-dependent inhibition of recombinant human (rh)ACE or rhACE2 activities with all three inhibitors [67]. The isomer B was less selective (near minimal-maximal rhACE inhibition range 10^{-6}M-10^{-4}M) than the racemate or the isomer A (near minimal-maximal rhACE inhibition range 10^{-6}M-10^{-2}M) and less effective (near maximal rhACE2 inhibition at 10^{-7}M) than the racemate or the isomer A (near maximal rhACE2 inhibition at 10^{-8}M) for rhACE2 versus rhACE[67]. Moreover, all three inhibitors exerted a significantly higher inhibitory activity against soluble than membrane-bound forms of (m)ACE2 (near maximal mACE2 inhibition at 10^{-6}M), being the isomer B more selective and effective than the racemate or the isomer A for mACE2 vs mACE expressed on the surface of mononuclear cells (or CD34 hematopoietic progenitors) [67]. A second ACE2 inhibitor, Dx600, produced similar results to those obtained with the isomer B [67]. Another report evaluated the inhibitory activity of both MLN-4760 and DX600 (either the linear conformational form or the disulfide bridged cyclic variant) inhibitors, however the experiments were performed at pH 6.5 [68], the pH at which rhACE2 proteolysis operates at maximal activity [54] and a condition that resembles hypercapnic acidosis which may occur during SARS. MLN-4760 was still able to strongly and specifically inhibit rhACE2 activity (near maximal inhibition at 10^{-8} M), preventing rhACE2-driven Ang II degradation into Ang (1-7), whereas DX600 (either linear or cyclic variant) inhibits rhACE2 at relatively higher concentration (near maximal inhibition at 10^{-6}M-10^{-7} M, respectively) [68]. Altogether the data indicate that the racemate or the isomer A are more effective in inhibiting soluble forms of ACE2 than isomer B and Dx600, suggesting that these inhibitors at opportune (low) concentrations are expected to preferentially reduce systemic sACE2 activity (catalytic activity of ACE2 was undetectable in human plasma of healthy subjects due to an endogenous ACE2 inhibitor [69]), while preserving the ACE2 activity of (local) membrane-associated forms of ACE2.

Since inhibitor (e.g. MLN-4760) binding to ACE2 promotes the closed conformation that is the preferential conformer for virus binding, ACE2 inhibitors are expected to not prevent viral entry, nevertheless their inhibitory function may work on the above described positive feedback loops, if proven, by restoring the normal immune responses and inhibiting ACE2 pathway upregulation and its consequent membrane expression ultimately reducing cellular accesses for viral entry. A potential risk factor of inhibiting the Ang II metabolization into Ang 1-7 could be the increase of blood pressure. Although ACE2 pathway inhibition might lead to hypertensive effects, treatment with MLN-4760 for 4-5 weeks had no effect on blood pressure when administered 10 mg/kg/day in drinking water in wild type mice [70] nor in male (mRen2)27 transgenic hypertensive rats (administered 30 mg/kg/day subcutaneously via mini-osmotic pumps) [71], suggesting that hypertensive activity mediated by ACE2 inhibition is promptly balanced by compensatory mechanisms either in normal or hypertensive blood pressure conditions. Of interest, a report showed that on day

28 post-myocardial infarction, adult male Sprague-Dawley rats that had received C16/MLN-4760 25 mg/ml/day by daily intraperitoneal injection (as a solution of 42mg/ml in distilled water) tended to have lower left ventricular pulse pressure, mean arterial pressures and left ventricular relaxation time constant-Tau compared to untreated group (see table 2 of the paper) [30], suggesting a possible protective role of ACE2 inhibition in post-myocardial infarction.

Altogether these reports on ACE2 inhibitor (MLN-4760) administration do not reveal any adverse impacts or mortality in experimental animals, which suggests its safety in chronic (toxicity) studies, this was also confirmed in clinical trials in humans (see later). Physiology teaches us that there is a range of normality for every biological parameter, below (defect) and above (excess) of which there is a dysfunction. Within ACE/ACE2 pathways, this is true for ACE as well as for ACE2, suggesting that MLN-4760 might be helpful not only for COVID-19 but also in targeted therapies for pathologies correlated with an excessive increase of ACE2 activity that may involve heart, lung, liver, colon or other tissues/organs expressing ACE2. For example, GL1001 (old name of MLN-4760) showed to produce an anti-inflammatory activity in a mouse model of colitis [72], highlighting the importance of the yin-yang balance of ACE/ACE2 pathways ("*in medio stat virtus*").

