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## Novel insights in the pathogenesis of renal interstitial damage during ACE inhibition

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## CHAPTER 4

### Emerging role of ACE2 in physiology and disease

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## Abstract

The renin-angiotensin-aldosterone system (RAAS) is a key regulator of systemic blood pressure and renal function and a key player in renal and cardiovascular disease. However, its (patho)physiological roles and its architecture are more complex than initially anticipated. Novel RAAS components were discovered in recent years which may add to our understanding. In particular, the human homologue of ACE (ACE2) added a higher level of complexity to the RAAS. In a short period of time ACE2 has been cloned, purified, knocked-out, knocked-in, inhibitors have been developed, its 3D structure determined and new functions were identified. ACE2 is now implicated in cardiovascular and renal (patho)physiology, diabetes, pregnancy, lung disease and remarkably, ACE2 serves as a receptor for SARS and NL63 coronaviruses. This review covers available information on genetic, structural and functional properties of ACE2. Its role in a variety of (patho)physiological conditions and therapeutic options of modulation will be discussed.

## Introduction

The renin-angiotensin-aldosterone system (RAAS) is cardinal in renal and cardiovascular physiology and pathophysiology. Its architecture and functions are more complex than previously assumed. In the classical RAAS, the protease renin, which is secreted from renal juxtaglomerular cells, acts on the circulating precursor angiotensinogen to generate angiotensin (Ang) I (Figure 1). Ang I is converted by the dipeptidyl carboxypeptidase angiotensin-converting enzyme (ACE) to Ang II, the main effector substance of the RAAS with potent vasoconstrictive, proinflammatory and profibrotic properties. Consequently, ACE inhibitors (ACEi) and Ang II receptor blockers (ARBs) are effective in hypertension, heart failure and progressive renal damage.

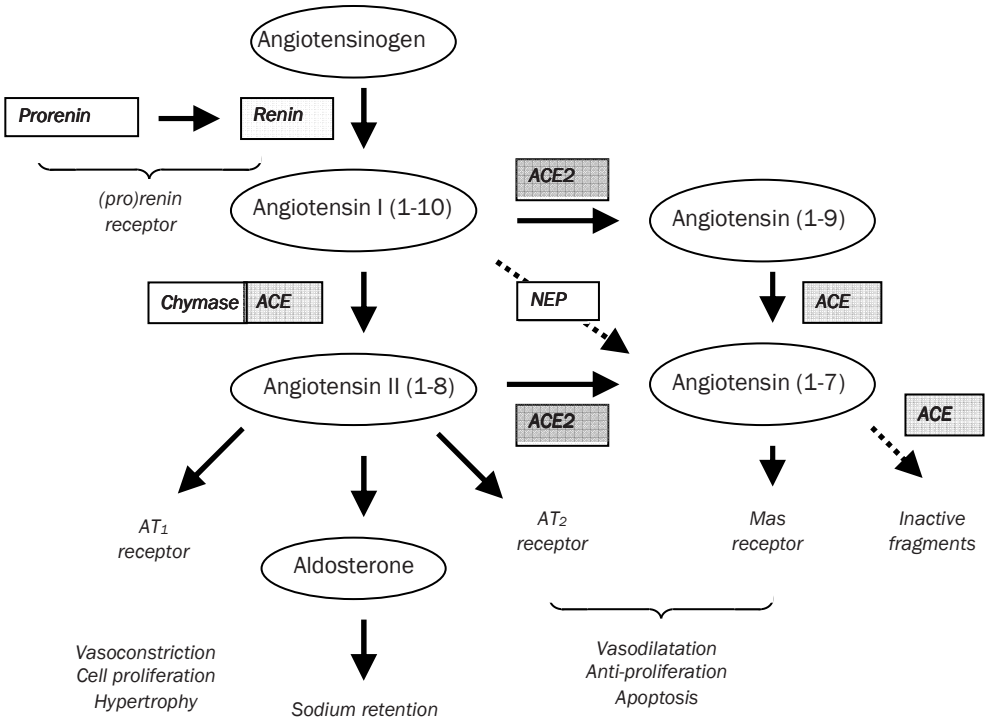
Recently, angiotensin fragments other than Ang II were also proposed to be relevant, in particular Ang(1-7) which mediates vasodilation, antiproliferation and apoptosis, thereby opposing effects of Ang II<sup>1</sup>. Further complexity was introduced by the discovery of an ACE homologue, ACE2. This enzyme cleaves Ang I into Ang(1-9), which can be converted to Ang(1-7) by ACE (Figure 1). Furthermore, ACE2 degrades Ang II to Ang(1-7). It has therefore been suggested that ACE2 acts in a counter-regulatory manner to ACE by shifting the balance between Ang II and Ang(1-7), thus acting as a functional clearance mechanism for Ang II.

