

REVIEW

Aging- and gender-related modulation of RAAS: potential implications in COVID-19 disease

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Abstract

Coronavirus disease 2019 (COVID-19) is a new infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 is frequently characterized by a marked inflammatory response with severe pneumonia and respiratory failure associated with multiorgan involvement. Some risk factors predispose patients to develop a more severe infection and to an increased mortality; among them, advanced age and male gender have been identified as major and independent risk factors for COVID-19 poor outcome. The renin-angiotensin-aldosterone system (RAAS) is strictly involved in COVID-19 because angiotensin converting enzyme 2 (ACE2) is the host receptor for SARS-CoV-2 and also converts pro-inflammatory angiotensin (Ang) II into anti-inflammatory Ang(1-7). In this review, we have addressed the effect of aging and gender on RAAS with emphasis on ACE2, pro-inflammatory Ang II/Ang II receptor 1 axis and anti-inflammatory Ang(1-7)/Mas receptor axis.

Key Words

- ▶ COVID-19
- ▶ aging
- ▶ gender
- ▶ cardiovascular disease
- ▶ ACE2

Introduction

Coronavirus disease 2019 (COVID-19) has been recently recognized as a new infectious disease caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). COVID-19 emerged in China in 2019 and rapidly spread throughout the globe; on March 11, 2020, the World Health Organization declared the COVID-19 outbreak a pandemic. Most COVID-19 patients are asymptomatic or have minimal symptoms, however, some patients develop severe pneumonia, acute respiratory distress syndrome (ARDS) and multiorgan damage frequently leading to the patient's demise (1, 2, 3, 4).

SARS-CoV-2 belongs to the Coronaviridae family that also includes severe acute respiratory syndrome coronavirus (SARS-CoV), first reported in China in 2002

(5), and middle east respiratory syndrome coronavirus (MERS-CoV), first described in Saudi Arabia in 2012 (6); both SARS-CoV and MERS-CoV can cause severe pneumonia.

The recent emergence of COVID-19 as a global health threat has prompted increased interest in the risk factors involved in COVID-19 poor outcomes. The first retrospective multicenter cohort study of COVID-19 in Wuhan, identified older age as a significant risk factor for in-hospital mortality (2). A CDC report from July 2020 describes the demographic characteristic of 52,166 deceased COVID-19 patients. Among these, 55.4% are males and 79.6% are older than 65 years (7). Further, the fatality rate is age-related: the highest fatality rate is

seen in persons older than 85 years (30.9%), followed by persons aged 75–84 years (27.1%) and persons aged 65–74 years (21.6%) (7).

Epidemiologic studies also identified male gender as a risk factor for poor outcome of COVID-19 disease. On August 7, 2020, an independent and internationally recognized initiative to advance action for gender equality (Global health 50/50), collected official data regarding sex prevalence in confirmed cases of COVID-19 in 66 countries. 32 countries reported a slightly higher number of cases in men, 9 had a similar incidence in both men and women, and 25 with a slightly higher number of cases in women (pages accessed on September 6th, 2020: <https://globalhealth5050.org/covid19/sex-disaggregated-data-tracker/>; <https://globalhealth5050.org/covid19/age-and-sex-data/#1589893682295-1abaac66-2013>). Although the gender-related infection rate can be different in each country, overall, male patients are highly prone to develop a more severe illness with a higher fatality rate, especially subjects older than 60 years (2, 4, 8, 9). Recently, a comparative analysis of COVID-19 case fatality rates (CFR) by sex and age in 38 countries showed that the average male CFR is 1.7 times higher than female (10). Moreover, it confirmed that both sexes exhibit an increased risk of death with advancing age, with males having a significantly higher fatality outcome than females (10).

The mechanisms responsible for the effects of aging and male gender, as independent risk factors (11, 12, 13), on morbidity and mortality of COVID-19 disease are still unknown. However, the renin-angiotensin-aldosterone system (RAAS) is strictly involved in COVID-19 and may play a role in aging- and gender-related differences in the severity of the infection.

Angiotensin converting enzyme 2 (ACE2) the virus gateway into the cells

In 2003, angiotensin converting enzyme 2 (ACE2), a pivotal anti-inflammatory component of the RAAS, was identified as the virus receptor on epithelial cells (14). ACE2 is the host receptor for SARS-CoV and plays an essential role in the development of the severe acute respiratory syndrome caused by SARS-CoV, both by facilitating virus internalization in lung epithelial cells and by acting as a protective mechanism against lethal lung failure (14, 15, 16). Similar to SARS-CoV, SARS-CoV-2 requires the binding of the virus with its spike (S) glycoprotein to the membrane-bound form of ACE2 and

the subsequent internalization of the complex by the host cell (Fig. 1). The host serine protease transmembrane serine protease 2 (TMPRSS2) cleaves the spike protein into S1 and S2 fragments, thus enabling fusion of viral and cellular membranes and facilitating viral entry (17, 18). The virus entry into the cell along with the membrane receptor ACE2, which is functionally removed from the external site of the membrane (15, 16). Interestingly, ACE2 affinity is ten-fold higher for SARS-CoV-2 than for SARS-CoV (19, 20).

Beyond the gateway ACE2 as a tool for cell injury

Virus-binding to ACE2 was also found to be implicated in cell injury and the development of ARDS. ACE2 is a key enzymatic component of RAAS, it converts pro-inflammatory angiotensin (Ang) II into anti-inflammatory Ang(1–7) (21) (Fig. 2). When bound by the virus, ACE2 is internalized by the cell leading to downregulation of ACE2 on the cell surface, in turn, increasing pro-inflammatory Ang II and decreasing anti-inflammatory Ang(1–7) (14, 15, 16, 22).