Based on ACE2 mechanism of action, it is possible to hypothesize alternative ways of ACE2 inhibition, for example concentration of cation/anion might also targets of intervention to inhibit the ACE2 zinc metalloprotease. To this regard, EDTA, a cation chelator, has been shown to be able to inhibit ACE2 activity [54]. Similarly, nicotianamine, a low-molecular weight metal chelator with high affinity for divalent metal cations that is extracted from plants (soybean), has been shown to inhibit activity of both ACE2 and ACE zinc metalloproteases [73]. Of interest, EDTA binds with 10^6- and 10^2-fold higher affinity to Zn^{2+} than to Ca^{2+} and Fe^{2+} and calcium-EDTA was approved by FDA in chelation therapy for lowering blood lead levels long time ago (1953). Then, different (commercially available) iron chelating agents, expected to work in chelating zinc ion as well, were approved by FDA. Chelation therapy comprises intravenous or oral administration of chelating agents that remove metal ions such as zinc from the body. Cells synthesising high amounts of ACE2 or ACE needs of high amounts of zinc as well, therefore they are expected to be particularly sensitive to reduction of zinc level in the plasma. For this reason, by limiting the availability of zinc to cells, metal chelating agents may have effects not only on ACE2/ACE activity but also on ACE2/ACE both synthesis and conformation, knowing that the closed conformer of ACE2 homodimer on the cell membranes both needs the presence of zinc in the catalytic site [65] and seems preferential for virus binding [63]. For all these reasons, cation chelating agents, administered alone or in combination with other therapies, could be effective to counter COVID-19 infection, in particular when, induced by hypoxia, both arms of the RAS are upregulated. However, some formulations of metal chelating agents carry a black box warning because they may cause serious and fatal renal toxicity and failure, hepatic toxicity and failure, gastrointestinal haemorrhage, and agranulocytosis that can lead to serious infections and death. As a result, treatments with

metal chelating agents require close patient monitoring, including laboratory tests of renal and hepatic function, and absolute neutrophil count should be monitored before and during treatment.

Of interest, a small (non-divalent) cationic inhibitor of ACE2 has been detected in plasma samples [69]. The endogenous inhibitor might play a compensatory fine-control (within a threshold limit) for normal fluctuation of ACE2 protein in plasma and it has been hypothesized to be a basic amino acid or a small peptide able to compete with ACE2 substrates [69]. Among the possible endogenous ACE2 inhibitors, agmatine, decarboxylated arginine, has an important role in down-regulating NO synthesis reducing NO overproduction by different mechanisms [74]. Of note, NOS pathway has been shown to be upregulated by both Ang (1-7)/MasR and Ang (1-9)/AT2 receptor pathways that are downstream ACE2 activity [21][22][24][26]. Moreover, Agmatine has a regulated plasma concentration in the range of 20-80 ng/mL and its chemical structure resembles that of an ACE2 inhibitor, NAAE [75]. NAAE is a small molecule that had demonstrated an anti-SARS-CoV activity, by acting on both ACE2 catalytic activity and ACE2 binding domain for spike protein of SARS-CoV [75]. Unfortunately, NAAE was never been used *in vivo*. Indeed, NAAE is a weak ACE2 inhibitor, it is in fact more than a thousand-fold less potent than MLN-4760; however, if agmatine will be proven to have ACE2 inhibitory activity, it might be helpful to prevent the trigger of the positive feedback loops in the first mild phases of the disease. To this regard, the use of dietary agmatine has been shown to be safe and effective in reducing neuropathic pain [76] and agmatine sulfate is regularly taken as a bodybuilding supplement.

Finally, since soluble and catalytically inactive forms of ACE2 have been shown to be potent inhibitors of SARS-CoV infection [14][16], a similar approach (in combination with other therapies) could be pursued for SARS-CoV-2 to both inhibit viral entry and avoid possible adverse effects using rhACE2 in this specific pathological condition. To this regard, a pilot clinical trial of rhACE2 in acute respiratory distress syndrome (ARDS, a SARS-CoV-like disease) started in 2017 [77]**;** unfortunately, no results are available yet.