High ACE2 gene expression was initially reported in testis, kidney and heart<sup>2,3</sup>. Later studies showed widespread distribution of both rodent and human ACE2 in lung, liver, small intestine and brain, albeit much lower than in kidneys<sup>4-7</sup>. Diverse roles have emerged for ACE2 since its identification in 2000<sup>2,3</sup>. Some 200 papers have subsequently addressed its structure, functions and its role in cardiovascular and renal disease, diabetes, SARS coronavirus infection and lung injury. This review covers available information on genetic, structural and functional properties of ACE2. Its role in a variety of (patho)physiological conditions and therapeutic options of modulation will be discussed.

## The ACE2 gene and protein

The 40kb *ACE2* gene is located on chromosome Xp22 and contains 18 exons, many of which closely resemble exons in the *ACE* gene<sup>3</sup>. Two alternative transcripts of the mouse *ACE2* gene have been identified which probably arise by alternative splicing<sup>8</sup>. Recently, an alternative 5'-untranslated exon of human *ACE2* and new polymorphisms have been reported<sup>9</sup>. The human ACE2 protein is a typical zinc metallopeptidase, which comprises 805 amino acids and is 40% identical in sequence with ACE, although it only contains a single catalytic domain. Critical active site residues, including the His-Glu-Met-Gly-His zinc-binding motif, are highly conserved. ACE2 is a type I integral membrane glycoprotein oriented with the N-terminus and the catalytic site facing the extracellular space (an ectoenzyme), where it can metabolise circulating peptides. The small C-terminal, cytoplasmic domain has a number of potential regulatory sites. The similarity with ACE relates only to its topology and much of the extracellular domain (Figure 2); the juxtamembrane, transmembrane and cytoplasmic domains of ACE2 share similarity with the

renal, transmembrane protein collectrin<sup>10</sup> (Figure 2), which stimulates insulin exocytosis and pancreatic beta cell proliferation<sup>11,12</sup>. Targeted deletion of the collectrin gene in mice suggests that it also plays a major role in regulating renal amino acid transport<sup>13</sup>.



**Figure 1.** Schematic diagram of the renin-angiotensin-aldosterone system which shows the role of ACE and ACE2 in the metabolism of the various angiotensin peptides. Modified from Warner et al <sup>14</sup>.

