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Game of "crowning" season 8: RAS and reproductive hormones in COVID-19 - Can we end this viral series?

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ABSTRACT:

The outbreak of a newly identified coronavirus, the SARS-CoV2, (alternative name 2019-nCoV), capable of jumping across species causing zoonosis with severe acute respiratory syndromes (SARS) has alerted authorities worldwide. Soon after the epidemic in Wuhan, Hubei province of China starting with late December 2019, the virus has spread over multiple countries in different continents being declared a pandemic by March 2020. The demographic characteristics of the infected patients suggest that patient's age, sex and comorbidities are predictive factors for the fatality of the infection. The mechanisms of viral entry into the human host cells seem to be in a close relationship with the mechanisms of regulating the renin-angiotensin system (RAS) which may explain the pathogenesis associated with the infection. This brings new insights on the possibilities of exploiting viral entry mechanisms to limit associated complications by means of enhancing the resistance of the infected patients using methods of regulating the RAS and strategies of modulating ACE2 expression. In this perspective article we exploit the mechanisms of COVID-19 pathogenesis based on the demographic characteristics of the infected patients reported in the recent literature and explore several approaches of limiting the initial steps of viral entry and pathogenesis based on viral interactions with ACE2 and RAS. We further discuss the implications of reproductive hormones in the regulation of the RAS and exploit the premises of using endocrine therapy against COVID-19.

Keywords: SARS-CoV2; 2019-nCoV; Covid-19; endocrine therapy; ACE2; ARBs; mineralocorticoid receptor antagonist; ADAM-17; miRNA.

Introduction

Coronaviruses are a large family of RNA viruses belonging to the Coronavirinae subfamily. Together with the Torovirinae, they constitute the Coronaviridae family in the order of Nidovirales. The first member of the family of coronaviruses was described in 1932 in birds[1]; since the severe acute respiratory syndrome (SARS) outbreak in 2002-2003, new family members have been defined, highlighting the capabilities of coronaviruses to jump across species. They are enveloped RNA viruses with the longest RNA genome amongst all RNA viruses consisting of 26.2-31.7 Kb. They are constituted of 4 genera (alpha, beta, gamma and delta - coronaviruses) out of which 2 (alpha and beta) contain the 7 subspecies that were known to be capable of infecting both animals and human hosts causing zoonosis. These are 229E-CoV, OC43-CoV, NL63-CoV, HKU1-CoV [2] the human enteric HECoV[3], the SARS coronavirus (SARS-CoV) and Middle East Respiratory Syndrome coronavirus (MERS-CoV). While most subspecies produce symptoms of common cold and enteritis, SARS-CoV and MERS-CoV are capable of causing important respiratory syndromes that in some cases can prove lethal. [4]

Infection of the host cells by coronaviruses relies on the interaction of the viral particle with specific proteins on the cell surface. Interactions between the virus and host cells are initiated following the binding of the spike proteins with host cell receptors. Spikes are surface proteins of the envelope that give the crown-like appearance to the virus from where its name derives. Coronaviruses and other budding viruses exploit the replicative machinery of host cells [5], shielded from host immune responses in double membrane vesicles [6] [7]. By budding through exocytosis for their egress, rather than inducing lysis or apoptosis and proinflammatory cytokine release, they aim to produce long-lasting infections with reduced associated pathogenesis. This is how they account for 15-30% of the annual common colds with mild symptoms, only occasionally affecting the lower respiratory tract [8] [9]. However much of the pathogenesis of SARS-CoV-2 seems to be related to the cytokine storm resulting from the excessive activation of adaptive immune responses in vital organs and from dysregulation of the renin-angiotensin system (RAS) [10] [11]. The adaptation to infect the human host cells by exploiting the angiotensin-converting enzyme 2 (ACE2) receptor in SARS-CoV and SARS-CoV-2 was not without consequences as this has led to increased pathogenesis by disrupting RAS regulation through the antagonization of ACE2. This is because ACE2 is a part of the depressor arm of the RAS, and accounts for counteracting vasoconstriction, proliferation, oxidative stress, fibrosis and pathogenesis of cardiovascular disease (CVD), all

