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SERIES XXXIV

The role of Angiotensin II and RAS inhibitors in SARS-CoV-2 infection: a mechanistic *in vitro* study on human bronchial epithelial cells

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1. ABSTRACT

Angiotensin converting enzyme 2 (ACE2) is the cellular receptor of SARS-CoV-2 that caused the COVID-19 pandemic. ACE inhibitors (ACEis) and angiotensin type 1 receptor (AT1R) antagonists (ARBs) are commonly used among the millions of patients with cardiovascular and renal diseases who present an activated renin-angiotensin system (RAS). Recently, these drugs were contended to raise ACE2 expression, thus facilitating the SARS-CoV-2 infection and representing a potential risk factor in patients with cardiovascular comorbidities, but evidences supporting this hypothesis lack. We investigated the effect of Angiotensin (Ang) II, ACEis and ARBs in the modulation of ACE2 expression and SARS-CoV-2 infection in human epithelial bronchial cells. We found that Ang II markedly increased the mRNA ($p<0.001$) and protein ($p<0.05$) levels of ACE2, while the ACEi ramipril and the ARB irbesartan did not. The effect of Ang II on ACE2 mRNA and protein was abolished by irbesartan ($p<0.001$) and unaffected by ramipril. In line with this, both experiments with wild type and pseudotyped SARS-CoV-2 showed that viral titre was enhanced by Ang II treatment ($p<0.001$) and blunted by irbesartan ($p<0.01$). Moreover, blockade of the ACE2-mediated Ang II conversion into Ang 1-7 with MLN-4760 did not affect the Ang II-induced ACE2 increase. Beside its effect on ACE2 mRNA and protein expression, Ang II did not modulate its shedding process. Thus, this study highlights that an activated RAS increases the expression of ACE2 and so the susceptibility to SARS-CoV-2 infection and the spread of COVID-19 in the human bronchial epithelial cells in patients with cardiovascular diseases. More importantly, it gives a mechanistic support to the beneficial effect of RAS blockers in patients infected by SARS-CoV-2.

2. BACKGROUND

2.1. The Renin-Angiotensin-Aldosterone System (RAAS)

2.1.1. Role and pathology: focus on renin-ACE-AngII-AT1R axis

The main pathway involved in the regulation of physiology and homeostasis in the cardiovascular system is the Renin- Angiotensin-Aldosterone system (RAAS). RAAS is responsible for the maintenance of vascular tonicity by regulating extracellular fluid volume and arterial pressure (Patel et al., 2017). Beside a systemic RAAS playing an endocrine role in the human body, the concept of a local RAAS was also demonstrated, having autocrine, paracrine and intracrine functions. Indeed, even locally, RAAS component can be produced and be active, limited to tissues or organs like kidney, heart, vessels, adipose tissue, lymphatic tissue, central nervous system, adrenal and pituitary gland, reproductive tissues and gastrointestinal tract, hematopoietic tissues (De Mello, 2017; Montezano et al., 2014; Paul et al., 2006). Nowadays, it is currently accepted the existence of two different branches of RAAS: the harmful one, ACE-AngII-AT1R-aldosterone, and the beneficial one ACE2-Ang 1-7-MAS1 axis, which is known to counterbalance the former and it will be better described in the next chapter. Unbalanced RAAS give rise to cardiovascular and renal diseases like hypertension, but also congestive heart failure, obesity, hepatic complications, diabetes, neuronal disease and miscarriage, endothelialitis, fibrosis, atherosclerosis (Patel et al., 2017). A schematic representation of RAS is shown in Figure 1 (by Souza-Santoz et al (Santos et al., 2019)).

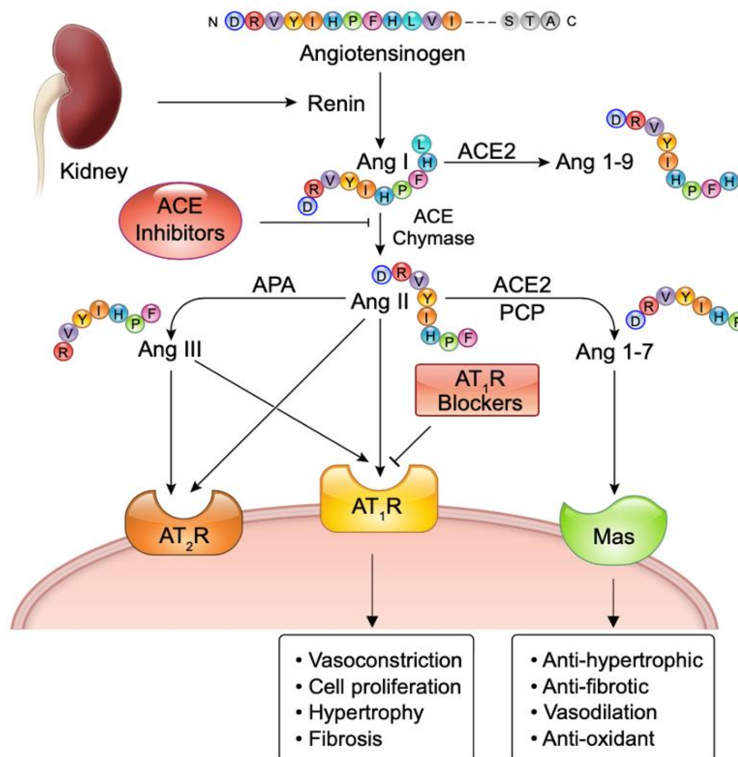

Figure 1. The Renin-Angiotensin-System enzymatic cascade. Ang I is cleaved by angiotensin-converting enzyme (ACE) to Ang II, which is metabolized by ACE2 to Ang 1-7. Ang II binds to Ang II type 1 (AT1Rs) and type 2 receptors (AT2Rs) inducing pro-hypertensive effects. Ang 1-7, instead, binds to Mas receptors (MasRs) and opposes Ang II/AT1R actions. Picture modified by (Santos et al., 2019).

Renin is a proteolytic enzyme synthesized in its inactive form (pro-renin) by the juxtaglomerular cells of the kidney. In response to low levels of intratubular sodium, hypotension in the afferent arterioles of renal glomerulus, and sympathetic activation it is then released into bloodstream and here is converted into its active form (renin), responsible for the conversion of Angiotensinogen into the decapeptide Angiotensin (Ang) I (Muñoz-Durango et al., 2016; Patel et al., 2017). Angiotensinogen is an inactive decapeptide mainly produced by hepatocytes, but production within other cell types may contribute to local or even circulating concentrations of Ang II in pathophysiological conditions (Putnam et al., 2012). Plasma levels of Angiotensinogen might increase in response to corticosteroid, estrogen, thyroid hormone stimulation, as well as to Angiotensin II (Ang II) (Kaschina et al., 2018). Angiotensin Converting Enzyme (ACE) hydrolyzes angiotensinogen into its active form,

Ang II. Beside RAAS, ACE plays an important role also in the kallikrein-kinin system (KKS) as it is the major intravascular peptidase of BK, producing des-Arg9-BK and several inactive intermediates (Chung et al., 2020). BK is a potent vasodilator that contributes to hypotension by increasing vascular permeability, plasma extravasation and bronchoconstriction (Sharma and Al-Banoon, 2012).

Ang II is the main player of RAAS. It is active in different tissues and cell types as endothelial cell, vascular smooth muscle cells (VSMCs), epithelial cells, it was found at high levels in hypertensive patients (Catt et al., 1969) and it acts mainly via two different receptors: Angiotensin Type 1 Receptor (AT1R) and Angiotensin Type 2 receptor (AT2R). AT1R is implicated in hypertension, pro-inflammatory response, vasoconstriction, increased heart rate, increased production of ROS species, release of prostaglandins, increase in intra-cellular calcium concentration (Bullock et al., 2001; Patel et al., 2017). By the contrary, AT2R plays a protective role, inducing anti-inflammatory, anti-fibrotic, anti-proliferative effect, vasodilation and therefore counterbalancing the effect mediated by AT1R (Bullock et al., 2001). Both Ang II receptors are widely expressed in the lung, particularly in epithelial cells, VSMCs and macrophages and are members of the seven transmembrane – domain G protein coupled receptor (GPCR) superfamily (Bullock et al., 2001; Montezano et al., 2014). BK receptors are known to heterodimerize with angiotensin receptors AT1R, AT2R, and Mas that may augment or diminish their activity (Chung et al., 2020). Ang II induces hypertension not only playing a role on vasoconstriction but also in sodium and water handling, since it activates sodium transporters in the proximal tubules and increased blood pressure is required to eliminate sodium excess. Moreover, it induces aldosterone production by the zona glomerulosa of the adrenal cortex. Aldosterone is a steroid hormone whose effects are mediated by the mineralocorticoid receptor (MR) which acts as a transcription factor for several genes involved

in the regulation of blood pressure and sodium-potassium balance (Nakamura et al., 2015). Abnormalities in aldosterone synthesis can be due mainly to aldosterone-producing adenoma or to genetic mutations (familial hyperaldosteronism) (Yang et al., 2018) leading to a pathological condition known as primary aldosteronism which is associated with hypertension, hypokalemia and metabolic syndrome, increasing cardiovascular risks (Bothou et al., 2020; Nakamura et al., 2015; Yang et al., 2018).

2.1.2. RAAS inhibition

Blockade of the detrimental branch of RAAS is of particular interest in the development of new pharmacological therapies. Four different classes of antihypertensive drugs are clinically used: ACE inhibitors (ACEis), Angiotensin type 1 receptor antagonist (ARBs), renin inhibitors and Mineralocorticoid receptor antagonists (MRA).

2.1.2.1. ACE inhibitors (ACEis)

The first class of antihypertensive drugs to be developed were ACEis. Taking these drugs, patients are protected by the deleterious effects of Ang II, because the production of this hormone from angiotensinogen is inhibited. Moreover, Ang 1-7 levels are increased, shifting RAAS activity to the beneficial axis (Luque et al., 1996; Romero et al., 2015). Moreover, ACE blockade leads to an accumulation of bradikynin (BK), which plays a vasoprotective role reducing blood pressure (Chung et al., 2020).

In the early 1980s, captopril was the first oral ACEi to be clinically approved for its antihypertensive properties and its improvement to the clinical status of patients with heart failure (Vlasses et al., 1982). In humans, captopril reduces blood pressure in patients with essential hypertension with low, normal, and high renin levels, and in patients with

renovascular hypertension and hypertension associated with chronic renal failure. Moreover, it can be easily metabolized because of its free sulfhydryl group and excreted by kidneys (Migdalof et al., 1984). Among all the ACEis currently used, perindopril and ramipril are known to have superior therapeutic qualities (Dinicolantonio et al., 2013). Like the other ACEis, they display anti-inflammatory, antioxidant, antithrombotic, anti-apoptotic, antifibrotic, NO-stimulating, and profibrinolytic properties, other than preservation of the endothelial function. However, perindopril ("Efficacy of perindopil in reduction of cardiovascular events among patients with stable coronary artery disease: randomized, double-blind, placebo-controlled, multicenter trial (the EUROPA study)," 2003), and ramipril (Gerstein et al., 2000; Lonn et al., 2001; The Heart Outcomes Prevention Evaluation Study, 2000), are the only ACE inhibitors associated with CV events prevention and lower CV mortality rates in patients with or at high risk for coronary heart disease, who have normal left ventricular function (Dinicolantonio et al., 2013).

Anderson and colleagues (Anderson et al., 2006) very well described the beneficial effects of ramipril: it is able to improve the hemodynamic function, to play an anti-trombotic and anti-inflammatory role, to reduce the incidence of postinfarction ischemic events, to prevent the development of heart failure and other cardiovascular comorbidities. Moreover, it is well tolerated, with few side effects including cough, dizziness, headache and fatigue while angioedema was observed in a very small percentage of patients (0,2% in HOPE-TIP study) (Sharpe, 2005).

Unfortunately, these side effects are typical of ACEis and they could be due to an increase in BK levels, since it has a higher affinity for ACE compared to Ang II.

2.1.2.2. AT1R antagonist (ARBs)

In the 1990s, subsequently to ACEis, AT1R antagonists were developed, assuming that an improved pharmacological specificity could lead to an improved tolerability compared to ACEis (Markham et al., 2000; Romero et al., 2015). Indeed, purpose-designed comparative studies have reported a significant lower incidence of adverse effects with ARBs and improved adherence to treatment compared to ACEis (Markham et al., 2000; Omboni and Volpe, 2019). AT1R is ubiquitously expressed in the human body and, particularly, high levels were detected in glomerular and juxtaglomerular cell of the kidney, in the zona glomerulosa of the adrenal gland, in the cardiac conduction system and the circumventricular organs of the brain. As a G-coupled receptor, many of the AT1R functions are mediated by signal transduction via selected G-proteins, but also G- protein independent mechanisms can be activated, like activation of multiple small non-receptors tyrosine kinases, transactivation of growth factor receptors, and induction of ROS production through the activation of NADPH oxidases (Nox enzymes) which in turn activates NFkB, a transcription factor responsible for the expression of several genes involved in atherogenesis, participating to cardiovascular damages induced by Ang II binding to AT1R (Eckenstaler et al., 2021). Therefore, inhibition of this receptor plays an important role in prevention and protection of the cardiovascular system from the deleterious effect of Ang II. Several clinical trials aimed to evaluate the effectiveness of these drugs in the amelioration of hypertension, heart failure, left ventricular hypertrophy, type 2 diabetes and metabolic syndrome (Borghi and Cicero, 2012; Markham et al., 2000). Moreover, ARBs improve glucose transport to insulin-sensitive tissues, increase insulin secretion by B cells, stimulate the insulin/glucose axis, and activate the peroxisome proliferator-activated receptor gamma (PPAR-g), leading to improved insulin sensitivity through increased levels of adiponectin (Borghi and Cicero, 2012). Based on their chemical structures, ARBs bind AT1R

with different affinity: candesartan showed the highest degree affinity for the AT1 receptor compared to the other ARBs analyzed (Van Liefde and Vauquelin, 2009). It efficiently inhibits the deleterious effect triggered by Ang II including vasoconstriction, hypertension, aldosterone release (Bulsara and Makaryus, 2022). The CHARM studies demonstrated that candesartan was generally well tolerated and reduced cardiovascular mortality and morbidity in patients with chronic heart failure and intolerance to ACEi (Granger et al., 2003). It also reduced hospital admissions for heart failure (Pfeffer et al., 2003). The addition of candesartan to other treatment (including ACE inhibitors, β-blockers, and an aldosterone antagonist) leads to a further clinically important reduction in relevant cardiovascular events in patients with CHF and reduced left-ventricular ejection fraction (McMurray et al., 2003; Young et al., 2004). It is also used off-label to treat conditions including cerebrovascular accident or stroke, diabetic nephropathy, left ventricular hypertrophy, and migraines (Bulsara and Makaryus, 2022). Moreover, it has been recently proposed as anti-inflammatory agent as it can inhibit the activation of the NLRP3 inflammasome (Lin et al., 2022). Also, irbesartan displays an optimal efficiency in lowering blood pressure, reducing serum creatinine and proteinuria and improving kidney function (Darwish et al., 2021). It is specific for AT1R and no interference with other receptors was observed (Borghi and Cicero, 2012; Darwish et al., 2021). Moreover, its pharmacokinetics allows it to be safely administered also in combination with other drugs since it does not interfere with the efficacy of these ones (Borghi and Cicero, 2012). Irbesartan reduces oxidative stress and inflammatory response induced by hypoxia (Boccellino et al., 2018) and stress (Yisireyili et al., 2018), inhibits Ang II-induced proliferation of cultured human aorta VSMC *in vitro*, displays anti-atherosclerotic effects and improves pulmonary capillary wedge pressure (Markham et al., 2000; Omboni and Volpe, 2019).

However, combinational therapy using both ACEi and ARBs was evaluated in several clinical trials (Böhm et al., 2011) but was not recommended, since it can enhance renal impairment and hyperkalemia (Momoniat et al., 2019; Romero et al., 2015).

2.1.2.3. Other RAAS inhibitors

Beside ACEis and ARBs, other RAAS inhibitors are widely used. Since renin is the rate-limiting enzyme for the activation of RAAS, inhibitors of its activity (renin inhibitors) aim to block the cascade leading to Ang II synthesis. Mineralocorticoid receptor antagonists (MRA) are widely used for the treatment of primary aldosteronism and aim at the suppression of all the signaling pathways ignited by MR activity. Also aldosterone synthase inhibitors were developed as well as agonists of the ACE2-Ang 1-7 - MasR axis (Romero et al., 2015).