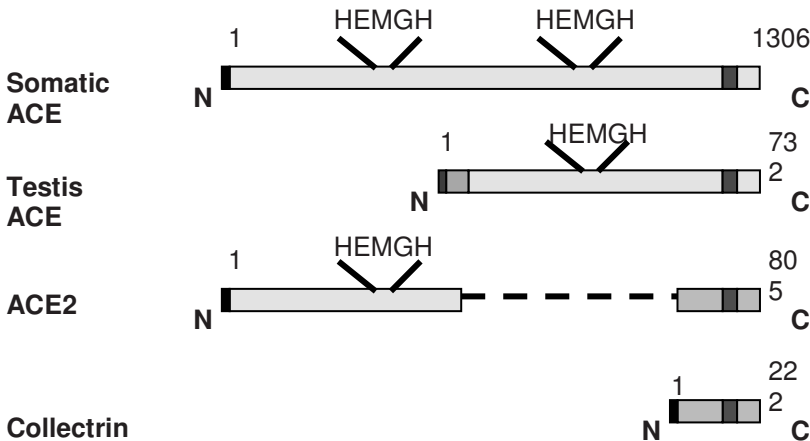
## Substrate specificity of ACE2

ACE2, a strict carboxypeptidase, hydrolyses its substrates by removing a single amino acid from their respective C-termini, rather than a dipeptide, as does ACE. ACE2 therefore has the ability to convert the decapeptide Ang I to Ang(1-9) and the octapeptide Ang II to Ang(1-7). A kinetic study evaluating the comparative roles of ACE and ACE2 in angiotensin metabolism<sup>15</sup> established that Ang II is hydrolysed two orders of magnitude more efficiently by ACE2 than Ang I. Hence, the major role of ACE2 in angiotensin metabolism seems to be the production of Ang(1-7) whose actions oppose those of Ang II. Different *in vivo* studies strongly support the concept that a major role of ACE2 is indeed the generation of Ang(1-7) from Ang II and that its conversion of Ang I to Ang(1-9) is not normally of physiological importance<sup>16-21</sup>, except possibly under conditions that raise Ang II levels, e.g. ACEi or ARB treatment<sup>22</sup>.

Although most studies have focused on the role of ACE2 in angiotensin metabolism, the enzyme has broad substrate specificity. In addition to Ang I and II, ACE2 hydrolyzes apelin-13, neurotensin-(1-11), dynorphin A-(1-13),  $\beta$ -casomorphin-(1-7) and ghrelin<sup>23</sup>. Although the ACE substrate bradykinin is not hydrolysed by ACE2, its metabolite des-Arg<sup>9</sup>-[bradykinin], which is an agonist for the B1 bradykinin receptor, is hydrolysed and a role for ACE2 in bradykinin metabolism cannot yet be dismissed. It is likely that other potential physiological substrates for ACE2 will emerge.

## Cell biology and shedding of ACE2

Knowledge of the basic cell biology of ACE2 still remains limited, partly because few cell models expressing ACE2 at high levels are available<sup>24</sup>. ACE2 is expressed as a cell-surface non-raft protein with little intracellular localization, and the protein does not readily internalize. However, binding of the SARS viral spike protein to ACE2 does trigger enzyme internalization, down-regulating activity from the cell-surface. In polarised cells, ACE2 is exclusively targeted to the apical surface<sup>24,25</sup> in contrast to ACE which distributes equally between apical and basolateral surfaces<sup>24</sup>. Another mechanism for downregulating ACE2 at the cell-surface is by proteolytic shedding of its extracellular domain. This shedding, which is also undergone by ACE, is stimulated by phorbol esters and is blocked by inhibitors of the ADAMs family of zinc metalloproteinases. ADAM17 (TACE) is implicated as the primary enzyme involved in the regulated shedding of ACE2<sup>26</sup> resulting in detectable levels of ACE2 in plasma and urine<sup>15,24,27</sup>.



**Figure 2. The domain structure of somatic and testis ACE, ACE2 and collectrin.** Each protein is a type I integral membrane protein with a cleaved signal peptide (black), an N-terminal ectodomain, a transmembrane domain (blue in ACE, red in ACE2 and collectrin) and a C-terminal cytoplasmic domain. Somatic ACE contains two HEMGH zinc binding active sites, while testis ACE and ACE2 contain a single HEMGH motif. Collectrin lacks a HEMGH motif. The ectodomain of ACE2 (yellow) is more similar to the N-terminal domain of somatic ACE, while its juxtamembrane stalk, transmembrane and cytoplasmic domains are more similar to collectrin. The numbers indicate the amino acid residues in each protein. Modified from Turner et al<sup>28</sup>.

## ACE2 and the heart

The importance of ACE2 in cardiac physiology and disease was initially suggested by two independent groups, based on cardiac ACE2 expression, particularly in endothelial cells<sup>2,3</sup>. Subsequent studies revealed ACE2 not only in endothelial cells and smooth muscle cells from intramyocardial vessels, but also in cardiac myocytes<sup>29</sup> (Figure 3).