effects of the pressor arm [12]. Disruption of the depressor arm, the ACE2/Ang-(1-7)/MasR and AT_2R will lead to exacerbation of the effects of the pressor arm, the ACE/AngII/AT_1R pathway, which seems to be responsible for the decompensation of pre-existent comorbidities in coronavirus disease 2019 (COVID-19) patients where the highest fatality is reported [13]. The resulting Ang-II increased levels will lead to cell apoptosis with the release of proinflammatory cytokines [14]. This stimulates adaptive immune responses with the possibility of triggering a cytokine storm from its excessive activation.[15]

The newly identified coronavirus SARS-CoV2

On January 7, 2020, following a recent outbreak of a new type of a highly contagious coronavirus,[16] the 8th subspecies of human infecting coronaviruses was described and characterized while a test method was developed.[17] It was initially named 2019-nCoV[18] and later SARS-CoV-2. In a relatively short interval of two months, the outbreak turned into an epidemic which spread over several countries and continents infecting more than 100.000 individuals by March 7, 2020.[19] On March 11, 2020 the outbreak was officially declared as a pandemic by the World Health Organization (WHO),[20] infecting almost four and a half million people at the time of the writing of this manuscript. [21]

The SARS-CoV-2, although distinct from its cousins MERS-CoV and SARS-CoV, it is part of the same betacoronavirus genre and sarbecovirus subgenre with SARS-CoV but from a related subgenre with MERS-CoV, which is part of the Merbecovirus.[22] Although the disease caused by the SARS-CoV-2 may be less severe than SARS and MERS, it seems more contagious having a death toll already 180 times higher than SARS and MERS epidemics combined.[21]

Coronaviruses are able to exploit many cell surface molecules—proteins and carbohydrates alike - in order to gain entry into target cells. Three receptors on the human cells have been shown to interact with the viral spike proteins that enable its fusion and incorporation within the host, and these include Aminopeptidase-N, ACE2 and Neu 5.9 AC2. While Aminopeptidase-N is the receptor used by the human coronavirus 229E-CoV,[23] the sialic acid Neu5.9 AC2 is the preferred receptor for the human OC43-CoV. Angiotensin converting enzyme 2 (ACE2), a type I integral membrane protein largely distributed in the vasculature, the endothelial cells of the heart and kidneys, brain and lungs [24] [25] is the preferred receptor for NL36-CoV, SARS-CoV and the new SARS-CoV-2, while MERS-CoV

uses a different receptor, DDP4 (**Figure 1**).[26] This distribution of the ACE2 in key organs might explain how patients with pre-existent cardiovascular system pathologies may be more susceptible to severe complications caused by the coronaviruses that use ACE2 to fuse with the human host cells, disrupting the physiological regulation of the RAS.

Implications of ACE2 in the RAS regulation

The receptor used by the SARS-CoV-2 to infect human cells, ACE2, is an important component in the regulation of RAS, as it is part of the counteracting arm against the vasopressor pathway, the ACE2/Ang1-7/MasR and AT_2R. Accumulating evidence shows that the RAS is regulated differently in women and men, the androgens and estrogens having different effects on the regulator arms of the RAS. Such differences allow the adaptation to increased total blood volume during pregnancy and confer a considerable degree of protection from cardiovascular disease in premenopausal women as compared to age-related men. This is done through the protecting arm of the RAS pathway, ACE2/Ang1-7/MasR and AT_2R which counteracts the vasopressor effects of the ACE/Ang II/AT1R axis. In men, the ACE/Ang II/At_1R is predominant. [27] This might at least in part, explain how lethality is lower in women than age related men and how exacerbation of pre-existent cardiovascular pathologies increased lethality. Although these observations rely mostly on indirect evidence, competitive mechanism may exist between RAS regulation through ACE2 and mechanisms of viral entry into the human host cells since antagonizing ACE2 by the virus is associated with exacerbation of the effects of the pressor arm - the ACE/Ang II/AT1R axis.