2.2. Angiotensin Converting Enzyme 2 (ACE2)

2.2.1. Main player in the protective branch of RAAS

2.2.1.1. ACE2-Ang 1-7-Mas-AT2R axis

The beneficial effects of RAAS are mediated by the ACE2-Ang 1-7-MasR-AT2R axis. Indeed, all these molecules play a protective role in several cardiovascular diseases and, in experimental disease models, Ang 1-7 / MasR signalling suppressed Ang II-induced pathogenesis (Karnik et al., 2017).

ACE2 is a carboxypeptidase mainly involved in the degradation of Ang II into Ang 1-7, making this enzyme probably more important than ACE in regulating local levels of Ang II and Ang 1–7, and therein the balance of RAS activation (Tikellis and Thomas, 2012). It was firstly discovered in 2000 by Tipnis and Donoghue (Donoghue et al., 2000; Tipnis et al., 2000). Experiments performed in ACE2 knockout mice models underline the importance of this enzyme in diabetes (Tikellis et al., 2008), cardiac function, hypoxia and cell contractility (Crackower et al., 2002; Oudit et al., 2007; Yamamoto et al., 2006), endothelial function (Lovren et al., 2008), liver (Wu et al., 2018) and kidney injury (Fang et al., 2013; Wong et al., 2007). Its overexpression is associated with an improved pressure and glycemic control, reduced oxidative stress and modulation of ER stress (Unger et al., 2015), prevention of cardiac hypertrophy and fibrosis induced by Ang II (Huentelman et al., 2005), and of acute lung injury by the modulation of pro-inflammatory molecules (Ye and Liu, 2020), while its loss leads to cardiac dysfunction, hypertrophy, fibrosis and a greater diastolic function (Santos et al., 2019). The protective role of ACE2 is due mainly to the action of Ang 1-7.

Beside ACE2, neprilysin (NEP) as well as prolyl endopeptidases and thimet oligopeptidases can participate in Ang 1-7 biosynthesis, depending on tissue or cell type (Serfozo et al., 2020; Unger et al., 2015). Ang 1-7 is an heptapeptide well known to counterbalance the effect of

Ang II through the activation of its receptor, Mas, which is a G-protein coupled receptor whose signal leads to vasorelaxation, anti-inflammatory, anti-fibrotic and anti-proliferative effects. Through its binding to Mas receptor (MasR), Ang 1-7 improves insulin sensitivity and glucose tolerance in experimental animal models (Unger et al., 2015). It also stimulates NOS in endothelial cells and the production of nitric oxide (NO) in platelets, preventing platelets activation and coagulation, as well as in VSMCs and cardiac cells, influencing vascular tone and cardiac contraction. Moreover, it promotes bradykinin production which, though bradykinin receptor type 2 (BK2R), leads to NO formation and it can antagonize AT1R-mediated activation of MAP kinases, key regulators in several cell signaling pathways and involved in vascular remodeling, renal fibrosis and cardiac hypertrophy (Unger et al., 2015). Ang 1-7 also attenuates LPS induced pulmonary fibrosis down-modulating AT1R and increasing MasR expression (Cao et al., 2019) and displays cardioprotective effects in heart failure condition (Patel et al., 2016).

Since MasR, as GPCR can heterodimerize with receptor subtypes of other GPCRs, it may interact both with AT1R and AT2R, as it does with BK2R inducing vasorelaxation (Karnik et al., 2017). However, interaction with AT2R has been already excluded as reported in this study from Zhang (Zhang et al., 2017), where the authors stated that, despite AT2R displays all characteristics of a GPCR, upon activation of the AT2R, intracellular helix 8 changes its orientation in a way that it interacts with intracellular helixes III, V, and VI, thereby sterically blocking binding of conventional G proteins and β-arrestins. Beside MasR, Ang 1-7 can also bind the AT2R and, via this receptor, it prevents aneurysmal rupture and plays a vasoprotective and atheroprotective role in experimental models of atherosclerosis (Karnik et al., 2017). A further evidence of the beneficial effects of ACE2/Ang1-7/MasR axis was reported by Abuohashish (Abuohashish et al., 2017), where inhibition of ACE by captopril lead to an

upregulation of this branch of RAS, improving bone metabolism, mineralization and mass and reduced RANKL expression which can be increased by Ang II stimulation. Also Savoia and colleagues (Savoia et al., 2020) reported that, in an *in vivo* model of spontaneous hypertensive rats (SHR), chronic AT1R blockade enhances expression and activation of MasR, contributing to improved vascular remodeling and this effect is associated with reduced ROS production, increased NO bioavailability and it is independent on AT2R.

2.2.1.2. ACE2 structure and function

ACE2 is a carboxypeptidase, discovered for the first time in 2000 by two different groups independently: Tipnis and colleagues (Tipnis et al., 2000) were the first to identify a metalloproteases which shared >40% homology with ACE sequence. They described a transmembrane carboxypeptidase with a catalytically active domain that can be shedded in the extracellular space and that it is responsible for the cleavage of one C-terminal residue of substrates like Ang I, Ang II and [des-Arg9]-bradykinin but not bradykinin itself and, moreover, its activity cannot be blocked by ACEis (Hamming et al., 2007; Tipnis et al., 2000). At the same time, also Donoghue and colleagues published the same conclusions (Donoghue et al., 2000). The 40 kb *ACE2* gene is located on chromosome Xp22 and contains 18 exons which are closely related to the 17 exons encoding for ACE, sharing > 42% homology for the metalloproteases catalytic domain (Donoghue et al., 2000). Several polymorphisms have already been identified for this gene, particularly involved in hypertension and other cardiovascular diseases (Bosso et al., 2020; Imai et al., 2010).

ACE2 protein is a type I transmembrane glycoprotein made of 805 aminoacidic residues with an extracellular catalytic domain and a weight of 120 kDa (Hamming et al., 2007; Imai et al., 2010). The homology with ACE enzyme is only related to the N-terminal catalytic domain

which has just one active site, the zinc-binding motif (HEXXH) encoded by the exon 9, while the juxtamembrane, transmembrane and cytosolic domain are more similar to collectrin, a protein particularly expressed in the kidney and involved in amino acid transport at renal level (Hamming et al., 2007; Imai et al., 2010; Warner et al., 2004). Indeed, also ACE2 has been found to interact with an amino acid transporter, B0AT1 (SLC6A19) and it has particular relevance for dietary neutral amino acid absorption in the intestine (Imai et al., 2010; Kuba et al., 2013). Analysis of the ACE2 protein sequence, revealed that two hydrophobic regions are present: a 18-residues signal peptide at N-terminus, and a 22-residues transmembrane domain near the short intracellular domain (C-terminus) (Towler et al., 2004; Warner et al., 2004). Therefore, ACE2 is defined also as ectoenzyme since its enzymatic activity is applied to extracellular substrates (Warner et al., 2004). The definition of carboxypeptidase is derived by the physiological activity of ACE2: indeed, it cleaves a single amino-acidic residue at the C-terminus of its substrate, since the binding pocket where proteolysis occurs, is physically small and does not allow accommodation of further residues. Also because of this, ACEis are not efficient in blocking ACE2 activity, since they are designed for the different binding domain of ACE. At least 12 substrates are known to be cleaved by ACE2 (Warner et al., 2004). Among these, ACE2 it is the main responsible for the generation of Ang 1-7 by degradation of Ang II (Donoghue et al., 2000; Unger et al., 2015; Warner et al., 2004), it cleaves a C-terminal Leu residue of Ang I to generate Ang 1-9, but also opioid peptide dynorphin A (1 – 13) and the hypotensive peptide apelin-13 (with comparable kinetics to that of Ang II), as well as des-Arg9 bradykinin, β-casomorphin and the growth hormone secretagogue ghrelin (Warner et al., 2004). Des-Arg9 bradykinin, by its binding to a bradykinin receptor B1, induces pro-inflammatory effects, blunted by its degradation by ACE2 (Scialo et al., 2020).

Usually, human ACE2 is found in glycosylated form: indeed, it has six potential N-glycosylation sites, as indicated by the presence of the Asn-X-Ser/Thr motif (at positions Asn53, Asn90, Asn103, Asn322, Asn432, Asn546) in its primary structure. The de-glycosylated form has a molecular weight of ~ 85 kDa (Warner et al., 2004).

ACE2 is widely expressed in heart, kidney, gastro-intestinal tract, testis but also in the lung, adipose tissue and brain (Beacon et al., 2021; Li et al., 2020).

A relevant aspect of ACE2 is that its catalytic domain can be cleaved by proteases such as a disintegrin and metalloproteinase domain-containing protein like (ADAM)10, ADAM17, and transmembrane protease, serine 2 (TMPRSS2), allowing its release in the bloodstream where it keeps its enzymatic activity (Heurich et al., 2014; Hong et al., 2009; Patel et al., 2014; Scialo et al., 2020; Warner et al., 2004). Ang II induced ACE2 shedding in myocardial cells by enhancing ADAM17 activity (Patel et al., 2014). ADAM17 has higher specificity in ACE2 shedding compared to other metalloproteases of the same family (Lambert et al., 2005). In order to define the cleavage site, Hong reported that the residue L-584, contained into the ectodomain, it is necessary for shedding and identified the cleavage site between residues 716-741, excluding a "recognition motif" inside the intracellular, transmembrane or juxtamembrane regions (Hong et al., 2009). However, also residues 708-709 are reported as a potential cleavage site while residues 652-659 may determine whether a downstream cleavage site is recognized by ADAM17 (Heurich et al., 2014). TMPRSS2 cleavage, instead, occurs within ACE2 residues 697-716 at extracellular level, resulting just in the release of a 13kDa C-terminal fragment detectable in cell lysate and no ectodomain (Heurich et al., 2014).

2.2.1.3. Control of ACE 2 activity and expression

In order to control ACE2 enzymatic activity, different inhibitors have been developed. Among this, (SS)2-{(1)-carboxy-2-[3-(3,5-dichloro-benzyl)-3H- imidazol-4-yl] ethylamino}-4-methyl-pentanoic acid (MLN-4760) has shown a very high specificity for ACE2 (Elased et al., 2006; Warner et al., 2004). Moreover, ACE2 activity, as well as ACE, can be modulated by pH and ion concentration. The native structure of ACE2 shows a single bound chloride ion, which can induce changes in the conformation of the active site facilitating binding. However, this effect is dependent on the type of substrate that is going to be hydrolyzed: Ang II conversion into Ang 1-7, indeed, does not require Cl^- (Warner et al., 2004). Calcium concentration can modulate ACE2 shedding mediated by ADAM17 as well (Lambert et al., 2005; Scialo et al., 2020). As previously stated, ACE2 activity can be modulated by other components of the RAS, since balancing between the arms of this system is fundamental to maintain fluid and electrolyte homeostasis. Higher ACE2 expression levels were found in females compared to males (Tonon et al., 2022), but its enzymatic activity does not differ according to sex. Moreover, controversial data are available concerning ACE2 and age (Li et al., 2020). ACE2 plays an important role in the modulation of acute lung injury and COPD, therefore several mechanisms have been investigated so far: Endothelin-1 (ET-1) decreases ACE2 activity by downregulating ACE2 gene transcription via the ETA receptor by a p38 MAPK-dependent mechanism in bronchial epithelial cells (Zhang et al., 2013). Hypoxia and cell adaptation to hypoxia can induce fluctuations in ACE2 expression in the lung, and it is found downregulated in mouse model of acute lung injury (Imai et al., 2005) and in patients with pulmonary arterial hypertension or acute kidney injury , suggesting a protective role of ACE2 in their pathogenesis (Li et al., 2020). Moreover, in a condition of cell energy stress, sirtuin 1 (SIRT-1) induces activation of ACE2 transcription (Scialo et al., 2020). ACE2 levels are increased in individuals

with pro-inflammatory conditions like obesity and autoimmune diseases (Beacon et al., 2021). ACE2 transcription can be modulated by several factors like miRNAs (like miR-200 family), DNA methylation and histone modifications, as well as stimuli like Ang II, which induces the expression of ACE2 in human cardiac fibroblasts, IFN-a/b which increases ACE2 expression in human tracheal cells, bronchial epithelial cells, small airway cells, large airway epithelial cells, and in primary human nasal epithelial goblet secretory cell. Moreover, *ACE2* gene can escape inactivation following X chromosome inactivation (Beacon et al., 2021). Among the therapeutic strategies targeting ACE2, beside ACE2 activators development, recombinant human ACE2 showed promising results, inducing beneficial effects in pulmonary injury, diabetic nephropathy and hypertension (Li et al., 2020).

2.2.2. ACE2 as viral receptor

2.2.2.1. Receptor for different coronaviruses

ACE2 appears like a double edged sword: beside the beneficial effects that ACE2 exerts in RAS, it is also known as the viral receptor for different coronaviruses as Severe Acute Respiratory Syndrome - CoronaVirus (SARS-CoV) (Kuba et al., 2005), NL63 (Milewska et al., 2018), SARS-CoV-2 (Hoffmann et al., 2020a). These viruses are characterized by the presence, on their surface, of the S protein, which is the one mediating receptor recognition and binding. After viral binding, syncytia are soon formed and ACE2 is down-regulated both for internalization and increased shedding by ADAM17 activity (Kuba et al., 2005; Wang et al., 2020). The first site of the infection is the lung. Downmodulation of ACE2, leave the organs unprotected by the deleterious effects of Ang II, but also lead to an accumulation of des-Arg9 bradykinin enhancing pro-inflammatory response.

2.2.2.2. SARS-CoV-2 binding

At the end of 2019, a new virus was identified in Wuhan (China), highly contagious and transmitted through direct contact, respiratory droplets, or possibly aerosols, causing symptoms such as dry cough, fever, headache, dyspnoea and pneumonia. Soon, it was identified as part of the beta-coronavirus genus. It is characterized by positive-sense, single-stranded RNA genomes and by an external envelope provided by several glycoproteins named Spike (S protein) responsible for the viral binding with its receptor (Yan et al., 2020; Zhou et al., 2020). It was named Severe Acute Respiratory Syndrome CoronaVirus 2 (SARS-CoV-2), the cause of the coronavirus disease 19 (COVID-19) (Hoffmann et al., 2020a; Lan et al., 2020), subsequently defined as pandemic by the World Health Organization (WHO).