Since ACE2 is a key player in SARS-CoV-2 infection, in this review we examine the effect of aging and gender on RAAS with emphasis on ACE2 and on the balance of pro-inflammatory Ang II and anti-inflammatory Ang(1–7).

RAAS signaling cascade

Activation of RAAS cascade begins with prorenin and renin secretion that occur mostly in the kidney (23) (Fig. 2). Renin is secreted as a precursor protein named prorenin.

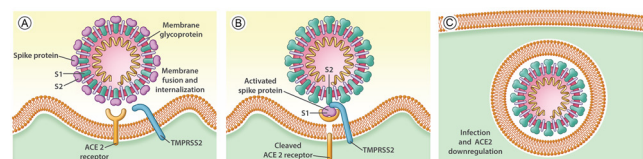


Figure 1

Mechanism of SARS-CoV-2 infection. (A) The virus infects cells through its spike protein by binding to ACE2 receptor with the complementary action of TMPRSS2. (B) The S protein is composed of two subunits: S1 is the receptor-binding subunit, S2 the fusion subunit. S1 binds ACE-2 receptor, that is cleaved by TMPRSS2, thus activating the spike protein, and promoting the viral entry. (C) SARS-CoV-2 RNA is then released into the cytoplasm and viral replication is efficiently processed. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Ang, angiotensin; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; TMPRSS2, Transmembrane protease, serine 2.

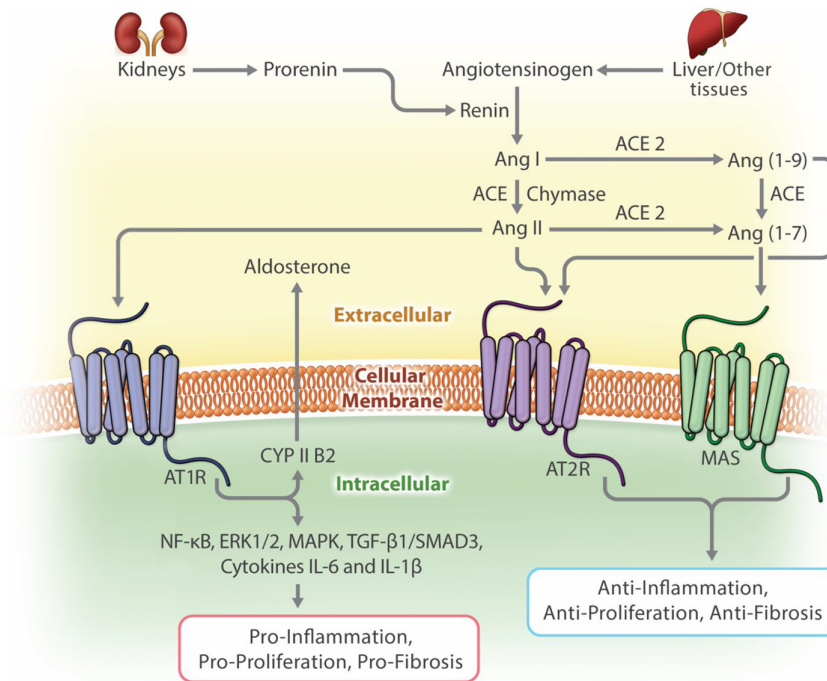


Figure 2

Renin-angiotensin-aldosterone system. Renin secretion is the first step in the activation of the RAAS pathway. Renin cleaves angiotensinogen to form Ang I, which is then transformed into Ang II by ACE and chymase enzyme. Specific receptors, AT1R and AT2R, can then bind Ang II. AT1R stimulates blood pressure, cardiac remodeling, and atherosclerosis, whereas AT2R has opposite effects. ACE2 cleaves Ang I and Ang II to form Ang(1-9) to Ang(1-7), respectively. Ang(1-7) induces vasodilation, anti-inflammatory, antifibrotic, and anti-remodeling effects, through MasR and AT2R. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Ang, angiotensin; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; MasR, Mas receptor.

Both prorenin and renin can bind to their specific receptor called prorenin-renin receptor (PRR) and activate intracellular pathways, triggering pro-fibrotic effects (24, 25). In the classical pathway, angiotensinogen produced primarily by the liver is converted into the decapeptide angiotensin I (Ang I) by renin (23). Subsequently, Ang I is cleaved by angiotensin converting enzyme (ACE) to produce the major effector of this system, the octapeptide angiotensin II (Ang II), which binds two G-protein coupled receptors, angiotensin II type 1 receptor (AT1R) and type 2 receptor (AT2R) (26). In the heart, the majority of Ang I is converted by the chymase enzyme (27).

AT1R stimulation increases the influx of extracellular Ca^{2+} inducing vascular smooth muscle cells (VSMCs) contraction and vasoconstriction (28), and enhances myocardial contractility (29). In the adrenal gland, Ang II induces aldosterone secretion, which increases tubular sodium reabsorption in the kidney and the effective circulating plasma volume by binding the mineralocorticoid receptor (30). Thus, under normal physiologic conditions, Ang II raises arterial blood pressure via a direct vasoconstrictive action and by inducing sodium and water retention via increased aldosterone production (28, 31).

