The rationale for administration of ACE2 pathway inhibitors in patients infected by SARS-CoV-2: Devising an administration strategy.

Corresponding author: Loris Zamai, Department of Biomolecular Sciences, via Ca’ le Suore 2, University of Urbino Carlo Bo, 61029 Urbino, Italy.
Tel. (+39) 0722 304319; e-mail: loris.zamai@uniurb.it

Abstract
The article describes the rationale for inhibition of the angiotensin-converting enzyme 2 (ACE2) pathways as specific targets in patients infected by SARS-CoV-2. 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 patients who are suffering from critical, advanced and untreatable stages of the disease.

Key words: Severe Acute Respiratory Syndrome Coronavirus-2; (soluble) ACE2, eosinophil, asthma, IL-10, IL-6, Lung fibrosis, Angiotensin, hypercapnic acidosis, hypoxia, infarction, hypertension.

Introduction
The clinical characteristics and allergy status of 140 patients infected by SARS-CoV-2 have been recently described [1]. Infection is known to produce either non-severe or severe symptoms, and in more severe cases, it may lead to severe acute respiratory syndrome (SARS) and even death [1][2][3]. Elder age and high number of comorbidities were associated with critical patients. Lymphopenia and eosinopenia were observed in most patients, and the critical ones have extremely low values of eosinophils, suggesting that eosinopenia may be a potential marker for diagnosis [1]. Moreover, a cytokine storm mainly involving IL-6, IL-8 and IL-10 upregulation is associated with most severe (SARS) cases [2]. Of note, hypertension, an age related disease, was the most common comorbidity, while asthma and other allergic diseases were not reported by any of the 140 infected patients [1], nor by any patients in other reports [2][3]. It is generally known that virus infections may increase the risk of allergic disease exacerbation; however, the reported data seem to suggest that the opposite is true. Indeed, the absence, in the examined cohorts, of asthmatic patients suggests that they might be protected from virus induced SARS, whereas pre-existent hypertensive disease and/or pre-existent antihypertensive treatments represent a risk factor for virus infection. 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-2 infection
It is known that SARS-CoV-2 virus shares about 80% sequencing identity with the original SARS-CoV virus [4]. Of note, angiotensin-converting enzyme 2 (ACE2) was identified as a receptor for the spike (S) protein of SARS-CoV after its priming by cellular serine protease TMPRSS2, finally facilitating viral entry into target cells [4].
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 [5]. Spike-Fc protein treatment (3h) resulted in a downregulation of ACE2 protein expression either in cell lines or in lung cells of mice in vivo [5][6], 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 [7] 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 [8][9]. Interestingly, ACE2 shedding is enhanced not only by binding with spike protein [8][9], but also by IL-1β and TNFα inflammatory cytokines [7], cytokines that are secreted at relatively high concentration in COVID-19 patients [2]. Moreover, sACE2 released from human airway epithelia has been demonstrated to retain its binding ability for spike viral protein, finally reducing spike protein-mediated viral entry into target cells [7]. 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, TACE/ADAM17-mediated ACE2 shedding or ACE2 enzymatic activity have been shown to intriguingly correlate positively with viral infection and disease complications [8][10][11]. In particular, HNL63-CoV binds to ACE2, infects ACE2-bearing cells but does not induce both ACE2 shedding and SARS [8] and a catalytically inactive form of soluble ACE2 can potently inhibit SARS-CoV infection [10], 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, ACE2 shedding does not lead to functionally inactive forms of soluble (s)ACE2 [7][8], therefore sACE2 cannot be considered a mere biomarker of disease, there is, in fact, evidence that sACE2 is not only enzymatically active but it is also associated with myocardial pathological conditions [12]. Moreover, spike protein has been shown to not interfere with ACE2 enzymatic activity that is retained by sACE-spike protein complex [7][8][13] and cardiovascular complications including hypotension (known to enhance both renin and Ang I ACE/Ace2 substrate) were common in SARS-CoV patients [14]. 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 [15]. In the same report, it has been shown that expression level of ACE2 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 [4]. 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 [4].

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

ACE2 and ACE are key enzymes of the renin-angiotensin system. ACE2 processes angiotensin (Ang) I and II into Ang (1-9) and (1-7), respectively, and it has also other known peptide targets, such as the Bradykinin metabolites. Ang (1-7) peptide, opposing the effects of ACE-generated Ang II, has been shown to mediate vasodilatative (hypotension), antiproliferative and apoptotic effects through Mas receptor [16] [17][18].