The importance of ACE2 in cardiac function was strengthened by Crackower et al. who described cardiac dysfunction in *Ace2* knock-out (KO) mice<sup>30</sup>. Specifically, there was a 40% decrease in fractional shortening with slight ventricular dilation. Interestingly, there was thinning of the left ventricular (LV) wall rather than LV hypertrophy and/or cardiac fibrosis. These changes progressed with age and were more prominent in male mice<sup>30</sup>. The hearts of these *Ace2* KO mice showed increased Ang II levels and upregulation of hypoxia-inducible genes. The authors suggest that cardiac function is modulated by the balance between ACE and ACE2 and that the increase in local cardiac Ang II was involved in these abnormalities. This is supported by the fact that the cardiac phenotype and increased Ang II levels were completely reversible by concomitant deletion of the ACE gene in *Ace2* KO mice. It remains unclear, however, why despite elevated Ang II levels, the hearts of these *Ace2* KO mice did not show any cardiac hypertrophy or fibrosis. This may partly be related to Ang II independent effects of ACE2, such as effects of the other substrates described above<sup>23</sup>, which also can influence cardiac contractility.

Gurley et al. generated an *Ace2* KO mouse<sup>31</sup> deleting the same exon as the Crackower group<sup>30</sup> and although demonstrating changes in blood pressure regulation, particularly in response to Ang II, these investigators could not detect a specific cardiac phenotype. Another study<sup>32</sup> also failed to identify cardiac abnormalities in their *Ace2* KO mice. However, they did demonstrate reduced cardiac contractility after transverse aortic constriction (TAC), a model of pressure overload. TAC was associated, when compared to wild type mice subjected to the same procedure, with a marked increase in cardiac Ang II levels and increased fibrosis, LV dilation and myofibrillar disarray. Indeed, in *Ace2* KO mice followed for a longer period, this reduction in myocardial contractility, led to pulmonary congestion and death<sup>32</sup>.

Recent studies suggest that ACE2 possibly influences the electrical pathways of the heart. In *ACE2* transgenic mice, cardiac conduction disturbances were present and some animals developed lethal ventricular fibrillation<sup>33</sup>. The level of ACE2 upregulation correlated with the severity of the conduction disturbance.

Accumulating evidence indicates that overactivity of cardiac RAAS and myocardial Ang II production contributes to the progression of heart failure. Several studies characterised ACE2 expression and activity in heart failure. In experimental myocardial infarction increased cardiac ACE2 expression was found in the infarct zone and the surrounding ischemic zone<sup>29</sup>. Moreover, local upregulation of ACE2 was also found in explanted human hearts with ischemic cardiomyopathy<sup>21,29,34</sup> and idiopathic dilated cardiomyopathy<sup>21,34</sup>. These findings may imply that the upregulation of ACE2 is a compensatory response to the ischemic insult and that the

consequent increase in the vasodilatory Ang(1-7) may confer cardioprotective effects in an attempt to counterbalance the effects of Ang II. Other groups did not observe upregulation of ACE2<sup>35</sup> or demonstrated a downregulation of cardiac ACE2 in experimental heart failure<sup>22,36</sup>, albeit in different rat strains.

Several studies noted a marked increase in cardiac ACE2 expression in response to RAAS blockade by ACEi<sup>22,37</sup>, ARB<sup>35-37</sup> or an aldosterone antagonist<sup>36,38</sup> (a diuretic with specific benefits in heart failure<sup>39</sup>). This was interpreted as Ang II receptor blockade conferring cardioprotection not only by reducing harmful Ang II mediated effects but also by locally increasing Ang(1-7) which, through binding to a putative Ang(1-7) receptor, Mas-1<sup>40</sup> is postulated to have beneficial effects on the heart<sup>41</sup>.