In 2000 a new form of ACE was described, the ACE2 [28] [29] first characterized by Crackower et al. 2002, [30] involved in heart function and development of arterial hypertension. ACE and ACE2 have similar protein structures with small differences, which lead to different substrate specificities. ACE is the enzyme present in the lungs responsible for converting Angiotensin I to Angiotensin II with vasopressor effects; ACE2 has been shown to exert counteracting effects on ACE action by inhibiting RAS through converting Angiotensin I to Angiotensin 1-9 which is further converted to Angiotensin 1-7 by the ACE. ACE and ACE2 are thus functionally different enzymes with opposite roles.[31] SARS-CoV2 spike glycoprotein binds to the cell membrane ACE2 to penetrate human cells. Further research confirmed the presence of ACE2 in the lung, heart, kidney and vessel endothelium. [32-34] Zhao *et al.* showed that 83% of ACE 2 expressing cells are alveolar epithelial, therefore the lungs appear the

most vulnerable target SARS-COV-2[35] while the heart, kidney and central nervous system[36] are the second most affected organs by the viral burden. It has been demonstrated that the binding of the SARS-CoV-2 spike protein to ACE2 leads to ACE2 downregulation, which results in excessive production of Angiotensin II by the related enzyme ACE, while ACE2 is not capable of converting Angiotensin I to Angiotensin 1–7. This results in increased pulmonary vascular permeability, lung and heart injury.[37] The large distribution of ACE2 to other organs could explain the end-stage multi-organ dysfunction in severely infected patients [38 11 39] including neurologic manifestations.[36]

A China CDC weekly report through February 11, 2020 has revealed the results of the demographic characteristics from 72,314 patient records admitted for the new coronavirus infection (COVID-19). Reported fatality in men was higher than in women (2.8 vs 1.7%), high age and underlying cardiovascular pathologies being associated with increased fatality. Furthermore, synthetic datasets generated from this report showed that older women had a similar fatality with age-related men, making it possible for some sex hormone influences to affect the viral entry and pathogenesis explaining some of the sex/age/associated pathology differences in lethality and morbidity. The datasets created from this report suggest that patient's high age was the most important risk factor of lethality from the disease, even greater than having any of the listed comorbidities.[13] These data relies mostly of indirect evidence since there is neither information about the correct prevalence of gender per age nor about comorbidities per age and gender in the CDC report. [11] At the current time, the reported sex ratio for the fatality men and women is still 1.6, but with higher fatality rates reported for men (4% in men and 2.5% in women)[40]

Differences in RAS regulation between men and women

Several lines of evidence show that intracellular RAS can operate independently of the circulating RAS and there is a close interaction between sex hormones and RAS regulation. Estrogens have been known to provide protective effects by modulating the RAS. One study on endothelial cells showed that physiological levels of estradiol increased ACE1 by 25% but not ACE2 protein expression in vitro through the estrogen receptor alpha (ERα). The same study showed that while ACE2 protein expression remained unmodified following estradiol exposure, the enzymatic activity of both ACE1 and ACE2 increased. Alternatively, the translation of ACE2 mRNA on protein could be down-regulated by estradiol, directly due to

increased ACE2 activity of increased Ang-(1-7) production. The increased activity of ACE2 without increased protein expression could be due to a direct estradiol-induced ACE2 activity, but an indirect activation could not be infirmed [41].