RAAS signal transduction is mediated and balanced by Ang II-induced activation of AT2R, which antagonizes the Ang II/AT1R axis by (i) activating phosphotyrosine phosphatases (PTP) and subsequent inactivation of their growth-promoting effectors (32) and (ii) by inducing nitric

oxide (NO) production and vasodilation (33). Further, the classical ACE/Ang II/AT1R axis is counter regulated by the antagonizing ACE2/Angiotensin(1-7)/Mas receptor (MasR) axis that opposes the classical actions of RAAS mediated via the AT1R (34).

Ang II/AT1R/AT2R in inflammation and fibrosis

Enhanced Ang II production induced by chronic activation of RAAS plays a major role in cardiovascular remodeling. In VSMCs, Ang II induces a phenotypic switch from a contractile to a proliferative and synthetic phenotype, that leads to vascular injury due to VSMCs hypertrophy/proliferation and to the production of pro-inflammatory and pro-fibrotic mediators (35).

Ang II also induces endothelial cell apoptosis and dysfunction via AT1 receptor (36). Different signaling pathways and mechanisms are responsible for the effect of RAAS on cardiac and vascular remodeling.

Ang II binding to AT1R activates phospholipase C (PLC), phospholipase A (PLA), and phospholipase D (PLD). $PLC\beta$ induces the formation of diacylglycerol (DAG) and inositol 3-phosphate (IP3), that activate sodium-proton (Na^+/H^+) exchange leading to Ca^{2+} release from the endoplasmic reticulum and intracellular alkalization, respectively. In addition, the increase in cytosolic Ca^{2+} induces pathological cardiac and vascular hypertrophy

through calcium/calmodulin-dependent protein kinase II (CAMK2) and calcineurin-nuclear factor of activated T cells (NFAT) cascades (37, 38).

AT1R signaling activates different mitogen-activated protein (MAP) kinases, including extracellular signal-regulated kinases (ERK1/2 and ERK5), c-Jun N-terminal kinases (JNK), and p38-MAP kinase. ERK1/2 and ERK5 are key growth signaling molecules both in VSMCs and fibroblasts, whereas JNK and p38-MAP kinase modulate cell survival, apoptosis, differentiation, and inflammation (39, 40, 41). Ang II induces phosphorylation of a variety of tyrosine kinases including c-Src, janus family kinase (JAK) and phosphatidylinositol 3-kinase (PI3K) (42, 43). Ang II also transactivates tyrosine kinase receptors including platelet-derived growth factor receptor (PDGFR), insulin-like 1 growth factor receptor (IGF-1R), and EGF receptor (EGFR) via activation of tyrosine kinase, production of reactive oxygen species (ROS), and activation of metalloprotease (MMPs) (44, 45). Among MMPs, A Disintegrin and Metalloproteinase 17 (ADAM17) contributes to RAAS signaling both by transactivating EGFR (46) and shedding/inactivating ACE2 (47, 48). In VSMCs, in endothelial cells, and in cardiac cells, Ang II-activated EGFR induces fibrosis by mediating fibronectin synthesis, MMPs production, breakdown of collagen IV and expression of plasminogen activator inhibitor-1 (49). In addition, AT1R directly increases transforming growth factor beta1 (TGFB1) resulting in nuclear translocation of SMAD proteins 2, 3 and 4, and expression of fibrotic marker proteins, that is, collagen, fibronectin, and connective tissue growth factor (CTGF). The latter is a critical profibrotic mediator implicated in fibroblast proliferation, cellular adhesion, and extracellular matrix protein (ECM) synthesis (50, 51, 52, 53).

Ang II directly increases oxidative stress by activating membrane dihydronicotinamide-adenine dinucleotide phosphate and hydrate oxidase (NADPH and NADH, respectively), mainly via AT1R, to produce ROS, superoxide and hydrogen peroxide (H₂O₂) (31). ROS mediate most of the pathophysiological effects of Ang II through the activations of growth-promoting and pro-inflammatory signaling molecules, including c-Src, EGFR, p38MAPK, and transcription factors including nuclear factor κB (NF-κB).

Ang II exerts its pro-inflammatory actions also by activating leukocytes, lymphocytes, and macrophages that in turn produce cytokines and chemokines. Through AT1R, Ang II mediates activation of CD34⁺ T helper cells, important for the adaptative immune response. Specifically, all three T helper cell subclasses, that is, Th1, Th2, and Th17, secrete Interferon (IFN)-γ, interleukin (IL)-4, IL-17, and, via these

mediators induce hypertension (54) and cardiac fibrosis (55). Ang II stimulation of mouse macrophage cell lines results in Nuclear factor κB (NF-κB) and activator protein 1 (AP-1) activation, ROS production, and secretion of inflammatory cytokines including tumor necrosis factor-α (TNF-A), IL-1B, IL-6, and the anti-inflammatory IL-10 (56). AT1R-dependent IL-6 production has been found in a rat lung macrophage cell line and mediates kidney injury (57). Moreover, Ang II infusion in the mouse increases plasma IFNG, TNF-A, IL-1, IL-6 (58). Interestingly, Ang II-dependent release of IL-6 induces microvascular injury leading to thrombo-inflammatory responses (59).

It is noteworthy that pro-inflammatory and pro-fibrotic mediators induced by Ang II are involved in pathophysiological lung remodeling (60). Transgenic mice with chronic activation of RAAS spontaneously develop progressive lung fibrosis, independently of blood pressure, with a marked decline in pulmonary function (61). Ang II-mediated activation of AT1R and AT2R in lung parenchyma, enhance lung remodeling and fibrosis (62). In lung fibroblasts, miR-21 induced by Ang II activates nucleotide-binding domain and leucine-rich repeat containing PYD-3 (NLRP3) inflammasome, a multiprotein complex implicated in the pathogenesis of inflammation and chronic inflammatory (63).