Zhou and colleagues were the first to isolate SARS-CoV-2 and to identify ACE2 as its cellular receptor (Zhou et al., 2020). Coronavirus entry into susceptible cells is a complex process that requires the concerted action of receptor-binding and proteolytic processing of the S protein to promote virus-cell fusion. A schematic representation of viral fusion steps is reported in Figure 2 (panel C). Indeed, the virus recognizes the extracellular domain of ACE2 with its transmembrane spike (S) glycoprotein that forms homotrimers protruding from the viral surface (Figure 2, Panel B). S protein comprises two functional subunits responsible for binding to the host cell receptor (S1 subunit) and fusion of the viral and cellular membranes (S2 subunit). Receptor binding domain (RBD) is part of the S1 subunit. The structural flexibility required for the membrane fusion reaction is due to the proteolytic separation of the S1 and S2 subunits, termed priming. S protein is cleaved at the interface between the S1 and S2 subunits, the S1/S2 site, and at a site located near the N-terminus of the fusion peptide, the S2′ site, generating the mature N-terminus of the fusion peptide, which is required for insertion into the target cell membrane and thus the successful execution of the membrane

fusion reaction (Böttcher-Friebertshäuser et al., 2018). S1 interacts with ACE2 via its RBD, stabilizing the pre-fusion state. Furin like-proteases cleave the S1/S2 site and TMPRSS2 protease, expressed on the host cell surface, is responsible for S2 priming (Bestle et al., 2020). All these events are necessary to allow membrane fusion, and therefore viral entry, occurring via extensive irreversible conformational changes (Barros et al., 2021; Hoffmann et al., 2020a; Lan et al., 2020; Raghuvamsi et al., 2021; Shang et al., 2020; Walls et al., 2020; Yan et al., 2020). Of note, the multibasic (furin) domain at the boundary between S1/S2 subunit is peculiar of SARS-CoV-2 and, indeed, it is not present in SARS-CoV S protein. Since it is processed by several furin-like proteases that are widely diffused, this domain strongly contributes to the expansion of viral tropism, enhancing viral transmissibility (Hoffmann et al., 2020a; Walls et al., 2020). Moreover, the S trimer can assume different conformational states: S glycoprotein trimer, present in highly pathogenic human coronaviruses, was usually found in partially opened states, while they remain largely closed in human coronaviruses associated with common colds. SARS-CoV-2 exhibits S glycoprotein trimers spontaneously sampling closed and open conformations, strongly enhancing its pathogenicity. These structural changes are necessary for receptor engagement and lead to initiation of fusogenic conformational changes (Walls et al., 2020). Likewise, ACE2 displays the ability to tilt and bend to accommodate S1 protein binding. As shown in Figure 2 (panel A), ACE2 presents, other than the catalytic zinc-binding peptidase domain (PD; residues 19–615), also a smaller neck domain (residues 616–726), which is involved into the homodimerization of ACE2, linked to the transmembrane domain (TM). ACE2 flexibility, also in the homodimerized form, is due to motions of the large head domain relative to the transmembrane helices, and tilt of the transmembrane helices in the membrane itself. Moreover, homodimers could potentially allow for even three ACE2 molecules per S, opening the possibility of multireceptor usage by the spike glycoprotein for

host cell infection (Barros et al., 2021). Furthermore, also the shedded ACE2 plays a role in SARS-CoV-2 infection. Indeed, the circulating ectodomain still contains the binding motif recognized by viral S protein, therefore allowing viral binding and entry via endocytosis. Indeed, sACE2/S-protein complex can be naturally internalized by the recognition of sACE2 by AT1R, as was previously reported by Deshotels and colleagues (Deshotels et al., 2014). This raised some concerns about the usage of sACE2 as a potential therapeutic strategy. However, it has been reported that super-physiological concentrations of sACE2 are actually beneficial for SARS-CoV-2 infections, since it can bind to the viral particles before they reach the membrane ACE2 and cannot be internalized because of the saturation of the system (Yeung et al., 2021).

Moreover, some ACE2 exons could participate in alternative splicing by producing a truncated ACE2, lacking the N-terminus that could not bind the Spike protein, or the C-terminus generating a soluble ACE2 that would again reduce virus/cell binding and infectivity (Scialo et al., 2020).

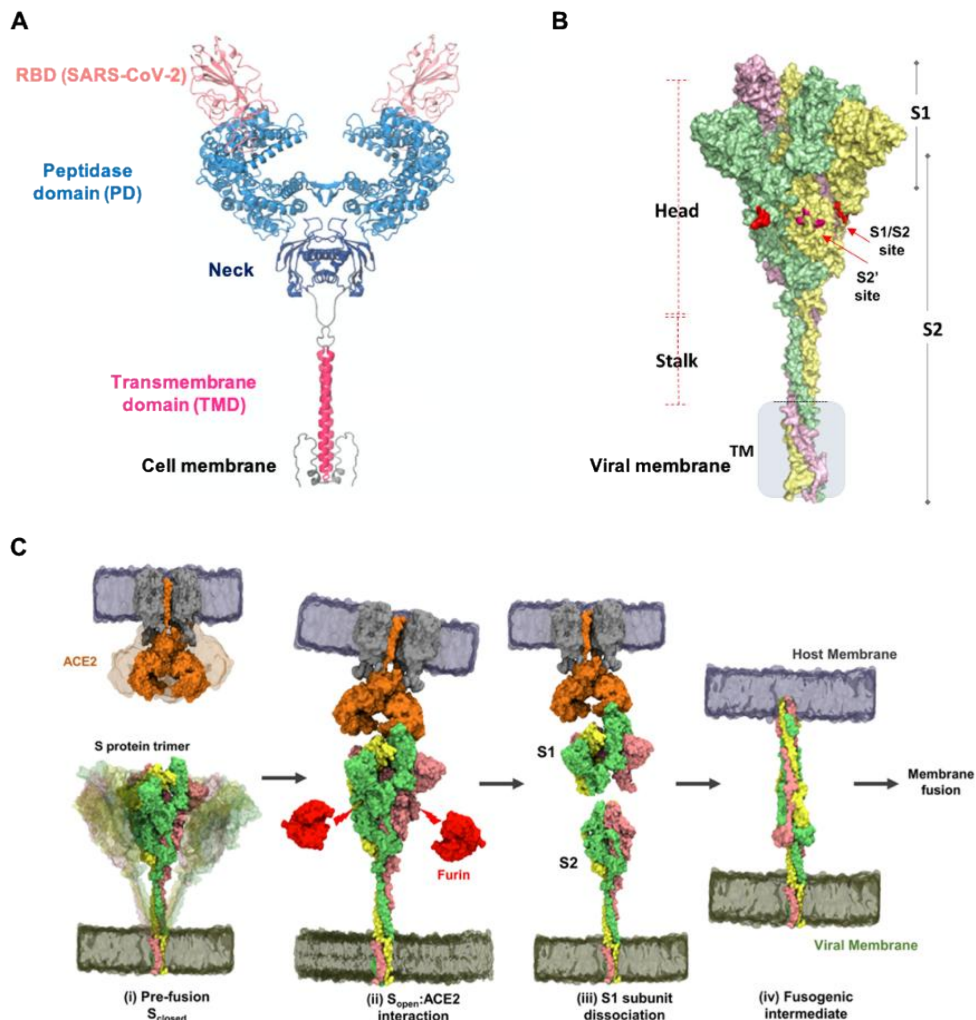

Figure 2. Schematic representation of ACE2 – SARS-CoV-2 binding and fusion. (A) Model structure of full-length ACE2 homodimer protein in complex with spike protein RBDs from SARS-CoV-2 (adapted by (Barros et al., 2021)). (B) Structure and domain organization of trimeric spike (S) protein in closed conformational state (prefusion phase) with monomers shown in yellow, green, and pink and highlighted priming sites in red. (C) Schematic of viral entry into host cell mediated by S:ACE2 interactions: (i) Intrinsic dynamics of prefusion S protein trimer and host ACE2 dimeric structure showing sweeping motions of S protein and ACE2 to facilitate S:ACE2 recognition. (ii) In the open conformation, receptor binding domain adopts an 'up' orientation to recognize and bind the host membrane-bound ACE2 receptor. ACE2 binding induces conformational changes promoting Furin* (red) proteolysis at the S1/S2 cleavage site (red arrows), leading to dissociation of S1 and S2 subunits, the mechanism of which is unknown. (iii) The residual ACE2-bound S1 subunit becomes stably bound to ACE2 and S2 subunits dissociate. (iv) Conformational changes in the separated S2 subunit promote formation of an extended helical fusogenic intermediate for fusion into the host cell membrane, membrane fusion, and viral entry into the host cell. Panels B and C are adapted by (Raghuvamsi et al., 2021).

2.2.2.3. Therapeutic strategies targeting or using ACE2

Soluble recombinant human ACE2 (rhACE2) has been tested on human kidney organoids displaying protection from SARS-CoV-2 infection (Monteil et al., 2020). Indeed, rhACE2 can act upstream by decreasing the binding between SARS-CoV-2/membrane ACE2, thus reducing infectivity and can also counter- act the increase in Ang II and DEABK/LDEABK preserving lung function (Scialo et al., 2020) and rhACE2 has already been tested in phase 1 in healthy volunteers (Haschke et al., 2013) and phase 2 in some patients with ARDS (Khan et al., 2017). IFNs stimulation upon viral treatment induced the expression of a truncated ACE2 isoform designate as δACE2, which lacks 356 N-terminal amino acids, is not able to bind SARS-CoV-2 and, therefore, does not contribute to the potentiation of the infection (Onabajo et al., 2020). Other potential therapeutic approaches include SARS-CoV-2 spike protein-based vaccine, TMPRSS2 inhibitors to block the priming of the spike protein (Hoffmann et al., 2020b), and blockade of the surface ACE2 receptor by using anti-ACE2 antibody or peptides (Scialo et al., 2020; Zhang et al., 2020). Hydroxychloroquine and chloroquine have been commonly tried for treatment of COVID-19, since they can impair terminal glycosylation of ACE2, influencing binding affinity between ACE2 and spike protein (Chung et al., 2020).

2.3. CoronaVirus Disease – 19 (COVID-19)

2.3.1. RAS, lungs and COVID-19

Soon after COVID-19 outbreak, it became clear that this disease was characterized by a relevant cardiovascular involvement. One of the commonly proposed underlying pathophysiological mechanisms of COVID-19 is a viral acute respiratory distress syndrome (ARDS) coupled with high levels of cytokines that subsequently lead to a cytokine-release syndrome (Rysz et al., 2021). As already explained in chapter 1, RAS is a fundamental regulator of cardiovascular physiology. Indeed, the lung is the first organ to be targeted by SARS-CoV-2 infection, causing severe pneumonia, and the RAS system is known to play a role in lung protection or injury. In acute lung injury characterized by uncontrolled oxidative stress, pulmonary edema, inflammation, and neutrophil infiltration, ACE2 is found to alleviate the inflammatory response and protect against LPS-induced lung injury, through the maintenance of the equilibrium between the two arms of RAS (Ye and Liu, 2020). Rysz and colleagues reported that manipulation of RAAS balance in swine, by infusion of supraphysiological levels of Ang II or by ACE2 blockade and low-level infusion of Ang II, leads to a pathological phenotype that shares several features of COVID-19 (Rysz et al., 2021). In lung physiology, upregulation of the ACE/Ang II/AT1R axis is associated with enhanced inflammation, through the activation of NF-κB and JAK2/STATs pathways and secretion of pro-inflammatory chemokines, induced apoptosis in alveolar epithelial cells, contributing to the acute respiratory distress syndrome (ARDS). Administration of ACEis and ARBs plays a protective role attenuating the lung edema, lung AECs apoptosis, and microvascular permeability caused by ARDS (Wang et al., 2019). Moreover, in knock-out mouse model, ACE2 deficiency resulted in enhanced vascular permeability, increased lung edema, severe inflammatory cell infiltration and impaired lung function, while supplementation with recombinant ACE2 was able to

improve these outcome measures. Consistently, Ang 1–7 alleviated lung edema, myeloperoxidase activity, lung injury, and pulmonary vascular resistance in mouse models of ARDS (Wang et al., 2019). Altogether, these data highlight the importance of RAS in lung physiology and function and give some hints about the understanding of COVID-19 outcomes. ACE2 and TMPRSS2 are needed for SARS-CoV-2 viral entry and they are found to be upregulated in COPD patients (Ryu and Shin, 2021; Saheb Sharif-Askari et al., 2020) and the pro-inflammatory environment promoted by Ang II may participate to the enhancement of the cytokine storm observed in COVID-19 patients. Beside the effect on the lung, several extrapulmonary manifestations have been observed: hematologic, cardiovascular, renal, gastrointestinal and hepatobiliary, endocrinologic, neurologic, ophthalmologic, and dermatologic systems can all be affected (Gupta et al., 2020). SARS-CoV-2 infection mediates endothelial injury with consequent inflammation (endothelialitis) and possible formation of thrombi and microvascular dysfunction (Gupta et al., 2020; Guzik et al., 2020). Pre-existing comorbidities may be associated with a severe outcome of COVID-19. Indeed, myocardial injury which is associated with cardiac dysfunction and arrhythmias, have been associated with fatal outcome in COVID-19 patients too (T. Guo et al., 2020). Moreover, endocrine disorders like diabetes and obesity are currently considered also risk factors for COVID-19. The elevated cytokine levels observed after SARS-CoV-2 infection may lead to impairments in pancreatic β-cell function and apoptosis, decreasing insulin production and, by directly targeting ACE2 expressed in β-cells, it might contribute to insulin deficiency and hyperglycemia, as shown previously for infection with SARS-CoV (Gupta et al., 2020). Hypertension was found to be prevalent in patients with a more severe COVID-19 (Guzik et al., 2020; Savoia et al., 2021) which is supported by the fact that ACE2 and TMPRSS2 are found to be upregulated in hypertensive patients (Saheb Sharif-Askari et al., 2020). However,

information about the contribution of hypertension on the manifestations and progression of cardiovascular complications during the course of COVID-19 is scarce (Savoia et al., 2021).

2.3.2. ARBs and ACEis in COVID-19: friends or foe?

At the beginning of COVID-19 pandemic, it was contended that ACEis and ARBs can increase the expression of the viral receptor ACE2 (Burrell et al., 2005; Igase et al., 2005; Ishiyama et al., 2004; Takeda et al., 2007), leading to hypothesize that RAS blockers could worsen the prognosis of COVID-19 patients (Fang et al., 2020; Zheng et al., 2020) and raising enormous concern in the medical community. However, this hypothesis stands on experimental models of cardiovascular disease, for example rodents where acute myocardial infarction and/or heart failure were induced with coronary artery ligation (Burrell et al., 2005; Ishiyama et al., 2004). This suggests that in these conditions the increase of ACE2 could reflect activation of the protective RAS as a compensatory process counteracting the AT1R-mediated proinflammatory mechanisms (Ishiyama et al., 2004). ACEis and ARBs are life-saving drugs for millions of patients with cardiovascular disease worldwide, like arterial hypertension, heart failure, atherosclerosis, kidney diseases, and/or survived a myocardial infarction, and thus have an activated RAS (Caldeira et al., 2012; X. Guo et al., 2020; Liu et al., 2012; Mancia et al., 2020; Mentz et al., 2013; Mortensen et al., 2008; Savoia et al., 2021). Accumulating data thereafter suggested that ACEis and ARBs could be neutral, or even beneficial in COVID-19 patients, as they could protect the lung against acute respiratory failure, as they do against pneumonia in different categories of fragile patients (Liu et al., 2012; Mortensen et al., 2008; Savoia et al., 2021; Wang et al., 2015). Moreover, RAS blockers were shown to be beneficial in multiple settings featuring an activated RAS (Rossi et al., 2020; Unger et al., 2015), both

experimentally (Huentelman et al., 2005; Imai et al., 2005) and clinically (Caldeira et al., 2012; Liu et al., 2012; Mortensen et al., 2008), not only because they blunt the AT1R-mediated vasoconstrictive pro-inflammatory arm of the RAS, but also because they enhance its protective arm comprising ACE2, Ang 1-7, and the Mas receptor (Melissa Hallow and Dave, 2021). To date, several clinical trials that randomized patients to continue or suspend treatment with ACEis and/or ARBs, reported similar rates of acute hospitalization (Cohen et al., 2021) and no significant differences in the mean number of days alive and out of the hospital between the treatment arms (Lopes et al., 2021). Retrospective analysis reported that ARB use was significantly associated with a reduced risk of mortality among patients with COVID-19 (Wang et al., 2021). Furthermore, no association between ACEis/ARBs use and likelihood of a positive COVID-19 test has been observed in different studies (Mehta et al., 2020a; Reynolds et al., 2020).

Knowledge of the effects of Ang II on ACE2 expression and activity in the lung is, therefore, a fundamental piece of information to be obtained, along with establishing whether Ang II affects ACE2 expression in endothelial cells (Yuyang Lei et al., 2021), because COVID-19 causes endothelial dysfunction, inflammation (‘endothelialitis’), damage and thrombosis (Barbosa et al., 2021; Varga et al., 2020).

3. AIM

Patients with an activated RAS displayed high risk for SARS-CoV-2 infection and a worse prognosis of COVID-19. It was hypothesized that these effects may be due to the administration of ACEis and ARBs, widely used in these patients, since they could induce an increase in ACE2 expression, the viral receptor. However, mechanistical studies in humans are lacking, especially concerning lungs, the first target organ for SARS-CoV-2 infection.

Therefore, this study aimed to answer to fundamental questions:

1) Does Ang II increase ACE2 expression and/or shedding in human endothelial and airway epithelial cells?
2) Can ACEis or ARBs blunt or enhance this effect of Ang II?
3) Does Ang II enhance the airway epithelial cell infection by SARS-CoV-2 and spread of the infection?
4) Do these effects occur via the spike protein interaction with ACE2?