Differences between men and women in RAS regulation could be attributed to a higher stimulation of ACE activity by androgens, as it has been demonstrated in male mice[42],in which testosterone increases ACE activity, and also in women with hyperandrogenism from polycystic ovary syndrome.[43] This is further supported by sex differences in the regulation of arterial pressure and renal function by the RAS[44] with the balance tipped toward depressor pathways in women[45]. Moreover, women are protected against cardiovascular disease relative to men, prior to menopause. [46] Androgens are known to be involved in the sex differences in the regulation of arterial pressure, with estrogen protecting against and testosterone exacerbating hypertension.[44] In the last two decades a special interest has been redirected towards the RAS with the discovery of additional receptors specific for angiotensin peptide fragments, suggesting the presence of a depressor arm of the RAS (ACE2/Ang(1-7)/ MasR and AT_2R), which counter-regulates the classical ACE/ AngII/AT_1R pathway[12].

Sex differences have been demonstrated in the regulation of blood pressure and renal function by the RAS. Significantly, the counter-regulatory arm of the RAS, including ACE2/Ang(1-7)/MasR and AT2R, is upregulated in females.[27] Estrogen regulates all components of the RAS by increasing angiotensinogen synthesis, while reducing the secretion of renin and synthesis of ACE.[47] In males, testosterone amplifies the pressor pathways of the RAS.[48] Regarding genetic sex differences, the SRY gene family, located on the Y chromosome in men, decreases promoter activity of ACE2 while upregulating promoter activity of ACE, angiotensinogen and renin respectively.[49] In addition, both the AT2R and ACE2 genes are located on the X chromosome, suggesting a greater role of these depressor RAS arm components in females. In males, the ACE/ AngII/AT1R pathways are enhanced, whereas, in females, the balance is shifted towards the ACE2/Ang (1-7)/MasR and AT2R pathways. Evidence shows that premenopausal women are at lower risk of developing cardiovascular and renal disease as compared to aged-matched men, and this differential regulation of the RAS between men and women likely has an important contribution. This cardiovascular protection in women lowers with reaching menopause and reaches the same incidence as seen in age-related men, likely being related to loss of estrogen in postmenopausal period. However, the mortality gender difference for COVID-19 is far more

outspoken than the gender difference in the occurrence of CVD. This creates the premises of using endocrine therapy for the management of COVID-19.

Strategies of limiting viral entry into the human host cells and associated pathogenesis

Several potential strategies of inhibiting viral entry into the human host cells to reduce lethality and associated morbidity are worth pursuing based on the ACE2 interaction with the SARS-CoV-2 spike proteins (**Figure 2)**.

Endocrine therapy in COVID-19

Regulation of the ACE2 could be attempted by several strategies, used alone or in combination. Endocrine therapy may prove useful in modulating ACE2 expression and regulating RAS in men and women. Androgen deprivation therapy (ADT) could potentially shift the RAS regulation towards the ACE2/Ang1-7/MasR and AT2R pathway in order to achieve a reduced lethality in men, at least to the extent reported in premenopausal women. To date, there is no study evaluating the effect of reproductive hormones and their effects on the infectious cycle of the SARS-CoV-2. This represents a direction worth pursuing since reproductive hormones (estrogen 1microgram/ml and testosterone 3micrograms/ml) were found to enhance the replication of the avian coronavirus in vitro, while progesterone had no effect. The same study found an enhancing effect of cortisone 3micrograms/mL on the replication of the avian coronavirus.[50] Another study on avian coronavirus has shown that the adaptive immune response and cytokine activity was enhanced probably due to the effect of estrogens, *in vitro*.[51] In SARS-CoV and MERS-CoV, estrogen receptor inhibitors were shown to be active against the infection *in vitro*.[52] However an *in vivo* mouse model showed that the severity of SARS is associated with enhanced accumulation of macrophages and neutrophils in the lungs leading to vascular leakage and alveolar edema, and male mice had an increased susceptibility to these complications, independently of B and T cell response. Furthermore, ovariectomy or treating female mice with estrogen receptor antagonists increased their lethality from SARS-CoV.[53] Together, these findings show that gender differences in the severity to SARS-CoV in murine models parallel those reported in patients and support the hypothesis of estrogen receptor signalling as protective against COVID-19 in women. Although this may in part explain the reduced fatality from COVID-19 in premenopausal women, the implications of reproductive hormones in the pathogenesis of COVID-19 are