Counterregulatory RAAS pathway ACE2/Ang(1-7)/MasR

In the last 10 years, new counterregulatory RAAS effectors have been identified, including ACE2, angiotensin(1-9), angiotensin(1-7) and MasR.

ACE2 is a transmembrane carboxypeptidase expressed in a variety of tissues including small intestine, testis, adipose tissue, kidney, heart, ovary, and lung (64). ACE2 exerts a protective role by converting pro-inflammatory Ang I and Ang II into anti-inflammatory Ang(1-9) and Ang(1-7), respectively. Moreover, Ang(1-9) can be converted by ACE into Ang(1-7) that activates intracellular signaling by binding Mas receptor (21). It is noteworthy that ACE2 acts at different steps in RAAS signaling cascade: (i) it mediates the degradation of Ang I, limiting its availability for ACE-mediated generation of Ang II, (ii) it induces the degradation of Ang II and (iii) it promotes the formation of both Ang(1-9) and Ang(1-7) that exhibit anti-fibrosis and anti-inflammatory actions. Additionally, ACE2, via its C-terminal domain collectrin-homologous, mediates amino acid absorption in small intestine, both by binding neutral amino acid

transporter and increasing its expression (65). Finally, as discussed above, ACE2 is the host receptor for virus spike (S) proteins of the coronaviruses SARS-CoV (14) and SARS-CoV-2 and is a key mediator of COVID-19 infection (17); further, ADAM17 is responsible for the shedding of membrane-bound ACE2 (47, 48). The biological and clinical significance of ACE2 ectodomain shedding is yet to be fully characterized and it is still unknown whether increased ACE2 levels in plasma are associated with higher or lower cell membrane-associated ACE2 in tissues and whether circulating ACE2 acts as a decoy, binding SARS-CoV-2 and effectively inhibiting cell infection.

Ang(1–9) protective effects are both direct and indirect. In 2011 it was first shown that Ang(1–9) antagonizes Ang II by binding AT2R, a receptor that opposes the sequelae of AT1R signaling (66). Further, Ang(1–9) is converted by ACE into Ang(1–7), the key agonist of the ACE2/Ang(1–7)/MasR pathway.

Ang(1–7) antagonizes many effects of the Ang II-AT1R axis. It binds MasR, a G protein-coupled receptor (34), and induces vasodilation, modulates endothelial function, and mediates anti-inflammatory and anti-fibrotic actions. In vessels and in the heart, Ang(1–7) counteracts Ang II effects by inactivating NOS2/ROS pathway, activating PIK3/Akt/eNOS pathway and therefore enhancing NO production (67, 68). Ang(1–7) plays an inhibitory effect on TGFB/SMAD pathway in liver, in skeletal muscle and in renal epithelial cells (69, 70, 71). In the mouse, exogenous administration of Ang(1–7) results in anti-fibrotic cardioprotective effects mediated by decreased TGFB and inflammatory cytokines IL-1B, IL-6 and TNF- α , and increased collagen degradation by MMP-2 and MMP-9 (72). Indeed, Ang(1–7) decreases inflammatory cytokines, including TNF- α and IL-6, and increases ACE2, bradykinin type 2 receptor (BK2R), and IL-10 (73).

ACE2/Ang(1–7)/MasR axis counteracts the Ang II-induced pro-fibrotic effects in the lung both by decreasing the pro-fibrotic miR-21/NLRP3 pathway responsible for lung fibrosis (63) and inhibiting the proapoptotic effect of Ang II in alveolar epithelial cells (74). Moreover, Ang(1–7) directly inhibits TGFB1-Smad signaling pathway in alveolar epithelial cells, a protective effect observed in idiopathic pulmonary fibrosis (75).

Aging- and gender-related modulation of RAAS

As discussed previously, recent epidemiologic data show that the severity and mortality of COVID-19 infection

increase as a function of age, with a steep increase in patients older than 60 years; further, COVID-19 infection is more likely to be severe and lethal in men than women (2, 4, 7, 8, 9). These age and gender effects occur independently of comorbidities making aging and male gender independent risk factors for severe COVID-19 infection (10, 11, 12, 13). In light of the key role of RAAS in the response to COVID-19 infection, here we will review the effect of aging and gender on key elements of the RAAS cascade with emphasis on ACE2 and on the balance between the Ang II/AT1R and Ang(1–7)/MasR axes.

Aging-related modulation of RAAS

There is a wealth of data showing that chronic RAAS activation plays a major role in age-associated arterial pro-inflammation and arterial remodeling (Fig. 3) (76, 77). The increase in Angiotensin II enhances AT1R activation, increases aldosterone production and, via this mediator, mineralcorticoid receptor activation, and it also increases endothelin-1, an agonist with pro-inflammatory and vasoconstrictor actions. Studies in rodents have shown that age-related cardiac and carotid remodeling depend on overexpression of pro-inflammatory effectors of the RAAS pathway, including NADH oxidase (Nox2), MMPs, MCP-1, and TGFB (78, 79, 80, 81). This pro-inflammatory profile was confirmed in the aortic wall of both humans and nonhuman primates (76, 82).

The ultimate result of increased RAAS activation and Ang II production is diffuse intima-media thickening, enhanced central arterial stiffening and endothelial dysfunction; these are the key features of arterial aging and the conditions underlying the development of atherosclerosis and heart failure. Several steps in the RAAS signaling exhibit age-related modulation and they are briefly summarized subsequently (Table 1).