The potential for ACE2 to modulate cardiac function and remodelling is additionally suggested by the finding that lenti-viral vector encoding mouse ACE2 injected intracardially in Sprague Dawley rats significantly attenuated cardiac hypertrophy and myocardial fibrosis induced by Ang II infusion<sup>41</sup>. Moreover, ACE2 overexpression after neonatal development provides protection from high blood pressure and cardiac pathophysiology in SHR rat<sup>42</sup>.

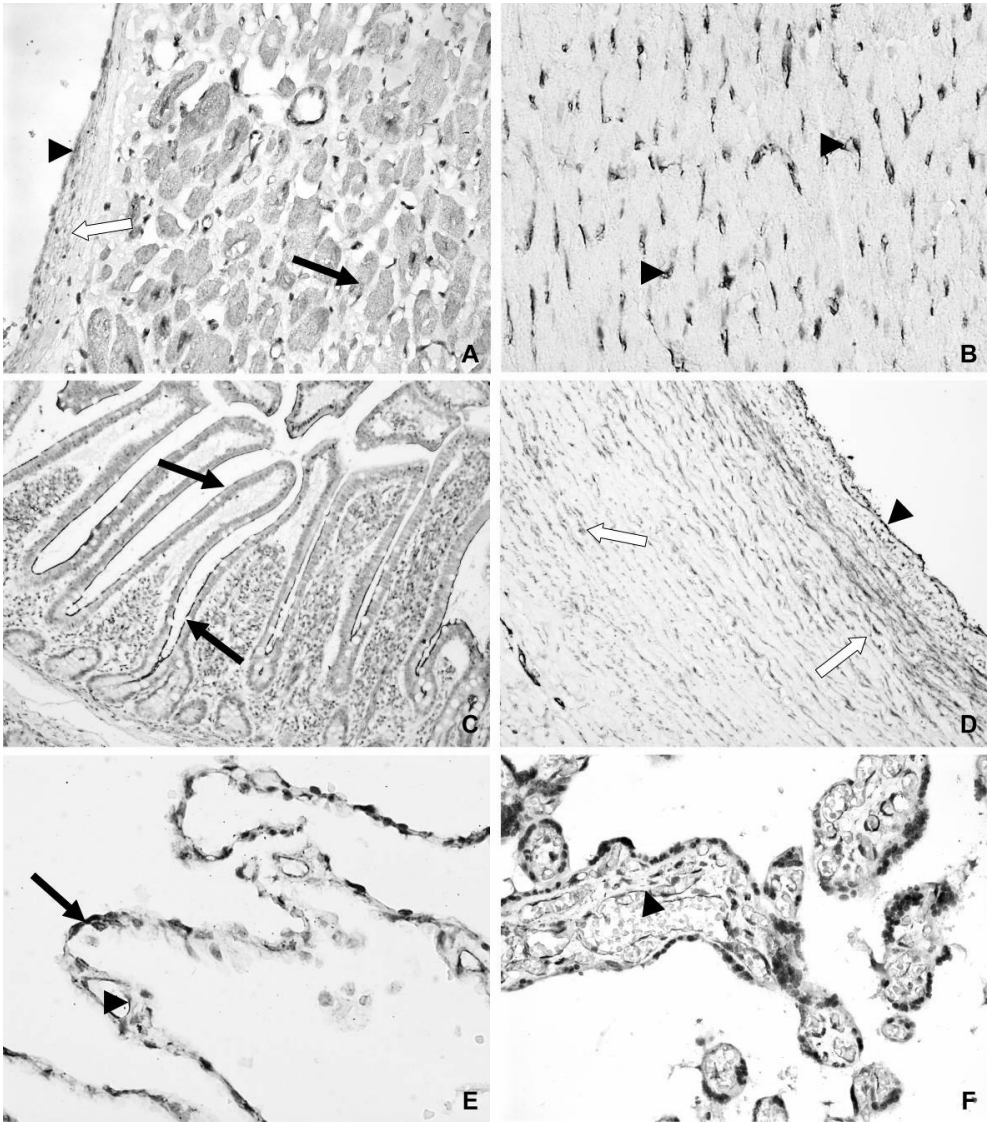
Altogether, these studies suggest that ACE2 is important in cardiac function and that ACE2 related effects contribute to cardioprotective effects of ACE inhibitors and ARBs. The increased ACE2 gene expression and activity during RAAS blockade suggest, that at least part of their mode of action results from hydrolysis of the vasoconstrictor, mitogenic Ang II to the vasodilator anti-proliferative Ang(1-7) through a feed-forward mechanism within the RAAS. Understanding the roles of ACE2 in heart failure may optimize current therapies and ultimately guide development of new therapeutic strategies.