even more compelling. Androgens have been shown to be a positive regulator of TMPRSS2 protease [54]. In lungs, TMPRSS2 (epitheliasin) is required for the activation of the spike viral protein for viral internalization and activation of the fusion peptide **(Figure 3)**, with alternative fusogenic mechanisms including furins, cathepsins and other trypsin-like proteases, accounting for potential therapeutical targets in COVID-19 [55-58].

The coding gene for the pulmonary epitheliasin is upregulated by androgen exposure, together with other genes involved in viral pathogenic mechanisms such as phospholipid metabolism, iron ion binding, oxygen binding, heme binding and clathrin-coated vesicle, and this was demonstrated *in vitro* on a culture of human lung adenocarcinoma-derived cell line - A549 [59]. The responsiveness of TMPRSS2 gene to androgen stimulation was also reported *in vivo* in mice and patient data correlated with these observations [60] while the implications of sex hormones in the immune response has been well documented [61]. Moreover, the protective effects of anti-androgens in SARS-CoV-2 infected patients was seen in an observational Italian study[62].

Based on these effects, androgen deprivation therapy (ADT), despite it's the most well recognized but reversible side effects including hot flashes, loss of libido and erectile dysfunction, could potentially be beneficial for reducing lethality in men, as well as women with hyperandrogenism from polycystic ovary syndrome [43]. While ADT represents an accessible option for aiding the treatment of COVID-19, a premise for its use should be the validation of these effects in controlled clinical trials including men, women and even transgender patients on endocrine therapy.

Several ongoing clinical trials are currently investigating the interactions of corticosteroid hormone derivatives in COVID-19 such as methylprednisolone (NCT04263402; NCT04323592; NCT04244591), dexamethasone (NCT04263402; NCT04325061), budesonide and formoterol (NCT04331470), while there are no clinical trials evaluating reproductive hormones in COVID-19. Testing endocrine therapy against SARS-CoV-2 could represent a close perspective. Nevertheless, several other strategies that can modulate the RAS and ACE2 expression are worth mentioning. The usage of recombinant ACE2[63] could benefit COVID-19 patients since ACE2 may bind SARS-CoV-2 spike protein in the plasma before reaching other cells, turning spike incapable of binding other membrane ACE2, thus avoiding internalization of the virus into cells. This is currently being evaluated in several clinical trials. (NCT04335136; NCT04287686)