Studies in aged rats show a decrease both in renal renin formation and secretion, which results in decreased plasma renin concentration (83); in contrast, PRR increase in the thoracic aorta of old mice (84). In agreement with these animal studies, normotensive humans older than 60 years have lower levels of plasma renin than younger adults (85, 86).

The local expression of angiotensinogen, the precursor of Ang II, increases in aortic VSMCs of old rats (87). Ang II plasma levels are higher in old than in young rats (76, 88) and also exhibit an age-related increase in murine (84) and human aorta (82).

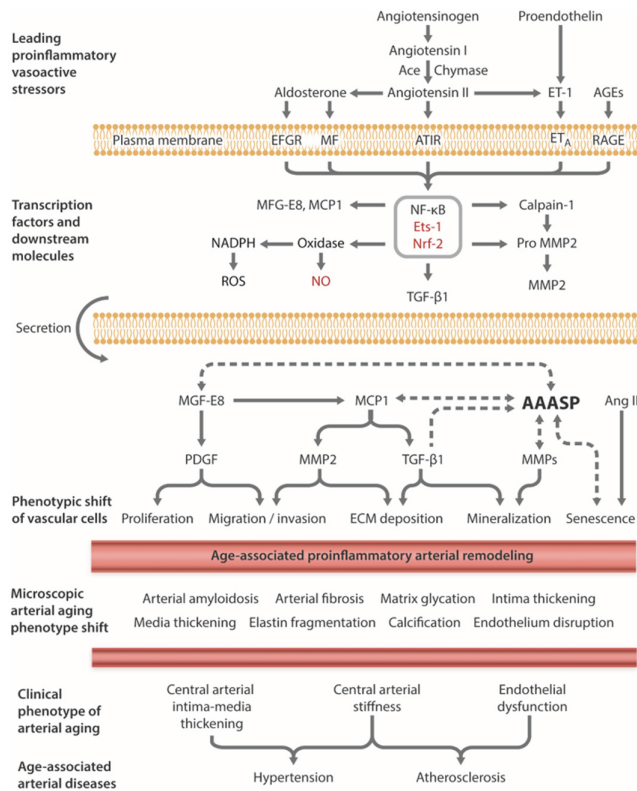


Figure 3

Age-associated arterial remodeling. Ang II promotes arterial remodeling via AT1R, MR, and ET-1/ETA signaling. AGEs recruit inflammatory molecules by interaction with receptor for AGEs (RAGE). NF-κB and Ets-1 are activated within the aging arterial wall, whereas protective factors such as Nrf-2 are reduced. Downstream signaling molecules include MFG-E8, MMPs, calpain-1, MCP-1, and TGF-β1. ROS are produced whereas NO bioavailability decreases with advancing age. Old VSMCs produce the AAASP, responsible for the underlying pro-inflammatory state, and exhibit enhanced proliferation, migration, senescence, and extracellular matrix deposition within the aged arterial wall. Disruption of the endothelium, intima-media thickening, arterial amyloidosis, fibrosis, calcification, elastin fracture, and matrix glycooxidative modifications are consequences of the enhanced signaling via these receptor signaling cascades. Ultimately, arteries develop endothelial dysfunction, increased stiffness, and atherosclerosis. Modified from (77). AAASP, age-associated arterial secretory phenotype; ACE, angiotensin converting enzyme; Ang II, angiotensin II; AT1R, angiotensin II type 1 receptor; AGE, advanced glycation end-products; ECM, extracellular matrix; EFGR epidermal growth factor receptor; ET-1, endothelin-1; ETA, endothelin-1 receptor A; Ets-1, v-ets erythroblastosis virus E26 oncogene homolog 1; MCP-1, monocyte chemoattractant protein-1; MFG-E8, milk fat globule epidermal growth factor-8; MMP, matrix metalloprotease; MR, aldosterone/mineralocorticoid receptor; NADPH dihydronicotinamide-adenine dinucleotide phosphate; NF-κB, nuclear factor κ light-chain-enhancer of activated B cells; Nrf-2, NF-E2-related factor 2; NO, nitric oxide; PDGF, platelet-derived growth factor; RAGE, receptor for AGE; ROS, reactive oxygen species; TGF-β1, transforming growth factor β1; VSMC, vascular smooth muscle cell.

Studies on 3- and 24-month-old rats show that cardiac Ang II levels increase with aging via an ACE-independent pathway mediated by enhanced expression of chymase enzyme (89). Chronic elevation of Ang II induces ROS

Table 1 Age-related modulation of RAAS signaling cascade.

	Age	Reference
Renin	Down	(83, 85, 86)
PPR	Up	(84)
Angiotensinogen	Up	(87)
Ang II	Up	(76, 82, 84, 88)
ACE	Up	(84, 91, 93)
ACE2	Down	(84, 91, 93, 94, 95)
AT1R	Up	(84, 88, 93)
AT2R	Down	(84, 88)
Ang(1-7)	Down	(84, 91, 93, 94, 95)
MasR	Down	(89)

ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Ang, angiotensin II; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; MasR, Mas receptor; PRR, prorenin renin receptor.

production (31), which triggers the release of chymase from mast cells (90) and activates a positive loop that further enhances Ang II production. Higher Ang II level directly contributes to imbalance RAAS by modulating ACE and ACE2 expression. In fact, *in vivo* infusion of Ang II for 4 weeks increases renal ACE/ACE2 ratio, both by increasing ACE expression and decreasing ACE2 expression (91). High levels of Ang II induce PRR expression resulting in increased renin signaling, thereby amplifying *de novo* production of Ang II, in a positive feed loop. Moreover, renin and prorenin binding to PRR triggers the activation of MAP kinases ERK1/2 thus leading to downstream activation of pro-fibrotic genes including TGF-β and fibronectin (25, 92).