## ACE2 and hypertension

It was initially hypothesised that disruption of the delicate balance between ACE and ACE2 would result in abnormal blood pressure control<sup>43</sup>; ACE2 might protect against increases in blood pressure, and conversely ACE2 deficiency might lead to hypertension. The localization of ACE2 in vascular endothelial cells and smooth muscle cells<sup>6</sup> (Figure 3) supports this.

Since hypertension was linked to loci on the X chromosome<sup>44,45</sup> and ACE2 was mapped to the X chromosome<sup>3</sup>, ACE2 became a candidate gene underlying the loci linked to hypertension. Crackower et al.<sup>30</sup> were the first to test ACE2 as the gene underlying the blood pressure locus on the X chromosome. They showed reduced expression of renal ACE2 in the salt-sensitive Sabra hypertensive rat compared to the normotensive rat. Both hypertensive SHR and SHRSP rats showed reduced renal ACE2 protein levels compared to the normotensive Sabra and WKY strains. Two other groups confirmed some of these findings by showing that SHR rats have lower renal ACE2 mRNA, protein and activity compared to WKY rats<sup>46,47</sup>.





**Figure 3. Immunohistochemical staining pattern of ACE2 in several organs.** In the healthy human heart **(A)** ACE2 is expressed in cardiomyocytes (closed arrow), vascular endothelium (arrow head) and smooth muscle cells (open arrow). In rat heart **(B)** ACE2 is predominantly expressed in vascular endothelium (arrow head). ACE2 expression in human small intestine (jejunum) **(C)**; abundant staining can be found in the brush border of enterocytes (arrow). In human aorta **(D)** ACE2 is expressed in the endothelium (arrow head) and vascular smooth muscle cells (open arrow). In healthy human lung **(E)** ACE2 is present in alveolar epithelial cells (arrow) and in capillary endothelial cells (arrow head). In human placenta **(F)** positive staining for ACE2 is found in the placental villi [syncytiotrophoblast, cytotrophoblast, vascular endothelium (arrow head) and smooth muscle cells].

However, others were unable to detect any differences in renal ACE2 mRNA, protein and activity between adult hypertensive rats and their normotensive controls<sup>48</sup>. On close scrutiny, in SHRSP rats the allele of the previously identified blood pressure locus on rat chromosome X contributes to a blood pressure lowering effect<sup>44</sup>, while in the salt-sensitive Sabra hypertensive rat this allele contributes to a blood pressure increasing effect<sup>45</sup>, suggesting that it is not the same gene that underlies the blood pressure locus. Surprisingly, while the allelic effects of the blood pressure locus are discordant between these hypertensive strains, a similar, i.e. concordant, reduction in renal ACE2 expression was reported<sup>30</sup>, which reduces the possibility that ACE2 is the candidate gene underlying the blood pressure locus.

Additional arguments came from studies in *Ace2* KO mice. *Ace2* KO mice on mixed B6/129 background had normal blood pressure compared to the wildtype<sup>30</sup>. However, a recent study which also studied *Ace2* KO animals on homozygous B6 and 129 backgrounds, showed that *Ace2* KO animals with a B6 background have a significantly lower blood pressure compared to wild type animals<sup>31</sup>, suggesting the genetic background with modifier genes present in the 129 strain may counteract the absence of *Ace2* or the 129 allele of another gene involved in blood pressure regulation moved with the KO into the B6 background and causes the difference in blood pressure in these animals.