4. EXPERIMENTAL DESIGN

To these aims, we measured the expression of AT1R and ACE2, in Calu-3 cells, a stable ACE2 expressing cell line derived from human bronchial epithelium (McCracken et al., 2021), which has been widely used to investigate the mechanisms of SARS-CoV-2 cell entry and replication (Lamers et al., 2021; Wyler et al., 2021), and in human umbilical vein endothelial cells (HUVECs) using digital droplet PCR. Calu-3 cells were found to express sizable amounts of AT1R and ACE2. We first investigated the expression of RAS component in our system. Then, we evaluated if ACE2 activity was modulated by the binding with the S1 protein from SARS-CoV-2. To gain insight on the potential protective role of Ang 1-7 from Ang II actions, we also measured the levels of Ang 1-7 produced by Calu-3 cells, and we tested the effect of the heptapeptide and the ACE2 inhibitor MLN-4760 on *ACE2* mRNA. We then treated Calu-3 cells with increasing concentration of Ang II, ACEi (ramipril and captopril) and ARBs (irbesartan and candesartan) and evaluated ACE2 expression, both at gene and protein levels. Furthermore, to investigate the functional relevance of ACE2 changes, we measured the effect of Ang II, ARBs and ACEis in the entry steps of SARS-CoV-2 Spike Pseudotyped Virus (pseudovirus) or the wild-type SARS-CoV-2 infection in Calu-3 cells. Finally, we explore ACE2 shedding in our cell model.

5. MATERIALS AND METHODS

5.1. Cell cultures

Since SARS-CoV-2 infection causes pneumonia and endothelial damage, we used Calu-3 (HTB55; ATCC, Milan, Italy) cells as a model of bronchial epithelial cells, since they stably express ACE2 and have been widely used to investigate the mechanisms of SARS-CoV-2 cell entry and replication, and HUVECs as a model of endothelial cells to evaluate the presence of *ACE2* and *AGT1R*, at basal levels. HUVECs were cultured in endothelial cell growth media (EGM^{TM}-2 Single Quots, Cod. #CC4176, Lonza, Milan, Italy), with fresh culture medium replenished every 2–3 days. When they reached confluence, 10^6 cells were used for gene expression analysis. Calu-3 cells were cultured in DMEM/F-12, GlutaMAX™ supplement, (Cod# 10565018, Gibco, Milan, Italy) supplemented with 10% FBS (Cod. # ECS0180L, Euroclone, Milan, Italy), 1% penicillin – streptomycin (Cod. #P0781, Sigma-Aldrich, Milan, Italy), with fresh culture medium replenished every 2–3 days. Before treatments, the cells were seeded in 12-well plates at a density of $3x10^5$ cells per well (for mRNA evaluation experiments) or in 6-well plates at a density of $4x10^5$ cells per well (for protein evaluation experiments), grown to sub-confluence (80%), and starved for 16 hours in serum-free medium.

For the viral infection, besides Calu-3 cells, other 2 cell lines were used: 293T (human, kidney, ATCC CRL-3216™) and VeroE6 (African green monkey, kidney, kidney, ATCC CRL-1586™) were maintained in in Dulbecco's' modified Eagle medium (Gibco, ThermoFisher Scientific).

5.2. Cell treatments

5.2.1. Drug dose response

Calu-3 cells were exposed to increasing concentration of Ang II (Cod. #A9525, Sigma-Aldrich, Milan, Italy) ranging from 10^{-7} M to 10^{-9} M and of the ARBs irbesartan (Cod. #I2286, Sigma-

Aldrich, Milan, Italy) and candesartan (Cod. #A10175-5, Adooq Bioscience, Guidonia Montecelio, Italy) as well as of the ACEis ramipril (Cod. #R0404, Sigma-Aldrich, Milan, Italy) and captopril (Cod. #211875-1GM, Sigma Aldrich, Milan, Italy) ranging from 10^{-5} M to 10^{-7} M for 12 hours or 48h to evaluate *ACE2* mRNA and ACE2 protein respectively.

We exposed Calu-3 cells also to increasing concentrations of Ang 1-7 (Cod. #A9202, Sigma-Aldrich, Milan, Italy) ranging from 10^{-8} M to 10^{-5} M for 12 hours.

5.2.2. Drug treatments in presence of Ang II

Calu-3 cells were exposed to Ang II 10^{-7} M alone or in combination with the ARBs irbesartan 10^{-5} M and candesartan 10^{-5} M or the ACEis ramipril 10^{-5} M and captopril 10^{-5} M for 12 or 48 hours to evaluate *ACE2* mRNA and ACE2 protein respectively. RAS inhibitors were added to medium at least 40 minutes before Ang II. Moreover, Calu-3 cells were treated for 12 hours with the ACE2 inhibitor MLN-4760 10^{-5} M (Cod. #5306160001, Merck-Millipore, Vienna, AT) added to fresh media 1h before exposure to Ang II.

5.2.3. Infection

For infection experiments with SARS-CoV-2, Calu-3 cells were treated for 48 hours with Ang II 10^{-7} M alone or in combination with the ARB irbesartan 10^{-5} M or the ACEis ramipril 10^{-5} M, other than an activator of the protease TMPRSS2, dihydrotestosterone (DHT) 10^{-7} M (Sigma-Aldrich, Milan, Italy).

5.3. Gene expression analysis

5.3.1. RNA isolation and reverse transcription

Total RNA was extracted from Calu-3 cells or HUVECs with the High pure RNA isolation Kit (Cod. #11828665001, Roche, Monza, Italy) following manufacturer’s protocol. Briefly, cells

were lysated directly in the well with 200 µl clean PBS and 400 µl of Lysis/Binding Buffer containing 4,5 M guanidine-HCl and 100 mM sodium phosphate in order to disrupt cells protecting nucleic acids from degradation. The lysate was applied to a silica-based filter that selectively binds RNA. Residual DNA was digested by DNAse I and contaminants were washed away. Finally, RNA was eluted in 35 µL Elution Buffer (clean water, PCR grade). The purity and the amount of extracted RNA was analysed using Nanodrop technology (ThermoFisher).

Five hundred ng of total RNA was reverse-transcribed, in a final volume of 20 µL, using the iScriptTM cDNA Synthesis Kit (Bio-Rad, Milan, Italy) following the manufacturer's recommendations and incubated at 25°C for 5 minutes, at 42°C for 30 minutes and at 85°C for 5 minutes. The RT-PCR reactions were performed in Delphi 1000TM Thermal Cycler (Oracle Biosystems).

One µg total RNA was then reverse-transcribed with Iscript (Cod. # 1708841, Bio-Rad Laboratories, Segrate, Italy) in a final volume of 20 µL, following manufacturer instructions. The reaction was performed using Delphi 1000TM Thermal Cycler (Oracle Biosystems): samples were incubated at 25°C for 5 minutes, at 42°C for 30 minutes and at 85°C for 5 minutes, then were kept at 4°C in the instrument or stored at -20°C.

5.3.2. Digital Droplet (dd) PCR

We obtained absolute quantification of angiotensin-converting enzyme 2 (*ACE2)* mRNA using Digital Droplet PCR system (ddPCR) (Bio-Rad Laboratories), which is an emulsion PCR whose basic principle is dilution and compartmentalization of templates in water droplets in a water-in-oil emulsion. Ideally, each droplet contains a single template molecule and functions as a micro-PCR reactor. At the end of the reaction, a droplet reader (Bio-Rad Laboratories) counts all cDNA amplified copies contained in each droplet present in the well. Primers (sequences

reported in Table 1) and probes for ddPCR were designed using ProbeFinder Software (Universal ProbeLibrary, Roche). Briefly, 2 µl cDNA (100 ng) and 18 µl of total reaction volume, containing primers, probes (if applicable) (Universal ProbeLibrary, Roche) and ddPCR Supermix for Probes (no dUTP) (Cod. #1863024, Bio-Rad Laboratories) or QX200 ddPCR EvaGreen Smx (Cod. #1864034, Bio-Rad Laboratories) were converted into droplets with the QX200™ Automated Droplet Generator (Bio-Rad Laboratories). Droplet-partitioned samples were transferred to a 96-well plate and PCR was performed on the Bio-Rad C1000™ (Bio-Rad Laboratories) with the following protocol: 95°C for 10 min (Ramp 2°C/s), followed by 40 cycles of 94°C for 30 s (Ramp 2°C/s) and 60°C for 1 min (Ramp 2°C/s) with a final step of 98°C for 10 min (Ramp 2°C/s). PCR plate was then incubated at 4°C for at least 30 min before the reading step. Using the QXDx Droplet Reader (Bio-Rad Laboratories), single droplets were analysed by QuantaSoft analysis software (Bio-Rad Laboratories) to count PCR-positive and PCR-negative droplets with an optical detector.

Gene	Primers	Probe (yes/no)
ACE2	For 5’-AAAGTGGTGGGAGATGAAGC-3’ Rev 5’-GAGATGCGGGGTCACAGTAT-3’	YES
AGT1R	For 5’-ATGATTCCAGCGCCTGAC- 3’ Rev 5’-GGTCCAGACGTCCTGTCACT-3’	YES
REN	For 5’- GGTCACCCCTATCTTCGACA -3’ Rev 5’- CTGCCAGACACCAGTCTTGA -3’	NO
AGT	For 5’-TCCAGCCTCACTATGCCTCT-3’ Rev 5’-GCGGTCATTGCTCAATTTTT-3’	NO
ACE1	For 5’- AGGAGCAGAACCAGCAGAAC-3’ Rev 5’- TCAGCCTCATCAGTCACCAG-3’	NO
KNG1	For 5’- GTGGTGGCTGGATTGAACTT -3’ Rev 5’- CGCAAATCTTGGTAGGTGGT -3’	NO
BDKRB1	For 5’- CAGCTCTTCCCTCAAAATGC -3’ Rev 5’- GCCCAAGACAAACACCAGAT-3’	NO
BDKRB2	For 5’- TCAGCTACCCATCCCTCATC-3’, Rev 5’- GAATAGCAGCAGCACAACCA-3’	NO
LDLR	For 5’- GAATTTGGCCAGACACAGGT -3’ Rev 5’- CACCGTACCCAGCTGATTTT -3’	NO
ADAM17	For 5’-CCTTTCTGCGAGAGGGAAC-3’ Rev 5’- CACCTTGCAGGAGTTGTCAGT-3’	YES
TMPRSS2	For 5’-CGCTGGCCTACTCTGGAA-3’ Rev 5’-CTGAGGAGTCGCACTCTATCC-3’	YES

Table 1. Primers sequences of the genes investigated.

5.4. Protein analysis

5.4.1. Protein extraction

After stimulation, cell medium was removed, and Calu-3 cells were washed with PBS to remove any residual. RIPA lysis and extraction buffer added with proteinase and phosphatase inhibitor (Roche) was used to lysate cells preserving total amount of protein. Samples were then sonicated with Amp 60 and cycle 0,5 and stored overnight at -80°C or kept in ice for at least 30 minutes in order to complete cell lysis. After centrifuge at 13000 rpm for 10 minutes at 4°C, pellets were discarded, and supernatants were stored at -80°C and then used for protein quantification.

5.4.2. BCA assay

Protein concentration was determined with BCA assay (Cod. #23225, Thermo Scientific, Milan, Italy), a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein, following manufacturer instructions. Exploiting reduction of Cu^{+2} to Cu^{+1} (cuprous cation) by protein in an alkaline medium, chelation of 2 molecules of BCA with one cuprous cation gives rise to a colorimetric reaction: the more the protein content is high, the more the colour moves from green to violet. This water-soluble complex exhibits a strong absorbance at 562 nm. Therefore, first the standards were prepared using Bovine Serum Albumin diluted in clean water, with concentrations ranging from 0 to 2000 µg/ml. Then, a 96 well plate (flat bottom) was loaded with standards, RIPA buffer as blank and samples diluted 1:5 in water, together with BCA reagent mix (supplied). Plate was incubated at 37°C for 30 minutes and the loaded in EnSight instrument for absorbance detection. Standard curve was designed plotting absorbance values on a cartesian graph against the known concentration of BSA and the equation of the interpolated linear curve was used to derive protein concentration in the cell lysates.

5.4.3. Immunoblotting

Immunoblotting for ACE2 was performed following a standard protocol. Lysate fraction (20 µg) was denatured with NuPAGE Sample Reducing Agent (10X, Cod. #NP0004, Thermo Scientific, Milan, Italy) and NuPAGE LDS Sample Buffer (4X, Cod. #NP0007, Thermo Scientific, Milan, Italy) and boiled at 70°C for 10 minutes. Finally, samples were separated in an 8% polyacrylamide gel and electro-blotted onto nitrocellulose membrane (Cod. #10600008, Amersham-Hybond EC, GE Healthcare Life Sciences, Milan, Italy). The membranes were blocked for 1 hour at room temperature with 5% Bovine Serum Albumin (BSA) (Cod. #A9430,

Sigma-Aldrich, Milan, Italy) in PBS - 0.1% Tween-20 (Cod. #P1379, Sigma- Aldrich, Milan, Italy) and then incubated overnight at 4°C with an anti-ACE2 antibody (diluted 1/1000) (Cod. #ab272690, Abcam, Milan, Italy). After washing with PBS-0.1% Tween, membranes were incubated for 1 hour with an antirabbit secondary antibody (Cod. #P0448; Agilent-DAKO, Milan, Italy), after which the band intensity was measured in ATOM UVITEC (Uvitec, Milan, Italy). Images were analysed with Nine Alliance Program (Uvitec). To adjust for differences in the amount of loaded protein ACE2 expression was normalized to β-actin (Cod. #A5441, Sigma Aldrich, Milan, Italy).

5.4.4. ELISA assay

The enzyme-linked immunosorbent assay (ELISA) was used to measure the ACE2 shedded in the culture supernatant by using Human ACE2 ELISA kit (Cod. # ab235649, Abcam, Milan, Italy), according to manufacturer instructions. Briefly, cell culture supernatants were collected, and cells were lysated using 120 µl 1X Cell Extraction Buffer PTR. Standard curve was prepared immediately before the assay with decreasing concentration of recombinant ACE2 (supplied). Using microplate strips already coated with monoclonal antibody against human ACE2, 50 µl of standards or samples were loaded in each well in duplicates, together with Antibody cocktail containing antibodies conjugated to an affinity tag that is recognized by the antibody used or plate coating. Cell media were incubated over night while cell lysates 3 h at room temperature in a plate shaker at 400 rpm. At the end of incubation, wells were washed with PT Wash solution and TMB development solution was added for 10 minutes in the dark in agitation. The reaction was stopped by adding Stop solution for 1 minute, then absorbance was immediately measured by EnSight instrument at 450 nm. Standard curve was designed plotting absorbance values on a cartesian graph against the known concentration of

recombinant ACE2 and the equation of the interpolated linear curve was used to derive ACE2 concentration in the culture supernatant or cell lysates.

5.4.5. LC-MS/MS

In order to measure the Ang 1-7 levels in the cell media, Calu-3 cells were seeded at a density of 150.000/well and cultured in DMEM/F12 added with 10% FBS and Pen-Strep 1%. Cells were grown to sub-confluence (80%), then washed extensively with prewarmed serum free medium and starved for 14 -16 hours with DMEM/F12 serum free medium. After starvation, Calu-3 cells were treated with AngII 10^{-7} M alone or together with SARS-CoV-2-RBD (Sino Biological, Milan, Italy) 10 ng/ml, 1 µg/ml, 10 µg/ml, or with MLN-4760 10^{-5} M. After 14 hours, media were collected and Ang 1-7 was measured with liquid chromatography tandem-mass spectrometry analysis (LC-MS/MS), as described (Domenig et al., 2016) by Attoquant Diagnostics GmbH (Vienna, Austria) in collaboration with Dr Marko Poglitsch and Dr Oliver Domenig.