Thus, aging-related decrease in prorenin and renin would be expected to decrease Ang II formation; however, the increase in chymase induces the conversion of angiotensinogen into Ang II and Ang II has several effects that include enhanced PRR expression. Under these conditions, in spite of lower renin levels, because of the increase in PRR induced by Ang II, there is increased activation of profibrotic genes and Ang II production.

In the thoracic aorta of 24-month-old compared to 2-month-old mice, ACE, and AT1R increase significantly, whereas expressions of AT2R, ACE2 and Mas receptor decrease (84). These findings on the age-dependence of ACE, AT1R, and ACE2 expression were confirmed in murine kidney when comparing 3- and 24-month-old mice (93). Further, it has been reported that AT1R expression increases and AT2R expression decreases in the old rat heart (88). *Postmortem* analysis of aortic samples of younger (20 ± 3 years) and older (65 ± 6 years) humans who did not succumb to cardiovascular diseases, has shown that older subjects have higher ACE and AT1R levels (82). ACE2 expression was also evaluated in the rat lung at 3, 12, and 24 months and it was found that ACE2

expression in the lung exhibits an age-related decrease (94). A significant reduction in cardiac ACE2 expression leads to lower Ang(1–7) formation. Further, aged rats exhibit lower expression of Mas receptor when compared to younger animals (89).

Recently, the analysis of a large Genotype-Tissue Expression (GTEx) Portal data showed that ACE2 expression decreases during aging in many tissues, including colon, salivary gland, brain and blood vessels, but no change was found in lung (95).

Interestingly, ACE2 is shed into the systemic circulation and its level was evaluated in the plasma of human participants of the InCHIANTI study; a total of 967 participants, 20–90 years old, were analyzed. ACE2 plasma levels exhibited a significant positive association with aging below age 55 and a negative association after age 55 (M AlGhatrif, T Tanaka, AZ Moore, S Bandinelli, EG Lakatta, L Ferrucci, unpublished observations). Further, a study in 23 healthy human subjects has shown an inverse association between ACE2/ACE ratio levels and aging in hematopoietic stem progenitor cells (96).

In summary, most studies show aging-related modulation of RAAS resulting in enhanced pro-inflammatory ACE/Ang II/ AT1R axis and diminished antagonizing anti-inflammatory response mediated by both AT2R and ACE2/Ang(1–7)/MasR axis.

Gender modulation of RAAS

There is evidence of an effect of gender on RAAS, however, the mechanisms that underlie gender-related differences are complex. RAAS is modulated both by sex chromosomes, XX and XY, and sex hormones, estrogen and testosterone (Table 2), with sex hormones production exhibiting a marked variability from birth through

senescence, in relation to gender, the menstrual cycle and, eventually, pharmacologic treatment of a variety of conditions including breast and prostate cancer, post-menopausal symptoms, and osteoporosis.

Sex chromosomes

ACE2 and AT2R genes are the only two RAAS components that are situated on X chromosome (97, 98, 99). Since females have XX chromosomes and males have XY chromosomes is plausible that this difference, *per se*, independently of hormonal differences, may account for their higher expression in females than in males. Further, the Sry (sex-determining region on the Y chromosome) gene on the Y chromosome modulates the activity of RAAS gene promoters and plays a key role in testes development and testosterone production.

Sry gene is a member of the high mobility group (HMG)-box family of DNA-binding proteins. It is located in the nonrecombining region of the Y chromosome and appears to initiate male sex determination. Sry evolved from the gene Sox3, located on X chromosome, during the process of Y-chromosome formation and is highly conserved in mammals: human and mouse have a single locus, whereas rats all rat strains have 6 Sry loci. Further, Sry3, the seventh Sry locus, is an exclusive additional locus of Spontaneously Hypertensive Rat (SHR) males (100). Its transcripts are expressed in the kidney and brain, two organs involved in blood pressure modulation.

The most relevant insights on the role of sex chromosomes in RAAS pathway regulation comes from studies in two different rodent models the Four Core Genotype (FCG) mouse model and from the Y consomic rat model (for an extensive review, see (101)).

Table 2 Gender-related modulation of RAAS signaling cascade.

	Higher levels observed in	Female			Male	Reference
		PM	POM	ERT	Androgens	
Renin	Male	Down	Up	Down	Up	105, 108, 109
Agt	Female (PM)	Up	Down	Up	Down	105, 107
Ang II	-	-	Down	Up	Up	107, 111, 113, 114, 116, 118, 120
ACE	Male	Down	Up	Unknown	Up	105, 110, 111, 112
ACE2	Female (PM)	Up	Down	Up	Down	94, 100, 103, 118, 120
AT1R	Male	Down	Up	Down	No effects	110, 115, 118, 120
AT2R	Female	Up	Down	Up	Down	100, 104, 111, 116, 117, 118, 119
Ang(1–7)	Female (PM)	Up	Down	Up	Down	94, 100, 103, 112, 118, 120
MasR	Female (PM)	Up	Unknown	Unknown	Down	100, 104, 118, 119, 120

ACE, angiotensin-converting enzyme; Agt, angiotensinogen; Ang, angiotensin; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II type 2 receptor; MasR, Mas receptor; PM, premenopause; POM, post menopause; ERT, estrogen replacement therapy (after menopause).