RAS blockers in COVID-19

While using ACE inhibitor drugs that lower blood pressure may seem a good approach to reduce the viral entry into the host, some of them are most likely not useful as treatment for COVID-19. Perindopril has little or no effect on ACE2.[64] Captopril, Enalapril, Lisinopril block ACE but not ACE2 and may be counterproductive. It is not clear if ACE inhibitors should be switched to another hypotensive drugs. [65-67] Knocking down the coding gene of ACE2 may seem as a proper approach to blocking viral entry into the host cells, but this was shown in animal models to severely impair heart function.[30] However angiotensin receptor blockers (ARB) as well as mineralocorticoid receptor antagonists (e.g. spironolactone), increase both ACE2 levels and Ang (1-7) levels.[68] [69] There is sufficient data showing that ARB treatment results in ACEs upregulation in humans and rats: losartan and olmesartan, were demonstrated to increase cardiac ACE2 expression three-fold following 1 month treatment after myocardial infarction in rats.[70] Losartan was also demonstrated to upregulate renal ACE2 expression in mice.[71] Olmesartan used in patients with hypertension induced high urinary ACE2 levels[72]. Therefore, higher ACE2 expression after treatment with ARBs, while seeming paradoxical, might protect against lung and heart injury. This may be the result of blocking the excessive angiotensin caused by the SARS-CoV infection, as well as, most importantly, upregulating ACE2, with increased production of Angiotensin 1–7. [73] A recent article suggested that ARBs might be beneficial for patients infected with SARS-CoV-2.[74] Several ongoing clinical trials are currently investigating the impact of RAS inhibitors in COVID-19 (NCT04335123; NCT04337190; NCT04331574; NCT04330300; NCT04312009; NCT04311177; NCT04335786) and look for arguments to continue or discontinue RAS inhibitors (NCT04338009). A possible way of treatment which would likely be resistant to SARS-CoV-2 mutations is to use available ARBs, such as losartan, telmisartan and olmesartan for reducing the binding of the virus to ACE2 and decrease aggressiveness and mortality from virus infections.[75] However, drugs that interfere with the regulation of the counteracting arm of the RAS directly or indirectly, have been shown to induce an increase in ACE2 expression and this was observed for ARBs,[76] [70] statins, [77-80] and the propionate derivative ibuprofen[81] in preclinical studies. Whether this increase is detrimental due to increasing binding sites for the virus or actually beneficial being part of the mechanisms of reducing the deletary effects of the pressor arm of RAS, remains elusive and requires further investigations. A recently published analysis found no association between ARBs or ACEI use and COVID-19 test positivity in a cohort of 18,472 patients tested for COVID-19 in a

single healthcare system[82]. In the absence of clinical evidence to the contrary, clinical consensus is to advise patients not to discontinue ACE inhibitors or ARBs in the setting of the COVID-19 pandemic[66] [83].

Other strategies of inhibiting viral entry in COVID-19

Other strategies of reducing ACE2 include ADAM metallopeptidase domain 17 (ADAM17) also known as TACE (tumor necrosis factor-α-converting enzyme) capable of shedding ACE2 from the cells through AngII[84] and miRNAs capable of regulating ACE2 expression in the lungs (miR-200c-3p[85], miR-21[86] miR-421[87]) while restoration of these RAS depressor pathways in older women or upregulation of these in males and females by gene therapy using adenoviruses for blocking angiotensin receptors could represent a therapeutic intervention to assist the treatment of SARS-CoV2 infected patients. Using such strategies could be followed by a destabilization of the blood pressure and should be done under specialized cardiologic observation.

An efficient active immunotherapy (vaccination) would be the cornerstone against COVID-19 but it could take many months to develop. This may also be limited in efficacy, or even prove ineffective since viral antigens can regularly change with adaptation mechanisms. Moreover, pre-existent antibodies from a recovered infection or vaccination may progressively fade away[88] [89] or may not exert sufficient protection against re-exposure to human infecting coronaviruses as shown in the studies of the SARS-CoV and MERS-CoV.[90] [91] Passive immunotherapy with monoclonal antibodies against antigens of the spike protein could represent a closer perspective and this was previously achieved in SARS-CoV *in vitro*[92].

Other treatment options in COVID-19 are under evaluation and while preliminary results seemed promising with antimalarials [93] and replicase inhibitors [94] several recent studies failed to show clinical benefits [95] [96] emphasizing the need of evaluating other potential therapeutic strategies against SARS-CoV-2.

Conclusions

While we may assert that there are multiple potential strategies against the early steps of viral entry into the human host cells and the pathogenesis associated with the dysregulation of

the RAS, evaluation of the effects of such agents in big cohorts is required to validate their efficacy in COVID-19. Drug repurposing can prove beneficial in controlling the extent of the disease while a specific treatment is being developed and exploiting endocrine therapy against SARS-CoV-2 could represent a unique approach, which is worth pursuing given the rapid spread of the pandemic. However, drug combinations may prove more effective than single agents and combining endocrine therapy with other strategies of inhibiting viral entry could represent a close perspective in the current pandemic as active immunization might require many months before being accomplished and readily available worldwide.