5.5. SARS-CoV-2 Infection assays

5.5.1. Pseudotyping of VSV

In collaboration with Prof. Sara Richter group (Department of Molecular Medicine of University of Padua) experiments using SARS-CoV-2 pseudoviral particles or active SARS-CoV-2 were performed. Expression plasmids for vesicular stomatitis virus (VSVΔG-fLuc), glycoprotein (VSV- G), and SARS-CoV-2 spike protein (pCAGGS_SARS-CoV-2_spike, provided by the National Institute for Biological Standards and Control, NIBSC), used for the generation of SARS- CoV-2 pseudovirus, were previously reported (Cegolon et al., 2021; Hoffmann et al., 2020a; Whitt, 2011). VSV-ΔG-fLuc and the VSV-G expressing plasmid were kindly provided by

Prof. Cristiano Salata (Dept. Molecular Medicine, University of Padua). Vesicular Stomatitis Virus (VSV) pseudotypes were generated according to published protocols (Cegolon et al., 2021; Rentsch and Zimmer, 2011). In brief, 293T, transfected with Lipofectamine 3000 (Cod. # L3000015, ThermoFisher Scientific, Milan, Italy) to express the viral surface glycoprotein under study, was inoculated with a replication-deficient VSV vector that contains the expression cassette of firefly luciferase in place of the VSV-G open reading frame, VSV-ΔG-fLuc (kindly provided by Prof. Cristiano Salata, Dept. Molecular Medicine, University of Padua). After an incubation period of 1 h at 37 ◦C, the inoculum was removed, cells were washed with PBS, and fresh culture medium was added to the cell monolayer. Pseudotyped particles were harvested 16 h post inoculation and clarified from cellular debris by centrifugation and used for experiments.

5.5.2. Infection with SARS-CoV-2 pseudovirus

Calu-3 cells were seeded in 24 wells plates at a density of $7x10^4$ cells per well and stimulated described in section 5.2.3. After 48 hours, culture medium was removed, and cells were inoculated with SARS-CoV-2 pseudovirus (in DMEM-F12 with no FBS) at a multiplicity of infection (MOI) of 0.05 for 1 h at 37°C in a humidified incubator. After this time, the medium containing the SARS-CoV-2 pseudovirus was removed, cells were washed in 1x PBS, and fresh compete medium was added to each well. In order to evaluate the infection efficiency, activity of firefly luciferase was measured 16 hpi in cell lysates using a commercial substrate (Cod. #6066766; Britelite plus, PerkinElmer Italia, Milan, Italy), and luminescent signal was detected using a plate luminometer (VictorX2, PerkinElmer, Milan, Italy). Luciferase values were normalized by the cellular protein content, quantified by BCA assay (Pierce™ BCA Protein Assay Kit, ThermoFisher Scientific) as previously described.

5.5.3. Generation of SARS-CoV-2 Viral Stock

SARS-CoV-2 was kindly provided by Prof. Sara Richter's Lab at Department of Molecular Medicine, Microbiology Unit. For infection experiments with SARS-CoV-2, the SARS-CoV-2 isolate Milan IT (NCBI sequence MW000351.1) was propagated in VeroE6 cells. Cells were seeded (3.5×10^6) in complete medium (DMEM supplemented with 10% FBS) in T-175 vented-cap flasks the day before infection. Complete medium was removed, and cells were infected with the SARS-CoV-2 virus (MOI 0.01, in DMEM no FBS) for 1 h at 37°C in a humidified incubator. The medium containing the virus was removed and replaced with fresh medium (DMEM supplemented with 2% FBS). Supernatants were collected, centrifuged at 2300 rpm for 10 min, and then stored in aliquots at −80°C.

5.5.4. Infection with SARS-CoV-2 virus

Calu-3 cells were seeded in T-25 flasks at density of $1x10^6$/flask. After 48h, cells were treated as previously described with Ang II 10^{-7} M alone or in combination with irbesartan 10^{-5} M and ramipril 10^{-5} M for 24 hours. Then, cells were detached and plated in a 96 wells plate at a density of $27.5x10^3$ per well, continuously stimulated as described above. After other 24 hours under stimulation, medium was removed, and cells were infected (in DMEM-F12 no FBS) with SARS-CoV-2 (strain IT, lineage B, Pangolin UK) for 1 h at 37°C at MOI 0.05, mock controls were included in each experiment. One hour post infection, the medium containing the SARS-CoV-2 virus was removed, cells were washed in 1x PBS, and fresh complete medium added with stimuli was added to each well. At 48 hpi, cell media were collected and used to measure viral titre (expressed as PFU/ml) by Plaque Reduction Assay in VeroE6 cells.

5.5.5. SARS-CoV-2 Plaque Reduction Assay (PRA)

Vero E6 cells were seeded in 24-well plates at a density of $9x10^4$ cells per well for 24 hours before the assay. 10-fold serial dilutions from 10^{-1} to 10^{-9} of infected Calu-3 cells media were added in a final volume of 250 µL per well. After 1h incubation at 37°C, medium was removed from each well and replenished by 500 µl DMEM with 2% FBS and 0,6% carboxymethyl cellulose (CMC) as “immobilization overlay” in order to block the spreading of viral infection. After 48 hours incubation, CMC was removed and 5% paraformaldehyde in PBS was added to each well for 1 hour at room temperature and then removed. Fixed monolayers were then washed with water and stained with crystal violet solution in 20% ethanol. Crystal violet very quickly binds to Vero E6 cell membrane, allowing to clearly identify plaque formation due to the cytopathic effect of SARS-CoV-2. Viral titer is measured by quantifying plaque formation on cell monolayer and recorded as Plaque Forming Unit (PFU)/mL.

5.6. Data analysis

Statistical analyses were performed with GraphPad Software™ (vers. 9.1, La Jolla, CA). Data were analysed with two-tailed non-parametric test (Mann Whitney) or Kruskall- Wallis test for multiple comparisons.

6. RESULTS

6.1. *ACE2* and *AGT1R* are expressed at high levels in Calu-3 cells

We measured the absolute amounts of *ACE2* and *AGT1R* mRNA copies with digital-droplet PCR in HUVEC and Calu-3 cells as a preliminary step for the following experiments. As shown in Figure 3, *ACE2* and *AGT1R* genes were abundantly expressed in Calu-3, but were barely detectable in HUVEC (Figure 3, panels A and B). Therefore, we decide to perform all the further experiments with Ang II and other agonists and antagonists just in Calu-3 cells.

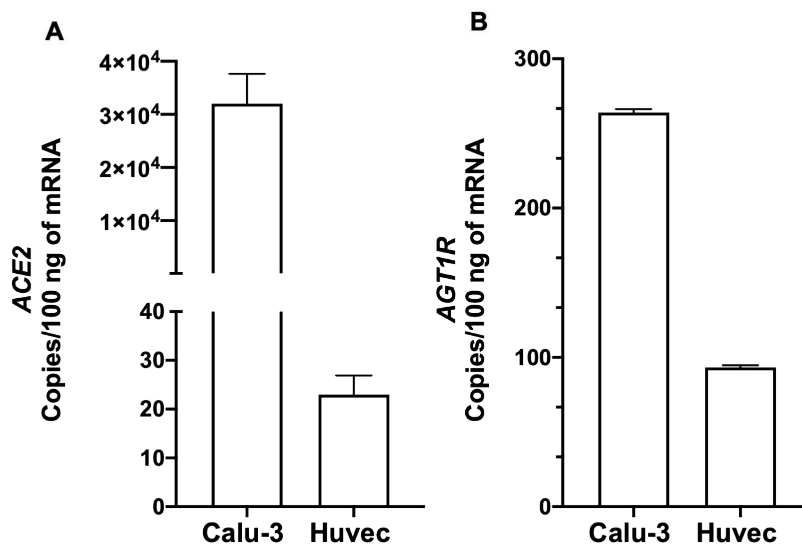

Figure 3. *ACE2* and *AGT1R* absolute mRNA levels in Calu-3 cells and HUVECs. *ACE2* and *AGT1R* absolute mRNA levels in Calu-3 cells and HUVECs were measured in basal conditions with ddPCR. Please note the much higher *ACE2* (Panel A) and *AGT1R* (Panel B) mRNA levels in Calu-3 cells than in HUVECs. Data are shown as copies/100 ng total mRNA (n=4, median ± QR, min to max).

6.2. Expression of RAS and KKS in Calu-3 cells

In order to characterize Calu-3 cells for the expression of RAS and KKS components, we performed ddPCR to investigate the expression levels of the following molecules: renin (*REN*), angiotensinogen (*AGT*), ACE (*ACE1*), AT1R (*AGT1R*), kininogen (KNG) and bradykinin receptors type 1 (*BDKRB1*) and 2 (*BDKRB2*) (Table 2). We found that Calu-3 express high levels of *AGT*

and *ACE1*, and small low but detectable levels of the other components investigated. Moreover, taking advantage of LC-MS/MS, we investigated the levels of angiotensin peptides present in Calu-3 cell media, with or without addition of exogenous Ang II 10^{-7} M (Table 3). We found that after 14 hours, Ang II was completely converted into Ang 1-7, and detectable levels of Ang 1-5, Ang 3-7 and Ang 2-7 were also observed in cell culture media from cells stimulated with Ang II 10^{-7} M. Instead, very low levels of Ang 2-8 and Ang 3-8 were found in the same supernatants. All the angiotensin peptides analysed were present at very low levels in untreated cell media.

Gene	Copies/100 ng total mRNA (mean ± SEM)
REN	67.4 ± 25.4
AGT	15008.0 ± 6079.0
ACE1	1687.0 ± 689.0
AGT1R	105.9 ± 26.5
KNG1	39.4 ± 16.2
BDKRB1	140.7 ± 90.0
BDKRB2	74.4 ± 41.8

Table 2. Gene expression levels of RAS and KKS components, evaluated by ddPCR (n=3).

Peptide	Vehicle	Ang II 10^{-7} M
Ang II (1-8) [nM]	<0.005	<0.005
Ang 1-7 [nM]	<0.005	10.3 ± 4.4
Ang 2-8 [nM]	<0.005	<0.005
Ang 3-7 [nM]	<0.005	0.3 ± 0,1
Ang 1-5 [nM]	<0.005	4.1 ± 3.5
Ang 3-8 [nM]	<0.005	<0.005
Ang 2-7 [nM]	<0.005	0.02 ± 0.009

Table 3. Angiotensin-derived peptides levels in Calu-3 cell media, evaluated by LC-MS/MS (n=3).

6.3. ACE2 activity is not modulated by SARS-CoV-2 RBD binding

ACE2, together with Ang 1-7, is the main actor in the RAS protective branch. To investigate whether the binding of RBD-S1 protein to ACE2 is able to modulate its activity, therefore the production of Ang 1-7, cells were stimulated with Ang II (as substrate) and RBD from SARS-CoV-2 at the concentration of 10 ng, 1 µg, 10 µg/ml for 14 hours and Ang 1-7 levels were measured by LC-MS/MS. At the same time, we performed a bioinformatic analysis based on the sequences recognized by S1-SARS-CoV-2 and the one responsible for ACE2 catalytic activity. Both analyses suggested that viral binding to ACE2 did not alter its own activity (Figure 4).

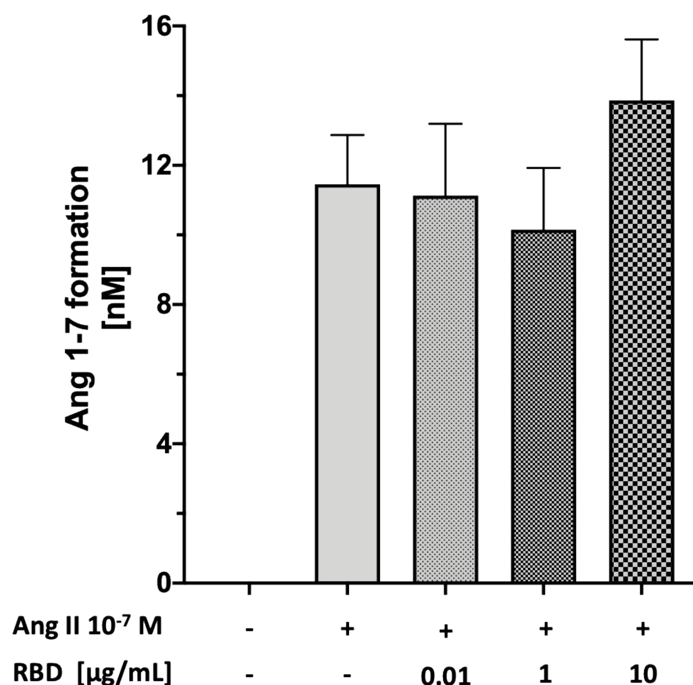

Figure 4. ACE2 activity is not modulated by the binding of the RBD-S1 protein in Calu-3 cells. LC-MS/MS data reported that Ang 1-7 formation can be detected only when ACE2 substrate, Ang II 10^{-7} M Ang II M is added and that its conversion is not modulated by the presence of different concentrations of RBD from the SARS-CoV-2 S1 protein. Data are reported as Ang 1-7 nmol/L [nM] detected in the cell media after 14 hours stimulation (n=3, all comparison were not significantly different).

6.4. Ang II, but not Ang 1-7, upregulates *ACE2* expression

In order to investigate whether Ang II modulates ACE2 expression, Calu-3 cells were stimulated with increasing concentration of Ang II, ranging from 10^{-9} M to 10^{-7} M, for 12h or 48h, to evaluate *ACE2* gene and protein levels, respectively. As shown in Figure 5, Ang II increases *ACE2* mRNA ($p<0.001$) and protein ($p<0.05$) levels in a dose-dependent manner, reaching significant difference at the highest concentrations analysed (10^{-7} M), over vehicle-treated control cells (Figure 5, panels A and B). The concentration of 10^{-6} M was also used, but, because of its cytotoxic effect, was not considered for the analysis.

Since data from LC- MS/MS reported that Ang II 10^{-7} M was fully converted into Ang 1-7 at the time point analysed (Table 3), we wanted to exclude that the effect observed on *ACE2* expression by Ang II was actually mediated by the product of its conversion, Ang 1-7. To this aim, cells were stimulated with concentrations of Ang 1-7 increasing 1000-fold, from 10^{-8} M to 10^{-5} M. The results showed that, even at the highest concentration, the peptide had no effects on *ACE2* expression, thus suggesting that the stimulatory effect of Ang II is not due to Ang 1-7 formation (Figure 5, panel C).

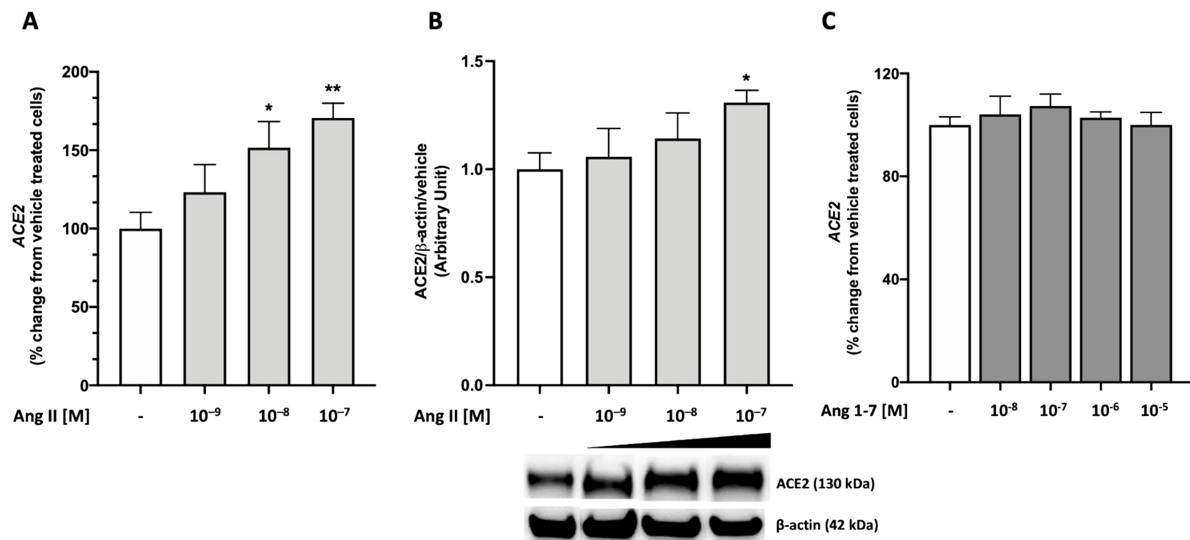

Figure 5. Ang II, but not Ang 1-7, modulates *ACE2* expression in Calu-3 cells. Panel A shows *ACE2* gene expression after 12 hours exposure of Calu-3 cells to Ang II concentrations ranging from 10^{-9} M to 10^{-7} M (n=3 in duplicate; mean ± SEM). Immunoblots and histograms show that 10^{-7} M Ang II strongly increased ACE2 protein levels in Calu-3 cells after 48 hours exposure. Data were normalized on vehicle and β-actin as a loading control (Panel B) (n=3 in triplicate; mean ± SEM. Panel C shows *ACE2* gene expression after 12-hours exposure of Calu-3 cells to increasing concentration of Ang 1-7 ranging between 10^{-8} M to 10^{-5} M. Ang 1-7 did not affect *ACE2* gene expression. Data were measured as copies/100 ng total mRNA and then normalized vs vehicle treated cells (n=5 in duplicate; mean ± SEM; * p<0.05 vs control, ** p<0.01 vs control).