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, 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, human cirrhosis, infarction and lung injury/fibrosis. For example, elevated plasma
sACE2 activity was associated both with greater severity of myocardial dysfunction and with an independent prediction of adverse clinical events [12][19]. Interestingly, in a rat model of myocardial infarction following coronary artery ligation, there is evidence that C16/DLM-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 [20]. Moreover, (s)ACE2 activity and Ang (1-7) concentrations in the plasma of patients with inflammatory bowel disease were higher compared to healthy subjects [21]. 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 [22]. To this regard, human pulmonary artery smooth muscle cells transiently but consistently upregulates ACE2 mRNA and protein expression under hypoxic conditions [23]. But even more interestingly, hypercapnic acidosis (pH 6.8/6.9, a SARS condition) induced in isolated rat lungs has been shown to induce a vasodilatator (compensatory) response mediated by CO2-dependent activation of cyclooxygenase (inhibited by indomethacin) [24], an activation that has been shown to be induced downstream of ACE2/Ang (1-7)/Mas receptor pathway in isolated rat hearts (again inhibited by indomethacin) [25]. Therefore, there is a high possibility that ACE2 pathway upregulation through a positive feedback loop might be induced by hypercapnia/hypoxia, a condition that occurs in SARS patient and that might later sustain SARS independently of the virus infection. If this is the case, indomethacin treatment might alleviate the symptoms. Of note, lung aspirates of acid- and/or spike-treated mice have revealed a synergistic mechanism in inducing lung injury, ACE2 cell surface shedding and high concentrations of Ang II that is supposed on the origin of increased lung microvascular permeability and pulmonary oedema [5][6]; however, this condition might subsequently favor diffusion in neighbouring lung tissues of both (s)ACE2 and Ang (1-7), the Ang II derived product of (s)ACE2 processing. Interestingly, in vivo administration of Ang-(1-7) alone (in Wistar rats) has been shown to promote morphological lung alterations, extracellular matrix accumulation and inflammatory cytokines (including TNF-alfa, and IL-6), characteristics of lung inflammation in pulmonary fibrosis [26]. Moreover, Ang-(1-7) alone either activated the bax/caspase–dependent apoptotic pathway or upregulated NF-kB signaling in lung fibroblasts in vitro [26]. To this regard, Ang-(1–7) has been also reported to promote eosinophil apoptosis in the lungs and in the bronchoalveolar lavage fluid (BALF) [18]. Interestingly, Ang-(1-7)/Mas receptor axis inhibits allergic airway inflammation and eosinophil cell counts in the BALF of a murine model of asthma, indicating that both an impairment of ACE2 pathway may favor asthma and ACE2 pathway activation can reduces asthma symptoms [27]. Moreover, a compound that mimics the angiotensin (Ang)-(1–7) actions has been shown to induce IL-10 upregulation via a Mas receptor-dependent pathway in BALF [28]. Of note, IL-10 is one of the cytokines downstream ACE2 pathway [15] and together with IL-6 is significantly upregulated in the most severe (SARS) cases [2], indicating an important correlation between ACE2/Ang (1-7) axis activation and SARS.

Correlation of circulating ACE2 activity with severe acute respiratory syndrome

As already mentioned, patients with cardiovascular disease and inflammatory bowel disease have an increased circulating ACE2 [12][19] [21], however, severe (SARS) symptoms have been described to be correlated with pre-existent hypertension, diabetes and age, [1][2][3], and in Europe with male gender. It is not still clear whether it depends on constitutive hypertensive conditions or/and on anti-hypertensive treatments or on other age-related conditions. To this regard, there is an interesting report describing ACE2 activity in blood samples of Spanish people, in which a total of 2572 subjects from a multicenter study (NEFRONA project, 2009-2011) were studied [29]. The report shows that male gender and advanced age were identified as independent predictors of enhanced ACE2 activity [29]. Furthermore, subjects with hypertension or diabetes or dyslipidemia
or plaques also had significantly increased circulating ACE2 activity as compared with those without these pathologies [29]. Instead, in chronic kidney disease patients without history of cardiovascular disease, significant decrease in circulating ACE2 activity was observed when compared with control subjects [29]. Notably, hypertensive (the most frequent comorbidity with SARS-CoV-2) and diabetic patients are often treated with ACE inhibitors, suggesting a possible positive correlation. Interestingly, circulating ACE2 activity significantly increased in subjects on therapy with angiotensin II receptor blockers (ARBs) or under oral antidiabetic agents as compared with non-treated patients, while treatment with ACE inhibitors had no significant influence on circulating ACE2 [29]. In addition, subjects on therapy with insulin or smokers tend to have an increased (although not significantly) circulating ACE2 activity as compared with control subjects [29]. Altogether the data suggest a strong correlation between circulating sACE2 activity and the predisposition to develop the most severe symptoms of SARS-CoV-2. Although more evidence is needed in humans, this observation suggests that ARBs should be avoided to reduce ACE2-mediated viral entry and that ACE pathway inhibitors may not help to face the disease.