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FIGURES' LEGENDS:

Figure 1. Schematic representation of the binding site for SARS-CoV2. Spike glycoproteins S on the viral surface bind to ACE2 on human cell surface, leading to internalization of the virus into the host cell.

Figure 2. Competitive mechanisms involving viral entry into host cells and RAS regulation through ACE2. Physiological mechanisms of regulating the renin-angiotensin system involve the pressor pathway (ACE/Ang II/AT_1R) and the counteracting arm – the depressor pathway (ACE2/Ang 1-7/MasR and AT_2R). The new coronavirus (SARS-CoV2) exploits ACE2 to ensure viral entry into host cells, antagonizing the counteracting arm of the RAS system. This leads to complications associated with exacerbation of the ACE/Ang-II/AT_1R axis as it`s been observed in the pathogenesis of the COVID-19. Possible strategies of limiting viral entry and pathogenesis involve modulating ACE2 expression and regulating the counteracting arm of the RAS system shifting the balance towards the ACE2/Ang 1-7/MasR and AT_2R axis.

Figure 3. Schematic representation of the mechanism of viral entry into the lung cells by exploiting ACE2 and epitheliasin (TMPRSS2). SARS-CoV2 enters the lung cells following the binding with the ACE2 and the activation of the fusion peptide in the spike protein by the membrane protease TMPRSS2. Androgen deprivation therapy decreases TMPRSS2 expression inhibiting the mechanisms of exploiting epitheliasin for the activation of the Spike.

Figure 1

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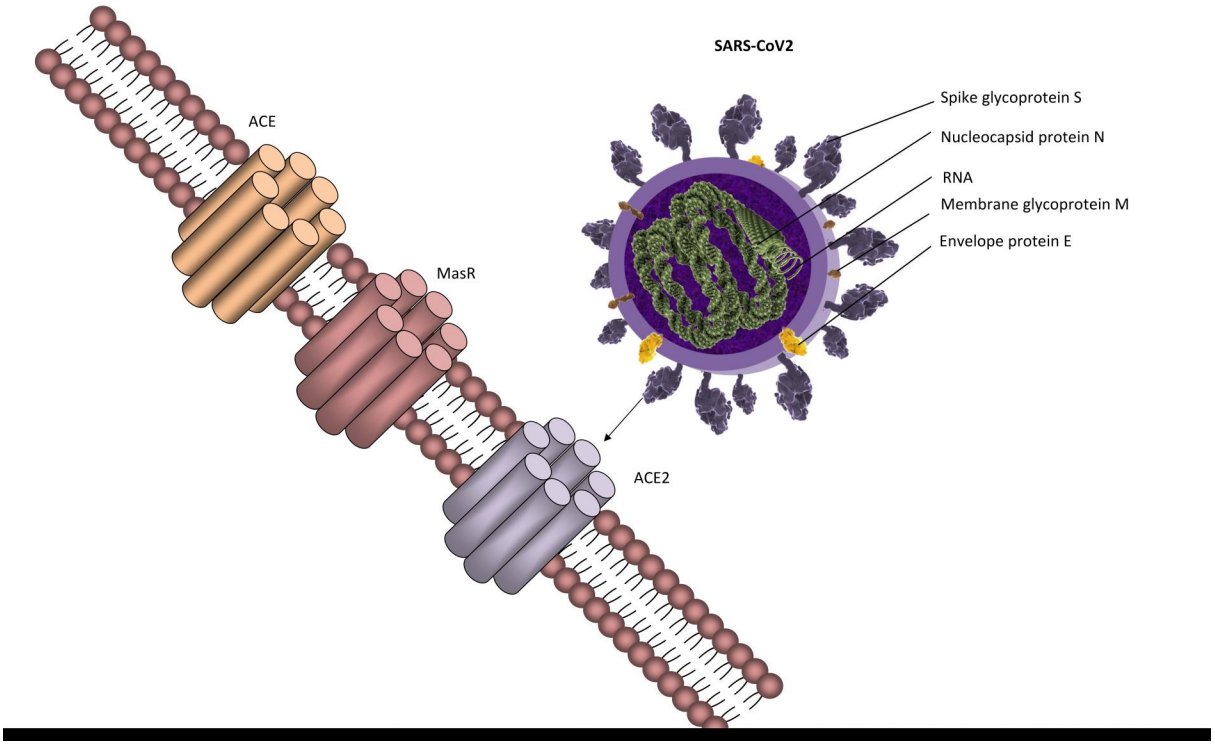