6.5. ACE2 blockade does not play an additive role in Ang II effects

ACE2 blockade, by blocking conversion of endogenous Ang II into Ang 1-7, might rise Ang II in the cell culture medium, strengthening the effect observed on ACE2 expression. To test this hypothesis, Calu-3 cells were stimulated with Ang II 10^{-7} M in presence of the ACE2 inhibitor MLN-4760. Results showed that MLN -4760 efficiently blocked Ang II conversion into Ang 1-7 (Figure 6, panel A) and, *per se,* it had no effect on *ACE2* mRNA neither on the increase of *ACE2* induced by Ang II 10^{-7} M (Figure 6, panel B), revealing that blockade of Ang 1-7 formation did not strengthen the effect of Ang II on *ACE2* mRNA.

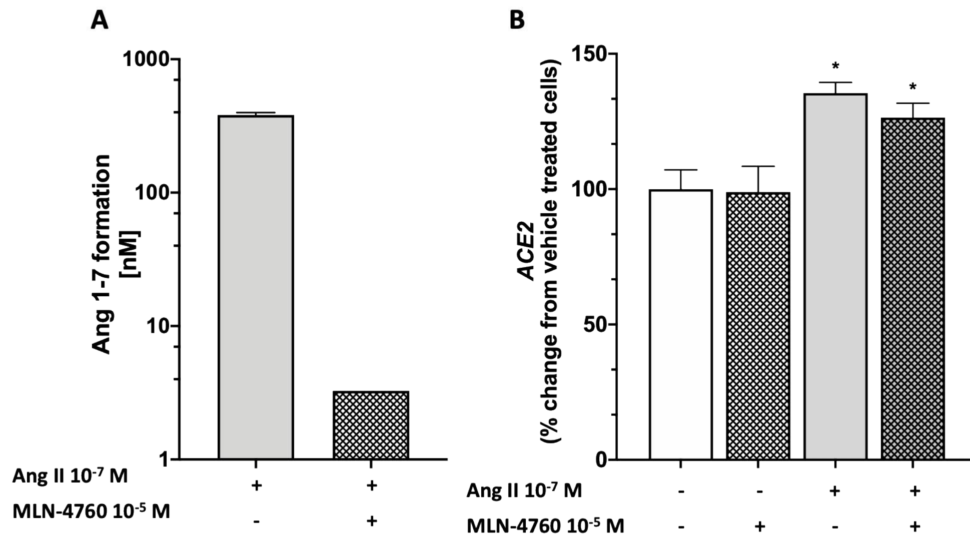

Figure 6. *ACE2* mRNA levels are not modulated by ACE2 blockade with MLN-4760. LC-MS/MS data reported that Ang II 10^{-7} M was fully converted into Ang 1-7 within the 12 hours stimulation, and this event was prevented by ACE2 inhibitor, MLN 4760 (panel A). Panel B showed *ACE2* mRNA levels after exposure of Calu-3 cells to Ang II 10^{-7} M in presence of 10^{-5} M MLN-4760. MLN-4760 treatment did not enhance the effect of Ang II on *ACE2* gene expression (n=5 in duplicate; mean ± SEM; * p<0.05 vs control).

6.6. ACEi and ARBs dose response on ACE2 expression

To better clarify whether the effect of Ang II on ACE2 expression was mediated by AT1R and/or can be modulated by ACE activity, Calu-3 cells were first treated with the ARBs irbesartan and candesartan or the ACEis ramipril and captopril, at different concentration ranging from 10^{-7} M to 10^{-5} M for 12 or 48 hours to assess *ACE2* gene and protein expression respectively. The concentration of 10^{-4} M for all the drugs was also considered, but cytotoxic effect was detected. As shown in Figure 7, both ARBs did not modulate *ACE2* gene and protein expression at either concentration (Figure 7, panels A and C). Similar effects were found with the ACEi captopril (Figure 7, panel B and D), whereas ramipril behaved differently: while *ACE2* gene expression was not modulated by this drug, its protein expression was increased in a dose-dependent manner.

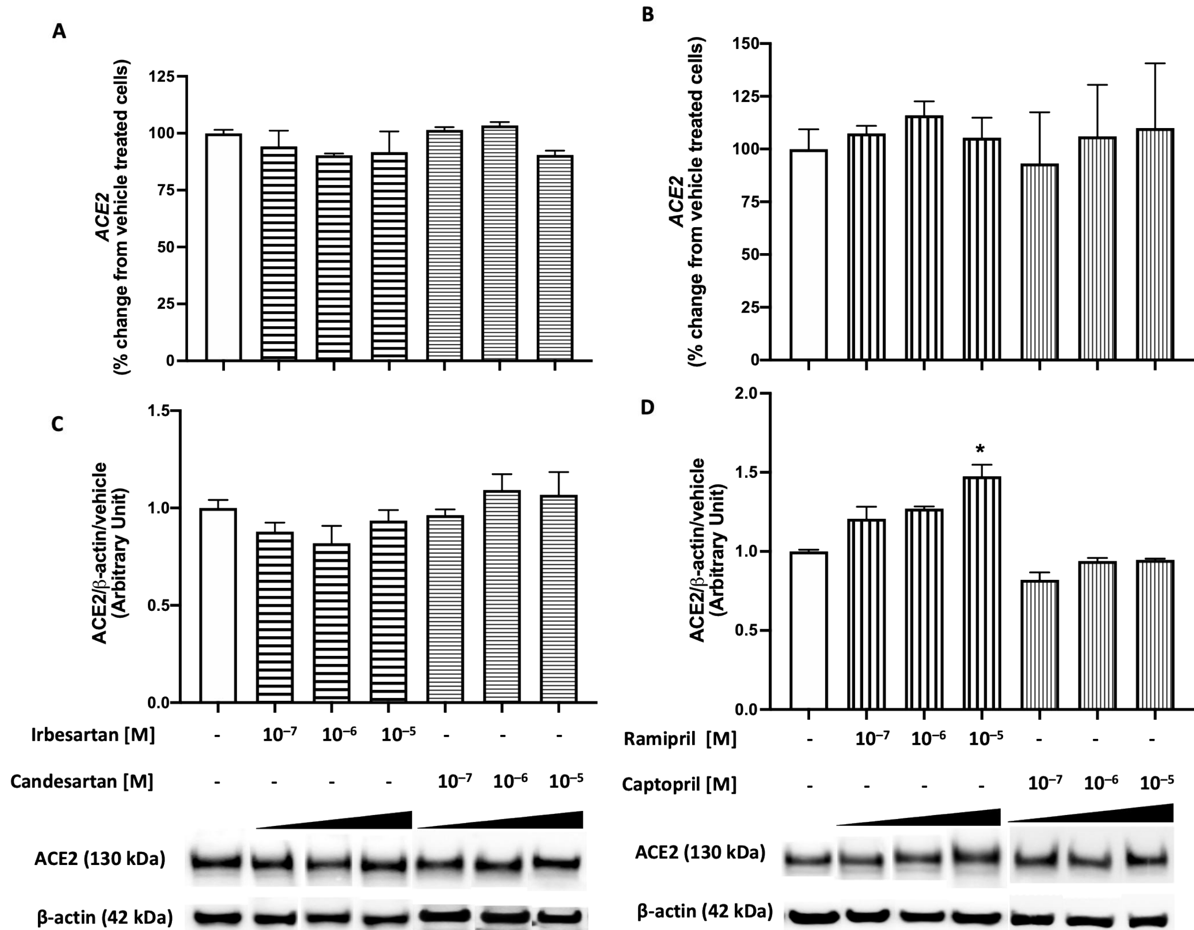

Figure 7. ***ACE2*** **mRNA and protein levels are not modulated by ACEis and ARBs** ***per se*****.** Both irbesartan and candesartan did not modulate ACE2 expression both at gene (panel A) and protein level (panel C), at none of the concentration analysed (10^{-7} M, 10^{-6} M, 10^{-5} M). The ACEis ramipril and captopril at 10^{-7} M, 10^{-6} M, 10^{-5} M did not alter ACE2 gene expression (panel B), but ramipril increased ACE2 protein levels at 10^{-5} M (panel D). ddPCR results are shown as a percentage over the vehicle-treated control (n=3 in duplicates, mean ± SEM). Immunoblotting data (from densitometric analysis) were normalized over vehicle and β-actin as a loading control (n=3 in duplicates, mean ± SEM) and representative bands are shown at the bottom of the panels B and D (*p<0.05).

6.7. Ang II effect on ACE2 expression is mediated by AT1R

Calu-3 cells were treated with irbesartan (10^{-6} M and 10^{-5} M) or candesartan (10^{-6} M and 10^{-5} M), ramipril 10^{-5} M or captopril 10^{-5} M alone or on top of Ang II (10^{-8} M and 10^{-7} M). As expected, Ang II significantly increased *ACE2* gene expression at both concentrations used (Figure 5, panel A and Figure 8, panel A), while only Ang II 10^{-7} M increased ACE2 protein levels (Figure 5, panel B). The ARBs irbesartan and candesartan at a 100-fold higher concentration (10^{-5} M) had no effect on ACE2 expression *per se* (Figure 7, panels A and C), but they abolished

the effect of Ang II (both at 10^{-8} M and 10^{-7} M) on *ACE2* mRNA (Figure 8, panel A). Moreover, candesartan inhibited the Ang II-induced *ACE2* upregulation starting from 10^{-6} M (Figure 8, panel A). Since the highest inhibitory effect of both ARBs is reached at 10^{-5} M, we decided to use this concentration for the further experiments (and similarly for ACEi). Since only Ang II 10^{-7} M significantly increased ACE2 protein levels (Figure 5, panel B), we analysed the effect of ARBs only with this concentration of Ang II, finding that both irbesartan and candesartan at 10^{-5} M reduced ACE2 protein levels after Ang II 10^{-7} M stimulation (Figure 8, panel B). All these data strongly suggest that the effect is AT1R mediated.

Both ACEis alone, ramipril and captopril, at 10^{-5} M did not modulate *ACE2* mRNA expression, neither they abolished the effect induced by Ang II on ACE2 expression (Figure 8, panel C). This effect was expected since ACEis block Ang II formation and therefore are not supposed to blunt Ang II downstream effect. According to our data, however, captopril seemed to play a role by decreasing ACE2 protein levels previously induced by Ang II, while ramipril did not (Figure 8, panel D). Instead, as already suggested by dose-response experiments (Figure 7, panel D), ramipril, but not captopril, raised ACE2 levels, even higher than Ang II (Figure 8, panel D). We hypothesized that this effect, not observed at mRNA levels, can be probably driven by molecular mechanisms regulating ACE2 expression at a post-transcriptional level, based on drug molecular structure, which is strongly different from captopril.

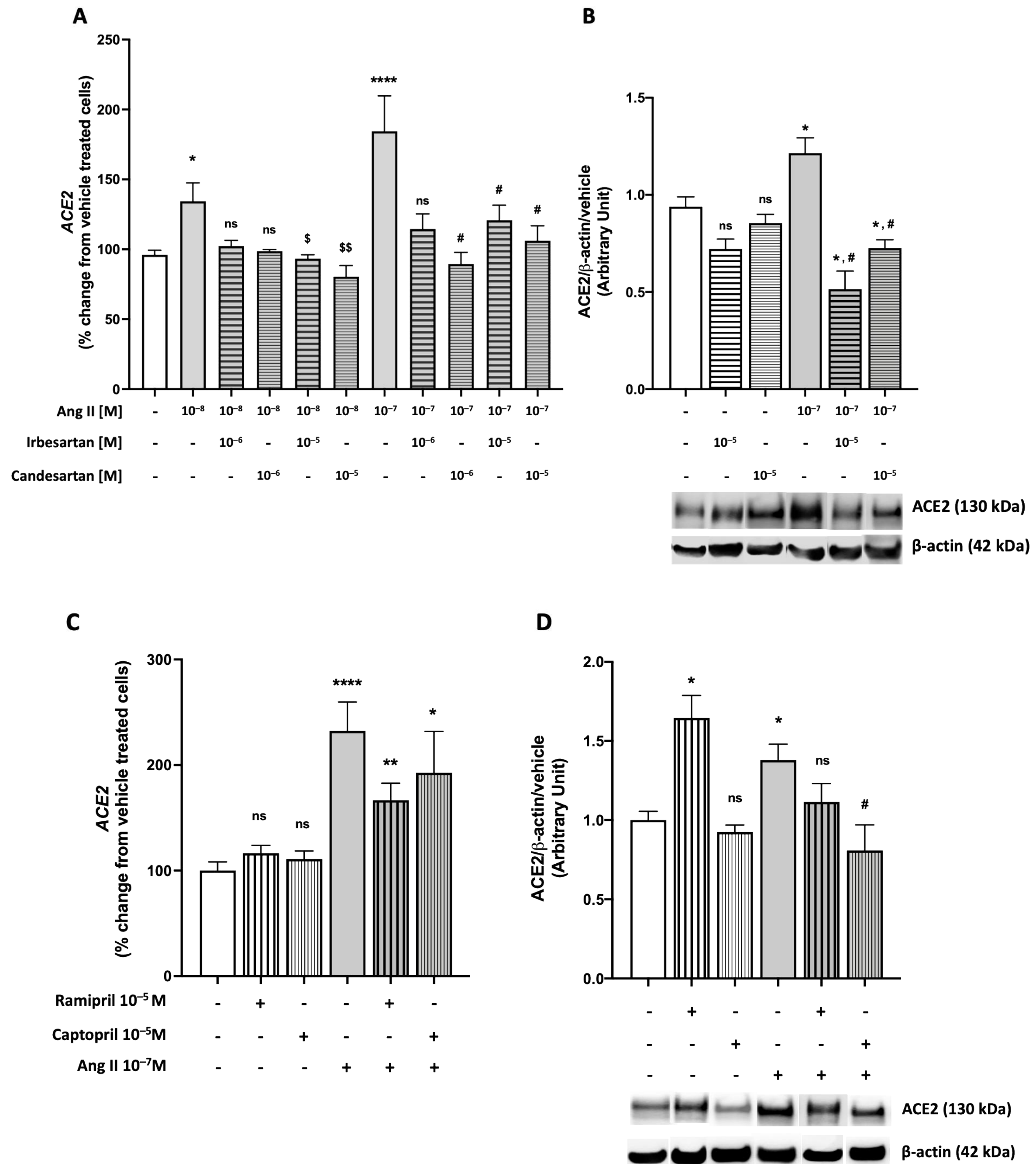

Figure 8. Ang II upregulation of ACE2 expression in Calu-3 cells occurs via AT1R signalling. ARBs 10^{-5} M abolished the stimulatory effect of Ang II both at 10^{-7} M and 10^{-8} M on *ACE2* mRNA (panel A) in Calu-3 cells. ACEis 10^{-5} M, *per se* had no effect on *ACE2* mRNA, neither abolished the stimulatory effect of Ang II 10^{-7} M on *ACE2* mRNA (panel C) in Calu-3 cells. Histograms from densitometric analysis of immunoblotting show that Ang II 10^{-7} M increased ACE2 protein levels in Calu-3 cells after 48 hours exposure and again ARBs abolished this effect (panel B), while ramipril, but not captopril, enhances ACE2 protein expression (panel D). Immunoblotting data (from densitometric analysis) were normalized over vehicle and β-actin as a loading control (at least n=3 in triplicate; mean ± SEM) and representative bands are shown at the bottom of the panels B and D. ddPCR data were measured as copies/100 ng total mRNA and here reported as a percentage over the vehicle control (at least n=4 in duplicate; mean ± SEM, * $p<0.05$ vs control, ** $p<0.01$ vs control, ***$p<0.001$ vs control, ****$p<0.0001$ vs control; $ $p<0.05$ vs Ang II 10^{-8} M, $$ $p<0.05$ vs Ang II 10^{-8} M; # $p<0.05$ vs Ang II 10^{-7} M, ## $p<0.01$ vs Ang II 10^{-7} M).