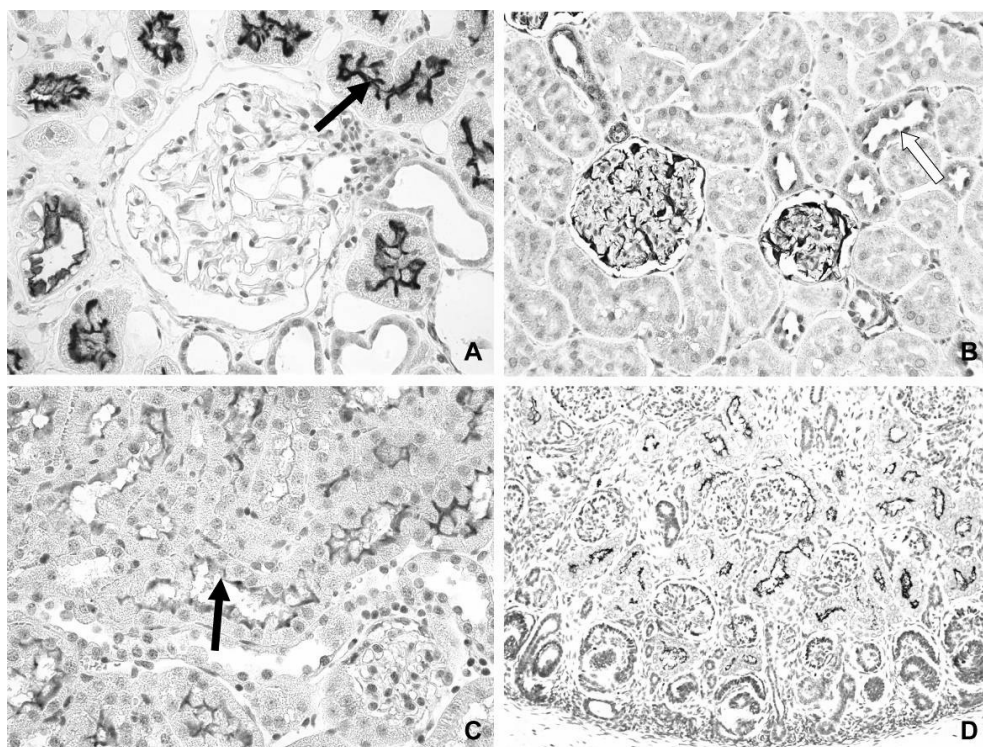
So far, three human association studies of ACE2 polymorphisms with hypertension were performed<sup>49-51</sup>. Two of these studies, using Chinese cohorts, reported an association between a SNP in intron 3 of ACE2 and blood pressure in females with metabolic syndrome<sup>51</sup> and females with essential hypertension<sup>50</sup>. However, the use of only one SNP cannot rule out the possibility of this SNP being in linkage disequilibrium with SNPs in neighbouring genes that could cause the difference in blood pressure.

Altogether, the role of ACE2 in hypertension is not conclusive. Functional studies that show blood pressure effects after administration of ACE2 inhibitors or stimulators are needed to further elucidate its significance in hypertension.

## ACE2 and the kidney

ACE2 is highly expressed in the kidney<sup>7</sup>, however its role in the kidney has not been fully elucidated. In the human kidney ACE2 is predominantly found in the proximal tubular brush border<sup>52,53</sup> where it co-localizes with ACE<sup>24,25</sup>. Moreover, ACE2 is found in endothelial and smooth muscle cells of renal vessels<sup>52,53</sup> and in glomerular visceral (in podocyte/slit diaphragm complex) and parietal epithelial cells<sup>53,54</sup> (Figure 4). The distribution of ACE2 is species specific. In human kidneys the ACE2 expression pattern is comparable to mouse kidneys<sup>5,55</sup>, whereas in rat kidneys, ACE2 is predominantly found in glomeruli and to a lesser extent in tubules (Figure 4). Several lines of evidence support a role for renal ACE in renal damage<sup>56</sup>. Individual differences in renal ACE activity predict the susceptibility for proteinuria-associated renal damage in experimental conditions<sup>57,58</sup>. Furthermore, Ang II is increased in damaged tubules as a possible