Correlation of pre-existing circulating ACE2 activity with the possibility to develop severe acute respiratory syndrome COVID-19

Severe (SARS-CoV-2) symptoms have been described to correlate with pre-existing hypertension, diabetes and age, [1][2][3] [4] [5], and in Europe with male gender. It is not still clear whether it depends on constitutive hypertensive conditions and/or on anti-hypertensive treatments or on other age-related conditions, considering that hypertension prevalence in Chinese adults is around 23% and that only about 41% take prescribed antihypertensive medications, being calcium channel blockers the most commonly used (around 50%) in China [7]. To this regard, there is an interesting report describing ACE2 activity in blood samples of Spanish healthy subjects and patients, in which a total of 2572 subjects from a multicenter study (NEFRONA project, 2009-2011) was studied [78]. The

report shows that male gender and advanced age were identified as independent predictors of enhanced ACE2 activity [78]. Furthermore, subjects with hypertension, diabetes, dyslipidemia, or plaques also had significantly increased circulating ACE2 activity when compared with those without these pathologies [78]. On the other hand, in chronic kidney disease patients without a history of cardiovascular disease, there was a significant decrease in circulating ACE2 activity when compared with healthy control subjects [78]. Since proteinuria was associated with lower blood levels of ACE2 protein [79], low ACE2 activity in chronic renal diseases and protection from SARS might derive from a higher sACE2 renal excretion. Indeed, ACE2 is detectable in urine of healthy subjects and urinary ACE2 protein levels are elevated in patients with chronic renal diseases and in hypertensive patients treated with the Ang II type 1 receptor blocker (ARB) olmesartan [24][80]. Notably, hypertensive (the most frequent comorbidity with SARS-CoV-2) and diabetic patients are often treated with ACE inhibitors (ACEIs) and/or with ARBs, suggesting a possible positive correlation. Interestingly, circulating ACE2 activity is significantly increased in subjects on therapy with ARBs or taking oral antidiabetic agents as compared with non-treated patients, while treatment with ACEIs and cholecalciferol had no significant influence on circulating ACE2 activity [78]. In line with this observations, losartan (an ARB), but not lisinopril (an ACEI), was able to upregulate ACE2 activity in left ventricle of Lewis rats [38]. In addition, smokers and subjects on therapy with insulin tend to have an increased (although not significantly) circulating ACE2 activity when compared with control subjects [78]. On the other hand, increased ACE2 protein expression was reported in plasma and/or urine of physically active men after acute aerobic training or in renal cortices of spontaneous hypertensive, but not normotensive, rats after chronic aerobic training [81][82]. To complete the picture, a recent work reveals that asthma and other allergic diseases, which protect from developing SARS-CoV-2, are associated with significant reductions in ACE2 mRNA levels in airway cells and that ACE2 expression is also significantly inversely associated with type 2 immune biomarker levels [83]. Altogether the data suggest a strong correlation between circulating ACE2 activity and the predisposition to develop the most severe symptoms of SARS-CoV-2, suggesting that circulating sACE2 might be a predictive biomarker of SARS development. Indeed, the correlation is extremely impressive and striking if we also compare the overall prevalence of chronic renal disease in China (10.2-11.3%) [84] with SARS-CoV-2 hospitalized patients suffering from the disease (1-3%) [1][2][3] [4][5] that might be protected by a higher sACE2 renal excretion. As already mentioned, some patients with cardiovascular diseases (and inflammatory bowel disease) have an increased circulating ACE2 [17][28], which might explain the higher probability of elderly heart patients to develop SARS-CoV-2 and this could occur independently on chronic treatments with RAS blockers. The surprising aspect is that circulating (differently from membrane bound) ACE2 is expected to protect from viral infection and clinical trials using recombinant ACE2 protein are being pursued (ClinicalTrials.gov number, NCT04287686). We can conclude that a basal