ACE2 pathway inhibitors experimented in vivo in rodents or in clinical trials in humans

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 [30]. 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, eosinopenia, hypotension (although many are “hypertensive” and/or receiving anti-hypertensive medications) and cytokine profile (e.g. IL-10) are compatible with downstream events stemming from an excessive ACE2 pathway activation. Altogether these data imply not only that the ACE2 is the “vehicle” of viral entry into the host cells but also that the virus sustains ACE2 pathway activation, finally promoting SARS-induced lung injury.

Since the clinical picture as a whole is consistent with a ACE2 gain of function (possibly due to increase of local active forms of soluble ACE2) rather than a ACE2 loss of function, as initially supposed, inhibition of ACE2/ANG (1–7)/Mas receptor axis or other ACE2 pathways to restore ACE/ACE2 balance is needed. Different strategies can be pursued through ACE2 pathway inhibitors (MLN-4760/C16/GL1001/ORE1001, Dx600, NAAE) and/or Mas receptor antagonist (A-779) [17] [27][28] and/or other ACE2 pathways (e.g. involving Bradykinin metabolites or other ACE2 substrates). For example, NAAE demonstrated an antiviral activity [31] and GL1001 showed to produce an anti-inflammatory activity in a mouse model of colitis [32], highlighting the importance of the yin-yang balance of ACE/ACE2 pathways (“in medio stat virtus”).

Based on this evidence, 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 Mas receptor 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 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 is possible to infer toxicity/efficacy for some human ACE2 inhibitory molecules that may be exceptionally exploited to face this exceptionally dramatic situation.

Conclusions: possible treatments
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 critical, advanced and untreatable stages of the disease, which are the most problematic cases to manage.

Briefly (more details will be provided in a near future work), the best candidate to treat patients with severe (SARS) symptoms, but neither to prevent nor to treat patients with mild symptoms of COVID-19 infection, is the small synthetic molecule MLN-4760 (specific ACE2 inhibitor, also known as C16, GL1001 or ORE 1001, [33]) for the following reasons:

1) It has been shown to bind/inhibit ACE2 enzymatic activity even at low/acidic pH (pH 6.5, [34]) typical of hypercapnia (as in case of SARS) when human ACE2 activity is maximal [35] and its binding to ACE2 does not perturb the S-protein-binding region of ACE2 [13], suggesting that it should bind and inhibit ACE2 activity regardless of the S-protein-mediated binding of CoV virus to (s)ACE2.

2) No adverse effects were described upon its administration neither in rodent experiments in vivo [20][36][37] 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 [20][36][37].
   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% H2O] [32]. Subchronic doses of GL1001 indicate no adverse effects up to 1,000 mg/kg (see [32]).
   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 [38]. Interestingly, the report underscores the role played 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; however, this inhibitor is less efficacious than MLN-4760 [39] and no clinical trials have been conducted. Finally, inhibition of Mas receptor activation through Mas receptor antagonists [A-779 and D-Pro7-Ang-(1-7)] could be an alternative approach to be pursued. Although we have less information regarding these two last compounds, I will discuss in detail their possible applications in a future work, but now it is urgent to start with this promising molecule, MLN-4760.

Finally, different route of MLN-4760 treatment administration can be pursued depending on the hospital condition/expertise. In this exceptionally critical situation, it can be delivered to critical untreatable patients as a controlled “compassionate use”, in particular by inhalation.

MLN-4760 is sold by different companies, that, in case it works, could be encouraged to produce it for free, 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.

**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.
References