The FCG mouse model was created to demonstrate that the chromosome complement, which defines gender, is not necessary for the determination of male sexual development (101). The insertion onto an autosome of transgenic Sry and the Sry knockout from the Y chromosome (producing the Y⁻ chromosome) induce four different genotypes: XX and XY^{Sry-}, female mice developing ovaries and producing estrogens; A^{Sry}XX and A^{Sry}XY⁻, male mice with Sry on autosomic chromosome, developing testes and producing testosterone. In physiological conditions, when compared animals with the same gonadal type, that is, in a similar hormonal environment (XX and XY^{Sry-}, and A^{Sry}XX and A^{Sry}XY⁻), no differences in blood pressure were detected (102). However, after gonadectomy in response to chronic angiotensin II infusion, the blood pressure is greater in A^{Sry}XY⁻ and XY^{Sry-} (XY genotype) than A^{Sry}XX and XX (XX genotype) (102). In this model, renal ACE2 activity is higher in males (A^{Sry}XY⁻ and A^{Sry}XX) (103). However, after gonadectomy enzyme activity increased in female mice (XX and XY^{Sry-}), although no differences are observed in the cardiac muscle or lung in mice or rats (103). Since ACE2 gene is located on the X chromosome, its expression and activity would be expected to correlate with the number of X chromosomes present. Therefore, the higher ACE2 renal activity in males suggests that it is hormonal-dependent rather than chromosome-dependent (103).

The Y consomic rat model was obtained from two different strains: the normotensive Wistar Kyoto (WKY) rat and the Spontaneous Hypertensive Rat (SHR). The male resulting progeny has a 'normotensive' Y chromosome or the 'hypertensive' one, derived from WKY and SHR, respectively. Since all the animals have a normal sexual development, it would be difficult account the testosterone's effects by its own nature, while chromosome effect is easy to evaluate. The Y consomic rat model demonstrated that the Y chromosome can regulate arterial pressure via the Sry gene family, specifically, Sry3. Intriguingly, Sry3 is not present in WKY normotensive rat but it is present in SHR. The *in vitro* overexpression of rat Sry3, as well as the co-transfection of human SRY expression constructs *in vitro* in Chinese Hamster Ovary cells (CHO), positively modulates angiotensinogen, renin, and ACE promoter activity, thus shifting RAAS toward vasoconstrictive responses, while the promoters of ACE2, AT2R, and MasR are downregulated (100, 104, 105). In WKY rats, Sry3 overexpression *in vivo* increases ACE expression by 40% and consequently Ang II concentration (101).

However, ACE2 and AT2R expressions are also sensitive to sex hormones as it will be discussed in the next section.

Sex hormones

Accumulating evidence suggests that different components of the RAAS pathway are modulated by sex hormones (106).

Angiotensinogen promoter contains an estrogen-responsive element: in fact, angiotensinogen circulating levels are higher in pre-menopausal women than in postmenopausal women and men, and estrogen replacement therapy can rescue angiotensinogen secretion in post-menopausal women (107). Conversely, plasma renin is higher in males than in females, an observation made in humans, rats, and mice (108, 109). Post-menopausal women without substitutive therapy exhibit higher renin levels than premenopausal women and postmenopausal women on hormonal replacement therapy (109). Further, plasma renin activity increases after ovariectomy and testosterone treatment in female rats and it decreases in male rats after castration (109).

ACE activity is downregulated by estrogens and upregulated by androgens. In post-pubertal children and in young healthy adults, ACE activity is lower in females than in males. After menopause, women have comparable ACE activity to age-matched men; estrogens replacement gives inconsistent results, with no change, increased or decreased ACE activity (110). However, a decrease in ACE activity in response to estrogen replacement therapy occurs in female rats and postmenopausal female monkeys (111, 112).

Plasma Ang II levels exhibit no gender difference prior to menopause. After menopause, plasma Ang II levels are upregulated in response to estrogen replacement therapy in women (113), in postmenopausal monkeys (111), and ovariectomized rats (114).

AT1R expression is downregulated by estrogens in several tissues, both in rats and beagles (110). A similar observation has been made in vascular smooth muscle cells isolated from rats (110). No effect of androgens on AT1R has been reported (115).

Regarding the effect of gonadal hormones on counterregulatory RAAS components there are limited data available. AT2R expression is upregulated by estrogens in adrenal and renal tissues, both in male and female rats, and downregulated by testosterone in mice and rat aorta

(116). AT2R gene expression and binding are upregulated in female mice with hormonal replacement therapy (117).

Several studies have examined the effect of gender and estrogens on ACE2. Renal ACE2 expression and activity, as well as MasR gene expression, are higher in female than in male rats, and Ang(1–7) is positively modulated by estrogens in female rats (118). In agreement with these observations Ang(1–7) plasma levels are low in males and in females after ovariectomy, whereas the administration of estrogens rescues ACE2 and, consequently, circulating Ang(1–7) (110). Moreover, Ang(1–7) plasma levels are higher in premenopausal than post-menopausal women and the *ex vivo* administration of estrogens to atrial myocardial tissue isolated from old men decreased ACE expression and increased expression levels of ACE2, AT2R, and MasR (119).

Interestingly, plasma ACE2 levels in the participants in the InCHIANTI study demonstrated no effect of gender on ACE2 plasma levels (M AlGhatrif, T Tanaka, AZ Moore, S Bandinelli, EG Lakatta, L Ferrucci, unpublished observations, work submitted for publication).