hyperactivity of ACE2 and consequent ACE2/ACE pathway disequilibrium in blood can predispose to the development of more severe SARS-CoV-2 symptoms. Although more evidence is needed in humans, these observations suggest that ARBs should be precautionarily avoided to reduce possible ACE2-mediated viral consequences. Unfortunately, clinical trials of losartan as a treatment for SARS-CoV-2, are actually under way among patients who have not previously been treated with ARBs and/or ACEIs (NCT04312009 and NCT04311177). Moreover, even if they might not be detrimental, neither ACE pathway inhibitors nor recombinant ACE2 (since circulating ACE2 upregulation correlates with severe symptoms) are expected to face the disease. A possible beneficial effect of ACEIs in COVID-19 patients could indirectly come by reducing ACE2 substrate, Ang II, and finally limiting Ang (1-7) [but not Ang (1-9)] production and its effects; however, this is only a hypothetical possibility since, in this case, the ACE2/ACE pathway is expected to be even more unbalanced. To this regard, on human pulmonary artery smooth muscle cells under hypoxia, either an ACEI or an ARB have been shown to upregulate membrane ACE2 protein expression by reducing the concentration of Ang II or by inhibiting its AT1-mediated ACE2 downregulation, respectively [36]. ACEIs and ARBs may therefore play a role in the upregulation of membrane ACE2 expression under hypoxic conditions such as SARS-CoV-2, knowing that the increase of membrane bound ACE2 (before its shedding) will also increase the probability of viral entry. Of note, ACEI and ARB antihypertensive medications are more commonly used in Europe/USA than in China.

Experimental evaluation of ACE/ACE2 activity in COVD-19 blood samples to prove ACE2 pathway upregulation and ACE2 inhibitors commercially available to blunt ACE2 pathway activity

No specific therapeutics are available for SARS-CoV-2. Animals immunized with inactivated SARS-CoV vaccines developed a severe (asthma-like) lung eosinophilic immunopathology when challenged with SARS virus, indicating a central role of eosinophil “balanced numbers” in this pathology [85]. Vaccines might generate antibodies against viral ligand/ACE2 complex that finally blocks ACE2 activity during SARS-Cov virus infection and consequent downstream asthma-like events/symptoms. On the other hand, eosinopaenia, tachycardia, normo/hypotension (although COVID-19 and hypoxia increase Ang II and many patients are “hypertensive” and/or receiving anti-hypertensive medications), hypoxia and cytokine profile (e.g. IL-10) in SARS-CoV-2 patients are compatible with downstream events stemming from both an excessive ACE2 pathway upregulation and activation of positive feedback loops (see **Fig.2**). Altogether these data imply not only that the ACE2 is the “vehicle” of viral entry into the host cells but also that virus-dependent and virus-independent mechanisms may sustain activation of both arms of the RAS, but in particular that of ACE2, finally promoting SARS-induced multi-organ injury.

Unfortunately, in COVID-19 patients, an exhaustive evaluation of circulating levels of ACE/ACE2 proteins and activities, together with ACE and ACE2 substrates and products i.e. Ang I, Ang 1-9, Ang II, Ang (1-7) and Ang (1-5), des-Arg-bradykinin and the inactive metabolite bradykinin (1-7) is lacking and has to be conducted to shed more light in support (or not) of the present hypothesis. However, in order to infer correct interpretations of data obtainable from blood sample analyses, it is important to consider that:

1) organ local microenvironment might not reflect the systemic one;

2) S1-sACE2 and COVID-19-sACE2 complexes, formed by viral-induced ACE2 shedding or by subsequent binding of sACE2 with viral particles or S1 fragments in the circulation, might not be detectable by some anti-ACE2 antibodies in ELISA. Therefore, since the complexes might prevent/mask sACE2 antibody recognition but not the enzymatic activity, sACE2 detection (and its real concentration) by ELISA in blood samples of COVID-19 patients might not be reliable;

3) circulating concentrations ACE/ACE2 substrates/products depend on:

a) level of ACE/ACE2 pathway activity and availability of substrates;

b) level of expression of the respective receptors (AT1R, AT2R and MasR) that bind and remove ligand products away from circulation.

For example, Ang (1-7) concentration in blood by ACE2 pathway upregulation is necessarily dependent both on the increase of the (antagonistic) Ang II (and its precursor, Ang I) and on MasR expression on surface of cells in the organs. To this regard, the detection of inactive metabolites, such as bradykinin (1-7), in COVID-19 patients and comparison with its concentration in healthy subjects could be the most indicative and reliable markers of ACE2 activity in the plasma.