6.8. Ang II, irbesartan and ramipril modulate SARS-CoV-2 infection in human lung cells

To determine the functional consequences of Ang II-induced ACE2 increase, we investigated the effects of irbesartan and ramipril on the cell entry of the SARS-CoV-2 wild-type virus in Calu-3 cells (Figure 9, panel A), since they are a susceptible and permissive human lung model for SARS-CoV-2 infection (Cagno, 2020).

Cells were infected with SARS-CoV-2 at MOI 0.05 after preincubation with Ang II alone or on top of irbesartan or ramipril. To test the hypothesis that Ang II could augment the cell receptors/mechanisms exploited by SARS-CoV-2 to enter the cells, the effects of the peptide were tested, alongside that of the known TMPRSS2 protease inducer DHT (Leach et al., 2021), by measuring the amount of new infective particles (viral titre) 48 hours post-infection (hpi) in plaque reduction assay (Figure 9, panel D). Results showed that both Ang II and DHT markedly enhanced (by 352% and 333%, respectively) SARS-CoV-2 viral titres (Figure 9, panel D). We found that irbesartan also slightly (by 158%) promoted viral infection over controls. More importantly, it drastically blunted the viral infection induced by Ang II (Figure 9, panel D) indicating that Ang II-induced increased cell infection by SARS-CoV-2 virus involves the AT1R. Likewise, ramipril alone was found to enhance viral infection (by 160%) but, like irbesartan, also ACEi treatment blunted the effect of Ang II, suggesting a beneficial role of this drug overall. To determine if the effects observed with SARS-CoV-2 virus depended on the entry of virus entry in Calu-3 cells, we also used the SARS-CoV-2 pseudovirus, a luciferase-expressing vesicular stomatitis virus (VSV) that was pseudotyped with SARS-CoV-2 Spike protein (Figure 9, panel B). This pseudovirus recapitulates the entry step of SARS-CoV-2 (Jonsson et al., 2009; Tai et al., 2015), while the later steps occurring after cell entry reflect a

general mechanism of viral infection rather than those specifically involving SARS-CoV-2 (Figure 9, panel B). After challenging Calu-3 cells with SARS-CoV-2 pseudovirus at MOI 0.05, we observed that both Ang II and DHT enhanced pseudoviral entry, as measured by the luciferase signal, by 160% and 180%, respectively, over control cells (Figure 9, panel E). In line with what observed with the wild-type SARS-CoV-2 virus, irbesartan and ramipril also enhanced pseudoviral entry, albeit to a lower extent than seen with Ang II and DHT. More importantly, these drugs effectively counteracted the pseudovirus cell entry promoted by Ang II (Ribstein et al., 2001) (Figure 9, panel E). To further understand the mechanisms by which Ang II, DHT, irbesartan and ramipril affected cell entry-enhancing, we examined the later steps of virus infection using the luciferase expressing VSV (named VSV-G pseudovirus) with its own natural attachment glycoprotein (i.e. G protein) (Kim et al., 2017), that recognizes low-density lipoprotein receptor (LDL-R) (Nikolic et al., 2018) and it is independent of ACE2 expression (Figure 9, panel C). We found that both DHT and Ang II had a negligible activity on VSV-derived luciferase signal (Figure 9, panel F), thus indirectly supporting the notion that these two compounds enhanced the wild-type SARS-CoV-2 virus and the SARS-CoV-2 pseudovirus infection via enhanced attachment and processing of the spike protein to ACE2 and ensuing involvement of TMPRSS2. Moreover, we checked whether DHT can also increase *TMPRSS2* expression in our system, since it is already well known in other cell types (Leach et al., 2021). We found that also in Calu-3 cells *TMPRSS2* expression is enhanced after DHT treatment after 12h and 24h stimulation (Figure 10, panel A). Irbesartan and ramipril also slightly stimulated VSV-G-pseudovirus infection by 124% and 121% respectively, while the co-exposure to Ang II did not affect its action (Figure 9, panel F). These data suggest that these compounds may enhance cellular permissiveness to some viruses *in vitro*, independently of ACE2 receptor and spike protein. We hypothesize that the enhanced VSV-G-pseudovirus infection could be due

to an increased expression of its receptor, LDL-R. Therefore, Calu-3 cells were stimulated with irbesartan 10^{-5} M or ramipril 10^{-5} M alone for 12h and *LDLR* gene expression was investigated. Data showed a slight significant increase in *LDLR* expression in treated cells over controls (Figure 10, panel B), supporting our hypothesis. Altogether, the experimental setting here reported, aimed at mimicking the pathological condition of the patients with an active RAS who are taking ARBs or ACEis therapy, highlighted that these drugs did not augment SARS-CoV-2 (or pseudovirus) infection in human lung cells.

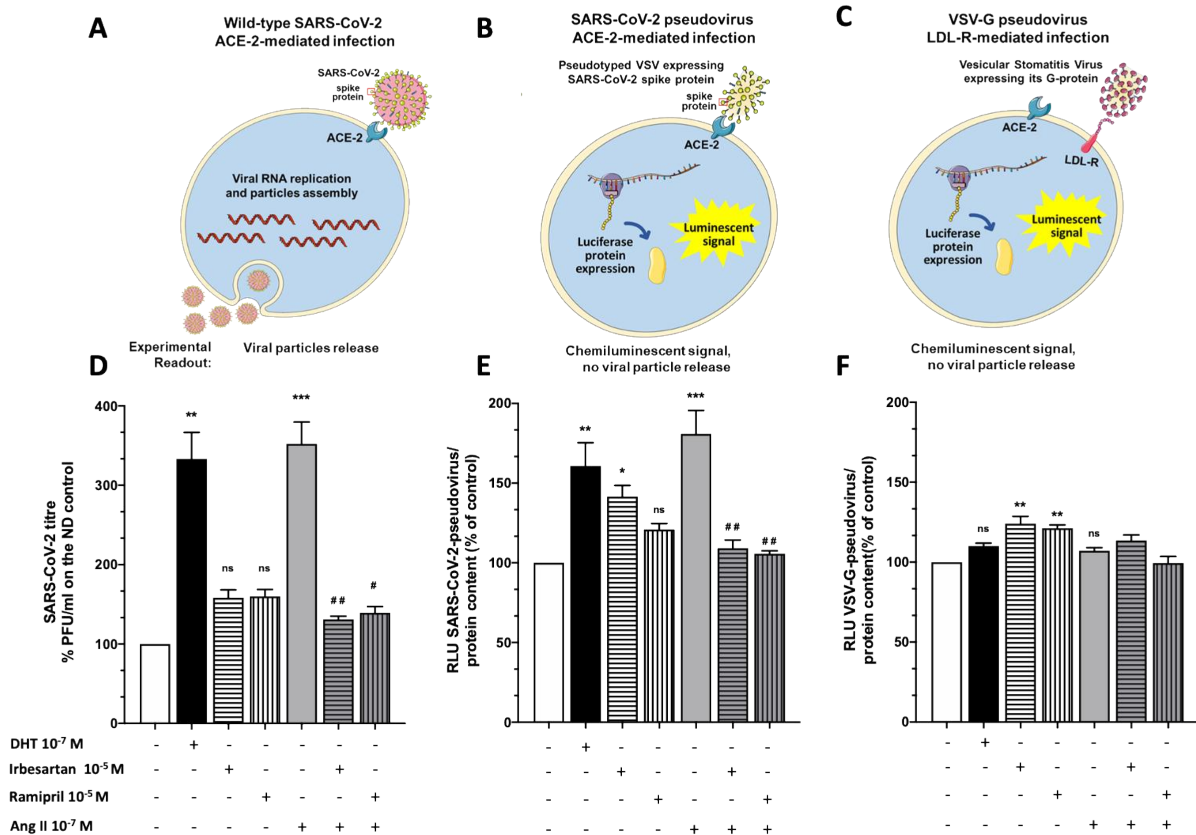

Figure 9. Effects of Angiotensin II and irbesartan on SARS-CoV-2 pseudovirus and virus infection in human lung cells. The cartoon illustrates the 3 different assays that were used to evaluate the effect of Ang II, irbesartan and ramipril on SARS-CoV-2 infection in Calu-3 cells: with wild-type SARS-CoV-2 virus (panel A), with pseudotyped vesicular stomatitis virus (VSV) expressing SARS-CoV-2 spike protein (panel B), and with a genetically modified VSV that binds to the low-density lipoprotein receptor and not to ACE2 (panel C). The three experimental designs were illustrated with Medical Art. Please note that the size of the Calu-3 cells and viruses are shown for the purpose of graphical illustration and not correspond to reality. Calu-3 cells were pre-treated for 48h with dihydrotestosterone (DHT) 10^{-7} M , Ang II 10^{-7} M, irbesartan 10^{-5} M, ramipril 10^{-5} M alone or on top of Ang II prior to infection with the SARS-CoV-2 virus (panel D), with the SARS-CoV-2-pseudovirus (panel E) or with the VSV-G-pseudovirus (panel F). SARS-CoV-2 infection in Calu-3 cells indicated that DHT and Ang II treatment enhanced viral infection. Irbesartan and ramipril slightly promoted viral infection over controls but they drastically blunted the viral infection induced by Ang II (panel D, n=3 in triplicate; mean ± SEM). SARS-CoV-2-pseudovirus infection in Calu-3 cells showed that both DHT stimulation on TMPRSS2 protease and Ang II effect on ACE2 enhanced pseudoviral entry. A similar effect was obtained in presence of irbesartan and ramipril. The concomitant administration of Ang II and irbesartan or ramipril diminished the percentage of infection, with respect to the administration of Ang II alone (panel E, n=3 in triplicate; mean ± SEM). VSV-G-pseudovirus infection in Calu-3 cells showed negligible effect upon DHT and Ang II treatment, whereas irbesartan positively influenced pseudoviral infection. The concomitant administration of Ang II and irbesartan or ramipril showed a reduction in pseudoviral infection, with respect to the administration of Ang II or irbesartan or ramipril (panel F) (n=3 in triplicate; mean ± SEM; * p<0.05 vs control, ** p<0.01 vs control, ***p<0.001 vs control ;# p<0.05 vs Ang II, ## p<0.01 vs Ang II).

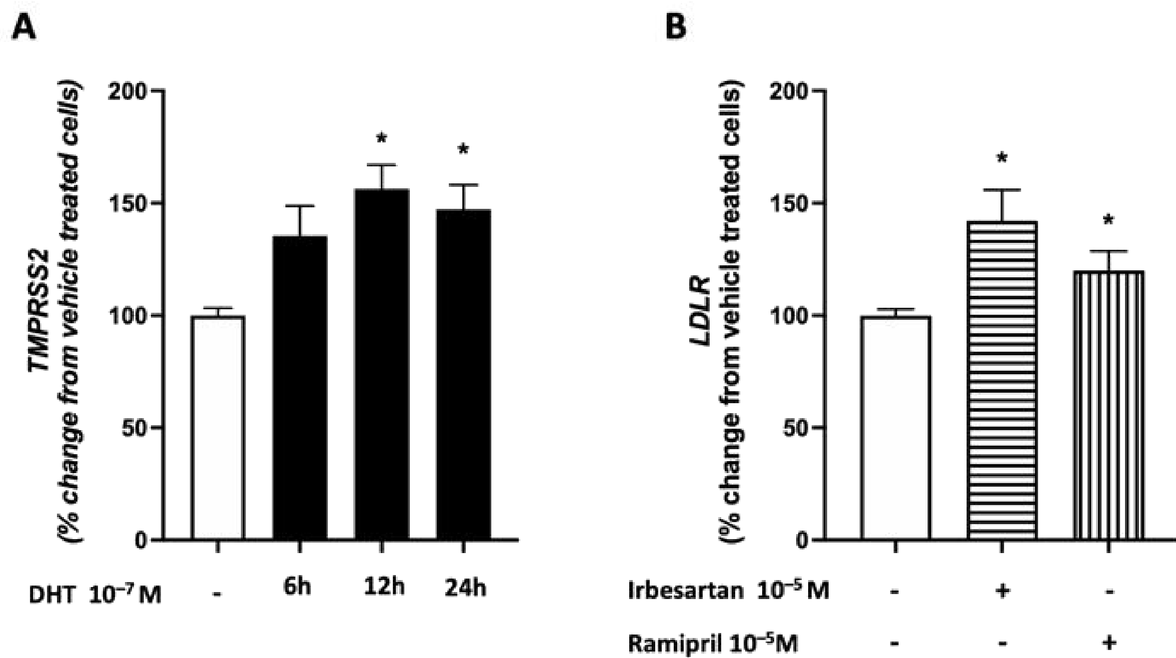

Figure 10. *TMPRSS2* expression is enhanced by DHT, while *LDLR* expression is enhanced by ACEi/ARBs treatment. DHT is known to enhance TMPRSS2 activity, therefore favouring SARS-CoV-2 S1 priming and cell infection. We found that DHT at 10^{-7} M is also able to increase *TMPRSS2* gene expression in Calu-3 cells, especially after 12h and 24h treatment (panel A). We also found that *LDLR* is highly expressed in Calu-3 cells and irbesartan 10^{-5} M and ramipril 10^{-5} M treatments can enhance its gene levels (panel B). ddPCR data are reported as a percentage over the vehicle-treated control (n=3 in duplicates, mean ± SEM; *$p<0.05$ vs vehicle).

6.9. Ang II does not modulate ACE2 shedding in Calu-3 cells

Ang II is known to enhance ACE2 shedding via upregulation of ADAM17 activity in cardiomyocytes (Patel et al., 2014). ACE2 ectodomain keeps its ability to be recognized by SARS-CoV-2, therefore contributing to the diffusion of viral infection. In order to investigate whether Ang II was able to activate the shedding process also in Calu-3 cells, we first investigated the effect of Ang II 10^{-7} M on *ADAM17* and *TMPRSS2* gene expression (Figure 11, panel A and Panel B). No differences were observed in *TMPRSS2* mRNA levels, while *ADAM17*

expression was found to be downregulated after Ang II stimulation compared to not-treated cells (Figure 11, panel A and B).

Moreover, we measured the levels of shedded ACE2 in the supernatant of Calu-3 cells after 3 hours treatment with Ang II 10^{-7} M. We found that shedded ACE2 levels were not modulated by Ang II at the time point analysed (Figure 11, panel C). However, further experiments are required since shedding process can be very fast and therefore also shorter time points should be considered.

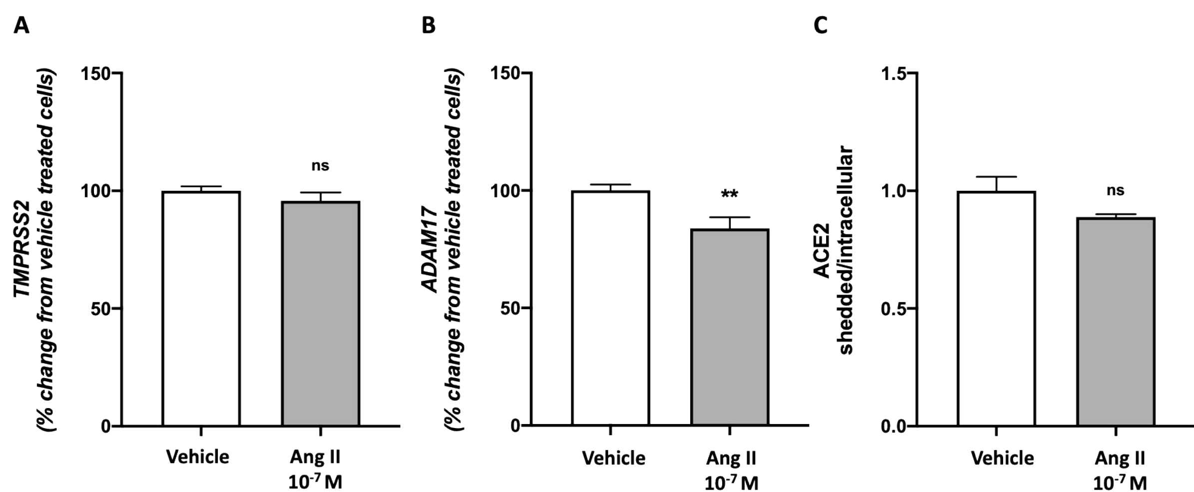

Figure 11. Ang II does not modulate ACE2 shedding in Calu-3 cells. Ang II 10^{-7}M had no effect on *TMPRSS2* mRNA (panel A), while significantly decreased *ADAM17* mRNA levels in Calu-3 cells (panel B). ddPCR data are reported as a percentage over the vehicle-treated control (n=3 in duplicates, mean ± SEM, **p<0.01). ACE2 levels in the supernatant of Calu-3 cells after 3h stimulation with Ang II 10^{-7}M, measured by ELISA, displayed no significant difference compared to the control (vehicle) (panel C). Data were measured according to the manufacturer instructions and normalized by the intracellular levels of ACE2, measured by ELISA as well (n=3, mean ± SEM; ** p<0.01 vs control).