In summary, ACE/Ang II/AT1R axis appears to be more active in males and ACE2/Ang(1–7)/MasR, and AT2R pathways are more active in females (118, 120). Estrogens shift the balance of the RAAS toward ACE2/Ang(1–7)/MasR axis from the ACE/Ang II/AT1R axis; in contrast, testosterone has the opposite effect and enhances ACE/Ang II/AT1R effects.

Conclusions

In this review, we have examined the effect of aging and gender on RAAS and on the potential role of RAAS in determining the severity and poor outcome of SARS-CoV-2 infection.

ACE2 appears to play a pivotal role because it is the SARS-CoV-2 host receptor and, following virus binding, it is internalized; this event is a key initial step in viral infection and intracellular viral replication. Further, once ACE2 is internalized, it can no longer convert Ang II into Ang(1–7), likely enhancing the pro-inflammatory Ang II/AT1R axis at the expense of the anti-inflammatory Ang(1–7)/MasR axis. Indeed, it is now established that a severe pro-inflammatory cytokine storm underlies the severe multiorgan failure and poor outcome frequently observed in patients with COVID-19 disease.

The events that lead to SARS-CoV-2 infection and modulate the inflammatory response remain to be fully elucidated. It is plausible that high ACE2 expression in

young people and females plays a role in the high incidence of COVID-19 infection; however, high ACE2 would also be expected to shift the pro-inflammatory Ang II/AT1R axis and anti-inflammatory Ang(1–7)/MasR axis toward the anti-inflammatory response and ultimately will be more likely to cause a COVID-19 disease with no or minimal symptoms. In contrast, advanced age and male gender are independent risk factors for more severe infection and worse outcome in patients with COVID-19 disease. The mechanism/s for these highly relevant clinical findings have not been fully elucidated; however, advanced age and male gender are associated with diminished ACE2 expression and a balance between pro-inflammatory Ang II/AT1R axis and anti-inflammatory Ang(1–7)/MasR axis shifted toward an enhanced inflammatory response and more severe disease.

It is noteworthy that after menopause gender differences remain in the severity of COVID-19 disease and poor outcome. These differences persist and need to be reconciled with the results of animal and human studies suggesting a shift toward a pro-inflammatory condition of the balance between the pro-inflammatory Ang II/AT1R axis and anti-inflammatory Ang(1–7)/MasR axis after menopause. Unfortunately, no studies have adequately compared ACE/Ang II/AT1R axis and ACE2/Ang(1–7)/MasR axis in age-matched males and post-menopausal females neither in humans nor in other species; therefore, it is possible that some gender-related differences in RAAS may persist. Finally, it is possible that after menopause gender may no longer represent an independent risk factor for the development of severe COVID-19 infection and poor outcome and that a higher prevalence of comorbidities may be the key factor responsible for the increased risk of elderly males.

Pharmacological modulation of ACE2 expression theoretically contributes to the predisposition for the COVID-19 disease. This notion has driven an earlier controversy around whether the increased ACE2 expression associated with the use of angiotensin converting enzyme inhibitors (ACEi) and angiotensin-2 receptor blockers (ARB), increases COVID-19 risk (121, 122). However, so far, observational data has shown no difference in COVID-19 occurrence or severity among those on these medications and those who are not (123).

Some additional aspects of ACE2 biology are relevant to the present study. Recently, electrical vagal nerve stimulation (VNS) has been proposed for COVID-19 treatment. Acetylcholine (ACh), the principal neurotransmitter of the vagus nerve, inhibits the production of inflammatory cytokines and macrophage-TNF

release specifically via $\alpha 7$ nicotinic ACh receptors ($\alpha 7$ nAChR) mediating the cholinergic anti-inflammatory pathway (124, 125, 126). Moreover, it has been reported that exposure to nicotine causes epithelial cells to increase ACE2 levels, via $\alpha 7$ nAChR (127, 128). Further experimental investigations and ongoing clinical trials will determine the potential therapeutic use of VNS for SARS-CoV-2.

While still limited, more data has become available on the epidemiological changes of ACE2 levels and their correlation epidemiological pattern of COVID-19. Earlier epidemiological data, which were predominantly from sick and hospitalized patient, have shown a greater COVID-19 impact on older individuals (page accessed on December 3, 2020: https://www.cdc.gov/bloodpressure/facts.htm?CDC_AA_refVal=https%3A%2F%2Fwww.cdc.gov%2Fdhdsp%2Fdata_statistics%2Ffact_sheets%2Ffs_bloodpressure.htm). However, with increase community testing of mild-to-moderate cases, it became apparent that younger adults have the greatest incidence, followed by older adults, while children have the lowest rates of occurrence (129).

Finally, although the relation between circulating and tissues-associated ACE2 is still unclear, it is noteworthy that the InCHIANTI study showed that ACE2 plasma levels exhibited a significant positive association with aging before the age 55 and a negative association after age 55 (M AlGhatrif, T Tanaka, AZ Moore, S Bandinelli, EG Lakatta, L Ferrucci, unpublished observations). In conclusion, our present knowledge of RAAS and COVID-19 infection leads us to formulate plausible experimental hypotheses but much remains to be done in order to establish the role of RAAS in COVID-19 infection, characterize the mechanisms leading to more severe infections and worse outcome in elderly patients and in males, and establish the role, if any, of drugs such as ACE inhibitors and angiotensin receptor blockers that modulate RAAS in ameliorating or worsening the severity of COVID-19 infection.

Declaration of interest

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Author contribution statement

L M, M C F, and M C C conceived the idea for the article. L M, M C F, and M C C performed the literature search and drafted the review. L M, M C F, M A, E G L, and M C C critically revised the work.

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