Of interest, a report that described the *ex vivo* effect of human rACE2 on the levels of several endogenous Ang peptides in plasma samples, thus mimicking an acute increase of systemic ACE2 activity in plasma [68] showed a strong reduction of Ang II (as expected), a moderate reduction of Ang I, a moderate increase of both Ang (1-7) and Ang (1-9) and finally a strong increase of Ang (1-5) [68], indicating that Ang (1-5) may also be an interesting surrogate marker of ACE2 activity in COVID-19 patients [68].

At the present time, it has been reported that plasma levels of Ang II in SARS-CoV-2 infected patients are markedly elevated as compared with healthy subjects and linearly associated to viral load and lung injury [86]; unfortunately, nobody has tested the levels of Ang (1-7), Ang (1-9) and bradykinin (1-7), which are expected to be at high levels as well, thus justifying both normo/hypotension and eosinopaenia in SARS-CoV-2 patients.

On the other hand, in the plasma samples from ARDS (a SARS-CoV-like disease) patients, only levels of Ang I have been shown to be significantly increased in non-survivors

compared to survivors but, unfortunately, no comparisons of the various patients with healthy subjects were reported [87]; this is important to consider since the plasma concentrations of the RAS peptides detected in the report by high pressure liquid chromatograph linked to a mass spectrometer were extremely low if compared to other reports that differently detect them by ELISA. For example, in ARDS patients, the mean values of Ang II were around 0.3-0.8 ng/ml (depending on survivors/non-survivors and days after study entry), instead in healthy subjects and SARS-CoV-2 patients, mean values of Ang II detected by ELISA were around 100 ng/ml and 300 ng/ml, respectively [86]. Of interest, the mean arterial pressure of ARDS patients was markedly low and significantly lower in non-survivors (65 mmHg) compared to survivors (71 mmHg), although in both survivors and non survivors, the ratios of Ang (1-7)/Ang II were low < 1, suggesting that the vasodilator Ang (1-7) might be quickly removed from circulation by binding with Mas receptors.

Since the clinical picture as a whole is consistent with a ACE2 gain of function (possibly due to both circulating active forms of S1-sACE2 complexes and local forms of COVID-19-sACE2 complexes) rather than a ACE2 loss of function, as initially supposed, inhibition of ACE2/Ang (1–7)/MasR axis or other ACE2 pathways to restore ACE/ACE2 balance is needed, at least in the first phases of the disease when hypoxia is not yet induced. Different strategies could be pursued through ACE2 pathway inhibitors (MLN-4760/C16/GL1001/ORE1001, Dx600, NAAE) and/or MasR antagonists and/or renin inhibitors and/or metal chelators.

Based on the above described hypothesis, inhibition of ACE2 pathway might be beneficial for SARS-CoV-2 patients. Several different molecules have been designed to specifically inhibit human ACE2 enzyme (both membrane bound and soluble forms) or human MasR signal transduction, but only a few have been studied *in vivo*. Some ACE2 pathway inhibitors have been widely used in mouse/rat models, as control of human and mouse ACE2 activity or in mouse models of colitis, unfortunately, to my knowledge, only one (MLN-4760/C16/GL1001/ORE1001) of the ACE2 pathway inhibitors has been tested *in vivo* in humans in a Phase I clinical trial long time ago (http://oreholdings.com/wp-content/uploads/2013/06/09.02.09-S-4-A.pdf). Thanks to mouse/rat *in vivo* experiments and clinical trial results in human participants it has been possible to infer toxicity/efficacy for some human ACE2 inhibitory molecules (MLN-4760/C16/GL1001/ORE1001, Dx600 and A779) that may be exploited to face this exceptionally dramatic situation.

Devising an administration strategy

In order to design a possible strategy for therapeutic administration, I have focused my attention on inhibitors of human ACE2 pathway that were consistently administered *in vivo*. Making use of a large quantity of published reports in which human/rodent ACE2 pathway inhibitors were administered *in vivo*, I have hypothesized a possible therapeutic pharmacological intervention through an inhibition strategy of ACE2 pathway for SARS-

CoV-2 for patients who are suffering from both mild and critical, advanced and untreatable stages of the disease (the most problematic cases to manage).