7. DISCUSSION

An activated RAS, and thus increased Ang II levels, are found in millions of patients suffering from arterial hypertension, malignant hypertension, renovascular hypertension, liver cirrhosis with ascites, left ventricular hypertrophy (Dahlof et al., 2002; Schwalm et al., 2019), heart failure, kidney diseases, and myocardial infarction as well as in COVID-19 patients (Camargo et al., 2022; Osman et al., 2021; Wu et al., 2020). RAS blocker drugs, as ACEis or ARBs, but also the mineralocorticoid receptor antagonists, proved to be life-saving drugs for these patients (Caldeira et al., 2012; X. Guo et al., 2020; Jering et al., 2021; Liu et al., 2012; Mancia et al., 2020; Mentz et al., 2013; Mortensen et al., 2008; Pitt et al., 1997). Nevertheless, few reports suggested that these drugs increased the expression of ACE2, the SARS-CoV-2 receptor, in experimental models and led to the contention that RAS blockers might be detrimental in COVID-19 patients (Fang et al., 2020; Zheng et al., 2020). However, remarkably sized observational studies thereafter suggested that ACEis and ARBs are neutral (Mancia et al., 2020) or even beneficial in COVID-19 patients (X. Guo et al., 2020; Mehta et al., 2020b; Rossi et al., 2020), and recent clinical trials, evaluating ACEi or ARBs or both (Asiimwe et al., 2022; Bauer et al., 2021; Cohen et al., 2021; Duarte et al., 2021; Lopes et al., 2021; Macedo et al., 2022; Sato et al., 2022; Yin et al., 2022) supported this view.

Lung endothelial cells are a major site of the conversion of Ang I to Ang II, suggesting that bronchial epithelial cells are exposed to high concentrations of the peptide (Morton, 1993). The human bronchial epithelial cells Calu-3 have been widely used as a model to explore the mechanisms of SARS-CoV-2 infection (Lamers et al., 2021; Wyler et al., 2021), considering that the lung is a main target of SARS-CoV-2 infection. Human endothelial cells were also contended to be a target of the SARS-CoV-2 spike protein (Y Lei et al., 2021). By investigating Calu-3 and umbilical vein endothelial cells (HUVEC), we found that while the latter presented

barely detectable levels of *ACE2* and *AGT1R* mRNA, the former cells expressed abundant mRNA amounts of both these genes (Figure 3, panels A and B), indicating that Calu-3 cells are a suitable model to investigate the role of RAS in SARS-CoV-2 infection. Moreover, taking advantage of the high sensitivity of ddPCR, we found also detectable levels of RAS and KKS components in Calu-3 cells, even in cases where they had not been reported before (Lang et al., 1995). Of note, angiotensinogen (*AGT*) and *ACE* gene were found to be highly expressed in our cell model (Table 2), suggesting that local Ang II can be produced, although low levels were found by LC-MS/MS analysis (Table 3). However, ACE2 levels are much higher and, as suggested by LC-MS/MS data, also all the exogenous Ang II is converted into Ang 1-7 (Table 3). Importantly, we herein showed that Ang II, at concentrations mimicking the biological and pathophysiological levels of the peptide in COVID-19 patients (Wu et al., 2020), acted as a potent enhancer of ACE2 expression (Figure 5, Panels A and B). We could also demonstrate that this augmenting effect was mediated by the AT1R, as it was abolished by the ARBs irbesartan and candesartan (Figure 8). In our view, these results are important in that they providea mechanistic explanation for the beneficial effects of the RAS antagonists documented in observational studies as well as in meta-analyses (Asiimwe et al., 2022; T. Guo et al., 2020; Jeon et al., 2021; Kotfis et al., 2021; Shi et al., 2020; Yin et al., 2022). On the contrary, Baba and colleagues (Baba et al., 2020) did not found an increase in *ACE2* gene expression after Ang II 10^{-7} M treatment: this is not surprising, since they used a different time point (3h) but, as shown in Figure 5, the Ang II effect on *ACE2* was found only at 12 hours and 48 hours on gene and protein expression, respectively. However, they also found no changes after ACEi or ARBs treatments in Calu-3 cells, as we reported.

Moreover, the present study provided novel information on the so-called “protective” branch of RAS. As up-regulation of ACE2 would be expected to increase the conversion of Ang II,

both endogenous and added to the cell culture, it could be argued that the effect of Ang II was mediated and/or modulated by its conversion into Ang 1-7. Our results unambiguously showed that Ang 1-7, the main effector of the protective RAS, did not affect *ACE2 per se* (Figure 5, panel C), either under baseline conditions or when ACE2 activity was blocked by MLN-4760 (Figure 6). Thus, Ang II upregulates ACE2 directly by acting via the AT1R, and, at least *in vitro,* this up-regulation did not imply enhanced conversion of Ang II to Ang 1-7. Moreover, we found that the binding of the RBD-S1 protein of SARS-CoV-2 to ACE2 did not modulate ACE2 activity (Figure 4). This finding is in line with the clinical studies reporting no changes in Ang 1-7 levels in COVID-19 patients (Camargo et al., 2022; Osman et al., 2021).

Also, it was reported that Ang II could enhance ACE2 shedding promoting ADAM17 activity (de Queiroz et al., 2020) and expression in cardiomyocytes (Patel et al., 2014). On the contrary, in our *in vitro* experimental model, we found that Ang II does not modulate the amount of shedded-ACE2 in cell media at 3h post-treatment (Figure 11, panel C), and it downregulates *ADAM17* mRNA levels compared to controls (Figure 11, panel B), suggesting that Ang II plays a different role in lung epithelial tissue. Moreover, also Osman and colleagues found low levels of sACE2 and high levels of Ang II in plasma of COVID-19 patients compared to healthy volunteers (Osman et al., 2021), suggesting that, also in this case, there is not a direct correlation with increased levels of Ang II and shedded ACE2. However, we just analyse a small controlled *in vitro* microenvironment, lacking of the complex physiological events occurring *in vivo*. Indeed, in contrast with the data from Osman and colleagues, growing body of evidences, suggested that sACE2 plasma levels are elevated in severe compared to moderate COVID-19 patients, but controversial results are available about its activity (Daniell et al., 2022; Fagyas et al., 2022; Mariappan et al., 2022; Patel et al., 2021) . sACE2 levels are usually very low in healthy people, but they can be increased under pathological conditions including

heart failure, sepsis and COVID-19 (Daniell et al., 2022; Fagyas et al., 2022; Ramchand et al., 2020). It was also hypothesized that sACE2 may be used as a non-specific biomarker of pathological situations involving an highly pro-inflammatory environment (Fagyas et al., 2022), but robust data in support of this hypothesis are still lacking.

It is worth noting that under our *in vitro* experimental conditions the ACEi ramipril had no effect on the Ang II-induced up-regulation of ACE2 expression (Figures 8). However, it enhances *per se* ACE2 protein expression but not mRNA levels, while captopril has no effect on ACE2 (Figure 7, panel D and Figure 8, panel D). At first, we hypothesized that ramipril, by blocking ACE, leads to BK accumulation that may be responsible for the enhanced ACE2 protein expression. In support of this hypothesis, we found that both bradykinin receptors (BK1R and BK2R) were expressed in our system (Table 2) and several studies reported increased levels of BK and its derivatives in COVID-19 patients exploring the protective role of BK2R antagonist like icatibant in lung inflammation and dry cough (Cooper et al., 2021; Garvin et al., 2020; Lesage et al., 2020; Mansour et al., 2021; Pecori et al., 2021; van de Veerdonk et al., 2020). In accordance to these studies, Jakwerth and colleagues reported that icatibant reduced SARS-CoV-2 infection, adding that this occurs via downregulation of ACE2 expression (Jakwerth et al., 2022), supporting our initial idea of an upregulation of ACE2 protein expression induced by BK levels. However, since captopril as ACEi is supposed to play the same role, but indeed it did not induce the same effect, we concluded that ACE2 upregulation by ramipril alone was due to an off-target effect of this molecule. Indeed, ramipril is a pro-drug and needs to be metabolized into its active form, ramiprilat, (Anderson et al., 2006) which is ≈6 times more active in terms of ACE inhibition than ramipril (Anderson et al., 2006; Grima et al., 1991). Usually, this conversion occurs in the liver through de-esterification, but whether this conversion occurs also in the lung is not known to our knowledge.

Instead, the other ACEi used in this study, captopril, is synthetized directly into its active form and therefore its effects should be consistent with what is occurring *in vivo.* Moreover, it is consistently different in the structure, bringing a sulfydrylic group which is not present in ramipril molecule (Anderson et al., 2006; Vlasses et al., 1982). Therefore, the different effect observed on ACE2 protein expression may be due to the difference in the structure and activation rate between the two ACEis analysed.

In order to investigate the functional consequences of Ang II-induced ACE2 increase, and the effects of irbesartan and ramipril on SARS-CoV-2 entry in Calu-3 cells, known to be a susceptible human lung model for SARS-CoV-2 infection (Cagno, 2020), we performed experiments using the wild-type SARS-CoV-2 virus, a human vesicular stomatitis virus (VSV) and a genetically modified VSV (Figure 9). When exposed to wild-type SARS-CoV-2 virus in the presence of Ang II or the TMPRSS2 protease inducer DHT (Leach et al., 2021), the Calu-3 cells showed a prominent increase of new infective SARS-CoV-2 particles (viral titre) (Figure 9, panel D), indicating that both agents promoted SARS-CoV-2 cell entry. In accordance to Leach and colleagues (Leach et al., 2021), we also found that DHT induced an increase in *TMPRSS2* gene expression in the bronchial adenocarcinoma cell line (Figure 10, panel A). Irbesartan and ramipril also slightly promoted viral infection (but not statistically significant); however, more importantly, they drastically blunted the viral infection induced by Ang II (Figure 9, panel D). This is highly relevant from the clinical standpoint as ARBs and ACEis are prescribed in the setting of an activated RAS. To further dissect the mechanisms of SARS-CoV-2 virus entry and infection, we used also a luciferase-expressing vesicular stomatitis virus (VSV) pseudotyped with SARS-CoV-2 spike protein (SARS-CoV-2 pseudovirus), widely used to investigate the early steps of viral entry into the target cell (Jonsson et al., 2009; Tai et al., 2015) that can be detected by measuring the cell luciferase signal (Figure 9, panel B). Analogously to what

observed with wild-type SARS-CoV-2 virus, by challenging Calu-3 cell with SARS-CoV-2 pseudovirus, we observed that both Ang II and DHT enhanced pseudoviral cell entry. Irbesartan and ramipril augmented the pseudoviral cell entry slightly, but effectively counteracted the Ang II-promoted pseudovirus cell entry (Ribstein et al., 2001) (Figure 9, panel E). We further explored the ACE2-independent cell entry-enhancing action of the different molecules by examining infection with the luciferase expressing VSV pseudovirus (Kim et al., 2017) that recognizes low-density lipoprotein receptor (LDL-R) (Nikolic et al., 2018) (Figure 9, panel C). Both DHT and Ang II had negligible effects on the VSV-derived luciferase signal in this setting (Figure 9, panel F), indirectly confirming that the enhancing effects of the two compounds on wild-type SARS-CoV-2 virus and the SARS-CoV-2 pseudovirus infection involves the spike protein attachment to ACE2 and downstream activation of TMPRSS2. According to our data, the ACEi ramipril and the ARB irbesartan can enhance cellular permissiveness to this VSV virus by acting independently of ACE2, at least *in vitro.* Indeed, both slightly (but significantly) stimulated VSV-G-pseudovirus infection but did not alter *ACE2* mRNA in Calu-3 cells; furthermore, co-exposure to Ang II, which enhanced ACE2 expression (Figure 8, Panels A and C), did not affect pseudovirus infection (Figure 9, panel F).

This effect of ACEis and ARBs on VSV-G-pseudovirus could be due to the effect of these drugs on LDL-R expression, as it is the receptor for this pseudovirus. Indeed, it is known that administration of both ramipril (Doggrell, 2001) and irbesartan (Onishi et al., 2013) reduced the levels of circulating LDL. Therefore, it can be speculated that this is a consequence of an upregulation of LDL-R expression, which promoted LDL intake in the cell and so reduced LDL circulating levels (VIRANI et al., 2014). We therefore investigated the effect of irbesartan and ramipril on *LDLR* mRNA expression, finding that these drugs slightly increased *LDLR* in our Calu-3 cell model (Figure 10, panel B). Notably, concomitant presence of irbesartan or ramipril

and Ang II, which mimics the condition occurring *in vivo* in patients treated with ARBs or ACEis (Catt et al., 1971; Kassler-Taub et al., 1998), did not augment SARS-CoV-2 (or pseudovirus) infection in human lung cells.

Both SARS-CoV and SARS-CoV-2 bind to ACE2 and enter the cells via endocytosis, such that the initial detrimental effect of viral infection begins with a loss of ACE2-mediated tissue protection against proinflammatory Ang II. For this reason, RAS blockade would be beneficial against the detrimental effects of SARS-CoV-2 (Gheblawi et al., 2020).

Hence, based on the present findings, this study would like to propose that patients with enhanced Ang II levels can display raised expression of ACE2 in tissues, including the lung bronchial epithelial cells, thus increasing binding of the SARS-CoV-2 virus, and facilitating the infection and its spread. It is therefore conceivable that, under these conditions, ARBs can prevent the Ang II-induced enhanced expression of ACE2. Accordingly, while activation of the RAS can be regarded as a detrimental mechanism contributing to excess morbidity and mortality in general, blunting of this system can ameliorate the course of the COVID-19 infection. In line with this contention, ACEis have been shown to decrease mortality not only in patients with cardiovascular disorders entailing an activation of the RAS, but also in fragile patients with pneumonia as those who survived a stroke, and those with Parkinson's disease (Liu et al., 2012; Mortensen et al., 2008; Shinohara and Origasa, 2012; Teramoto and Ouchi, 1999; Wang et al., 2015).

Beside the strong impact of our data, there are some limitations. First, we used Calu-3 cell as a model, but, being adenocarcinoma cells, they may behave differently from non-tumoral cells. Moreover, we have no data from microvascular endothelial cells of the lung that, differently from the HUVEC analysed, could give another point of view on the impact of RAS in SARS-CoV-2 infection, especially in the early step of the infection. Importantly, it is not

known how much Ang II is present in the lung, either under normal conditions or under ACEi treatment. This information would be of particular relevance to translate our data to the *in vivo* conditions.

8. CONCLUSIONS

In summary, the observation that Ang II potently upregulates the expression of ACE2 and subsequently SARS-CoV-2 infection in human bronchial epithelial cells is, in our view, a fundamental novel piece of information, which supports the hypothesis that an activated RAS can increase the susceptibility to SARS-CoV-2 infection and aggravate the prognosis of COVID-19 patients.

The fact that blockade of the AT1R or ACE with ARBs or ACEis, respectively, abolishes this action of Ang II can explain the protective effects of these agents in patients with several conditions who are being treated with these drugs (Granger et al., 2003; Lindholm et al., 2002; Pitt et al., 1999). Therefore, even considering some limitations, our data strongly encourage to not discontinue ACEi/ARBs treatment in COVID-19 patients as they can ameliorate the course of the disease as already confirmed by clinical trial results (Bauer et al., 2021; Cohen et al., 2021; Lopes et al., 2021; Macedo et al., 2022; Sato et al., 2022).

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