Figure 2

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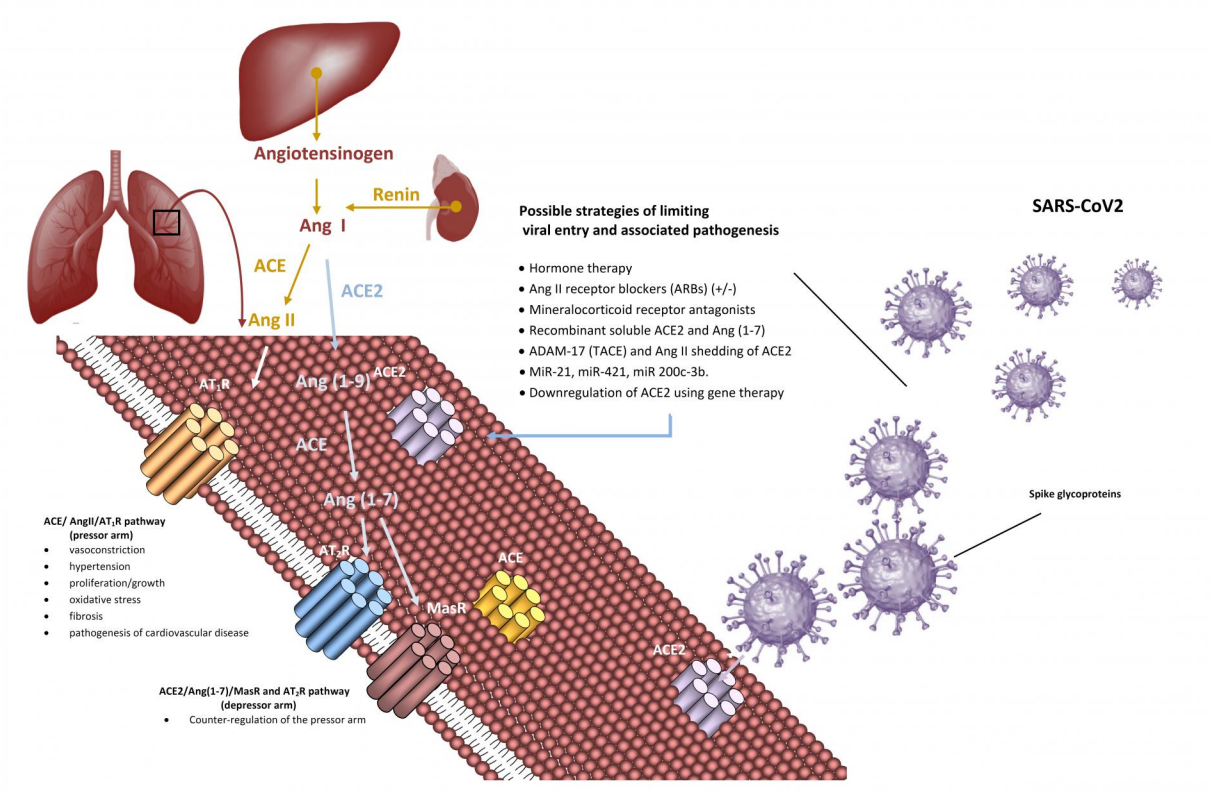

Figure 3

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