Briefly, one of the best candidate to treat COVID-19 patients, but not to prevent viral entry, is the small synthetic molecule MLN-4760 (specific ACE2 inhibitor, also known as C16, GL1001 or ORE 1001, [66]) for the following reasons:

1) It has been shown to bind/inhibit ACE2 enzymatic activity even at low/acidic pH (pH 6,5, [68]) typical of hypercapnia (as in case of SARS) when human ACE2 activity is maximal [54] and its binding to ACE2 does not perturb the S-protein-binding region of ACE2 [19], indicating that it is able to bind and inhibit ACE2 activity regardless ACE2 binding to COVID-19 viral particles or to S1 fragments.
2) No adverse effects were described upon its chronic administration neither alone nor in combination with ACE2 activators (while inhibiting their activating effects) nor after inducing functional impairment of ACE2 activity in rodent experiments *in vivo* [30] [70] [71] [72][88][89] nor in a clinical Phase I trial in humans (http://oreholdings.com/wp-content/uploads/2013/06/09.10.09-425.pdf);
3) Its administration by different route is well described in rodents and humans. In particular:
   a) Chronic administration (about 4 weeks) of C-16/DLM-4760 in combination with ACE2 activating treatments was performed by daily intraperitoneal injection at a dose of 25mg/kg in distilled water (as a solution of 42mg/ml) or 0.9% sterile saline (as a solution of 84 mg/ml using a 0.5-ml insulin syringe) freshly prepared [30][88][89].
   b) Alternatively, chronic administration (about 8 days) of GL1001/DLM-4760 disodium salt in combination with an ACE2 activating treatment was performed by subcutaneous injection (5ml/kg) containing up to a dose of 300 mg/kg, twice a day, formulated in a vehicle solution [15% 2-hydroxypropyl-beta-cyclodextrin (HPBDC)/85% H_2O] [72]. Subchronic doses of GL1001 indicate no adverse effects up to 1,000 mg/kg (see [72]).
   c) In humans ORE1001/GL1001/MLN-4760 was already proposed and tested in clinical trials. Its pharmaceutical indication was for digestive tract inflammations (Inflammatory bowel disease, gastritis and colitis) that are correlated with overexpression of ACE2. In a Phase I clinical testing up 14 days dosing, ORE 1001 was well tolerated. Subjects received drug (dosing up to 2100 mg) with no side adverse effects reported. In particular, 47 subjects received single-dose from 2.1 to 2100 mg and 24 subjects received 14 day multiple doses from 50mg to 1800 mg; all doses were well tolerated, with no significant side effects including blood pressure. Pharmacokinetics of orally administered capsules was consistent with once-daily dosing. (http://oreholdings.com/wp-content/uploads/2013/06/09.10.09-425.pdf). 300 mg (active drug) oral capsules were used in a Phase Ib/IIa clinical trial that was, however, abandoned (https://clinicaltrials.gov/ct2/show/NCT01039597).
   d) Finally, MLN-4760 was also administered (2.5 mg/kg per day) by nasal inhalation for 2-3 days in lung-infected mice by Pseudomonas bacteria [90]. Interestingly, the report underscores the role plaid by local concentration of molecules (ACE2) in modulating lung inflammation and disease. For these reason, in diseases involving respiratory tract, like

SARS, inhalation treatment is preferable, even for the lower concentration (and hopefully lower toxicity) of MNL-4760 needed for this route of treatment administration.

Extensive experiments have been also performed with DX600, a specific peptide ACE2 inhibitor that exhibited a mixed competitive and non-competitive type of inhibition [91]. Actually, several reports describing Dx600 inhibitor administration in mice suggest its safe use. Of interest, a report described its (safe) use alone (1 mg/kg per day) by nasal inhalation for 3 days in a mouse model of endotoxin-induced lung inflammation [92]; however, this inhibitor is less efficacious than MLN-4760 in inhibiting the soluble forms of human rACE2 [67] [68] and no clinical trials have been conducted.

Finally, inhibition of MasR pathway through MasR antagonists [A-779 and D-Pro7-Ang (1-7)], whose safe use was already discussed (see Box 1), could be an alternative approach to be pursued.

Going back to first ACE2 inhibitor, different route of MLN-4760 treatment administration can be pursued depending on the hospital condition/expertise. In this exceptionally critical situation, it could be delivered to critical untreatable patients as a controlled "compassionate use", in particular by inhalation. However, when, under hypoxic conditions, both arms of the RAS are upregulated, it might have a limited action and, by shifting the balance of ACE/ACE2 ratio in favour of ACE, might be even dangerous. Instead, specific inhibition of ACE2 enzymatic activity might effectively work in preventing the establishment of positive feedback loops in COVID-19 patients who are suffering from mild symptoms of the disease. MLN-4760 is sold by different companies, that, in case it works, could be encouraged to manufacture the molecule, actively contributing to face this global threat. On the other hand, the drug could be also synthesized in University chemistry labs because, to my knowledge, is no longer under patent restriction. MLN-4760, whose clinical development was abandoned after phase I trials (clinical name, ORE1001), is actually an interesting compound that could be useful for all patients in which a specific ACE2 upregulation is proven and associated to different (heart/lung/liver/intestine/kidney/immune system/blood/coagulation etc…) pathological conditions related to ACE2 pathway downstream events, as it seems to occur in COVID-19 patients.

Regarding the patients with severe symptoms of COVID-19 infection, in particular those in which the establishment of hypoxia upregulates both arms of renin-angiotensin system, aliskiren, the unique renin inhibitor commercially available, could be a useful "tool" since it reduces production of Ang I, the necessary fuel for both ACE and ACE2 hyperactivity and their detrimental effects. Aliskiren treatment in rats has been shown to upregulate both AT1R and MasR expression probably as consequence of a compensation mechanism when both Ang II and Ang (1-7) ligands are reduced by renin inhibition [93]; moreover, of particular interest in the context of SARS-CoV-2 infection, the treatment has been shown to reduce expression of both AT2R and ACE2, thus possibly performing a multiple action in inhibiting both arms of the RAS [93]. Similarly, cation chelating agents such as calcium-EDTA or nicotianamine may also act at different levels. As already mentioned, chelating agents, by limiting zinc cellular availability, can influence hyperactivity, synthesis and conformation of

both ACE2 and ACE, which may impair not only ACE2 enzymatic activity but also its availability on cell surface for viral entry. For these reasons, chelating agents could be particularly effective to counter COVID-19 infection, and in particular when, induced by hypoxia, both arms of the RAS are upregulated. Differently from aliskiren renin inhibitor, more chelating agents are commercially available, both classes of drugs can be administered, alone or in combination with other therapies not only for SARS-CoV-2 but also for other disease in which both arms of the RAS are upregulated, as for example ARDS or pulmonary hypertension associated to chronic hypoxia, which, notably, is also associated with cardiac failure.

However, before beginning any therapy with medication, particularly in the case of new diseases (such as COVID-19) or new treatments, a careful patient selection for these new treatments is essential to prevent unnecessary toxicity. In order to decide who to treat, and when to start treatment, it is important to define the entity of dysfunctions, probability of treatment success and probability to develop adverse effects. To promptly intervene in order to prevent severe forms of SARS-CoV-2, we need of early markers of disease progression. To this regard, if Ang (1-5) and bradykinin (1-7) will be proven to be reliable markers of ACE/ACE2 activity *in vivo*, they could be exploited in order to decide who and when to start treating COVID-19 patients, alternatively, eosinopaenia and hypotension may be also exploited as signs of disease progression. In any case, since the conditions of some patients can be critical, it is highly recommended to administer the minimal effective dose in order to reduce possible side effects, starting with low and increasing doses, while at the same time monitoring patient status and early signs of disease progression (e.g. blood pressure, eosinophil counts and molecular concentrations of RAS peptides). The aim of the project was not to stop viral entry (the epithelial cells take already care of it shedding ACE2) but to block positive feedback loops following the cellular response to COVID-19 which are the main cause of the severe symptoms associated with SARS-CoV-2. Doing so, the disease could hopefully become like a simple flu, a spontaneously eradicable disease. Unfortunately, diseases of different aetiologies may present similar final dysfunction/alterations, while diseases with similar aetiologies may present different final dysfunction/alterations depending on patients (COVID-19 is a clear example), this confounding aspect of diseases can lead to erroneous interpretations, and only defining the correct molecular origin of diseases is possible to reach the solutions.

Competing interests

The author declares that he has no competing interests.

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Author Contributions

LZ is sole author and sole investigator. The author conceived of the article and wrote it. LZ read and approved the final manuscript.

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