mediator of further renal damage in experimental and human renal disorders<sup>59,60</sup>. A disrupted balance between intrarenal ACE and ACE2 with consequent high levels of Ang II might therefore contribute to progressive renal damage. Indeed, in experimental hypertension and diabetes, renal ACE2 expression is decreased<sup>30,61</sup>. Moreover, Tikellis et al. showed that in kidneys of SHR rats ACE2 expression follows a developmental pattern with declining expression during development and onset of hypertension<sup>47</sup>. In line with the beneficial effects of RAAS blockade on cardiac ACE2 as mentioned earlier, renal ACE2 activity is also increased in response to ACEi and ARB<sup>61,62</sup>. In contrast, further enhancing the therapeutic efficacy of ACEi resulted in an unexpected reduction in renal ACE2 expression compared to ACEi alone<sup>63</sup>. It looks like despite extensive research the mechanisms of the effects of ACEi are still not completely understood<sup>64</sup>. Further studies on the regulation of renal ACE2 during RAAS blockade might help to elucidate this.



**Figure 4. Immunohistochemical staining pattern of ACE2 in the kidney.** In the healthy human kidney (A) ACE2 is predominantly present in the brush border of proximal tubular cells (arrow) and to a lesser extent in the glomerular visceral and parietal epithelium. In rat kidney (B) ACE2 is predominantly found in glomeruli and to a lesser extent in distal tubules (open arrow). In mouse kidney (C) the distribution pattern of ACE2 is comparable to human kidney with predominant expression in proximal tubules (arrow). ACE2 is also expressed during human nephrogenesis (gestational age 16 weeks) (D).

Initial reports on *Ace2* KO mice did not show any renal structural and functional abnormalities<sup>30,31</sup>, but recent studies showed that male *Ace2*<sup>-/-</sup> mice develop age-dependent glomerulosclerosis and albuminuria whereas the renal vasculature and interstitium were relatively protected<sup>65</sup>. The authors hypothesized that the glomerular abnormalities were caused by chronic exposure to increased circulating and tissue Ang II, as these abnormalities abolished by ARB treatment. The glomeruloprotective role of ACE2 is supported by studies in which chronic infusion of the ACE2 inhibitor MLN4760 increased albuminuria in db/db mice resulted in increased deposition of glomerular fibronectin<sup>54</sup>. Albuminuria could be prevented by ARB, indicating Ang II dependency. Moreover, these diabetic female db/db mice have decreased glomerular ACE2 staining compared to db/m heterozygous littermates<sup>54</sup>. The authors suggest that glomerular ACE2, present in the podocyte/slit diaphragm complex, could normally be renoprotective by favouring rapid degradation of Ang peptides and thereby preventing exposure to high levels of Ang II.

Other studies support the assumption that increased ACE2 activity tied with a decreased ACE activity may reflect a protective mechanism by limiting the renal accumulation of Ang II and favouring Ang(1-7) formation. Increased ACE2 expression is indeed coupled with profound reduction of ACE expression in renal tubules of young db/db mice<sup>55</sup>. The authors speculate that this might be an early renoprotective response. The kidneys of streptozotocin-induced diabetic mice also show an increased ACE2 expression at the post-transcriptional level<sup>66</sup>. In humans *de novo* expression of ACE2 is found in glomerular and peritubular endothelium in biopsies in patients with primary and secondary renal disease as well as in renal transplants<sup>53</sup>. In renal biopsies of nondiabetic and diabetic patients a significant upregulation of the ACE gene and not the ACE2 gene was found in diabetic nephropathy<sup>67</sup>. Moreover, no associations between polymorphisms in the ACE2 gene and diabetic nephropathy could be established<sup>68</sup>. Altogether, ACE2 appears to be involved in the pathogenesis of renal damage, but its precise role is unclear and further studies are needed in particular during renal disease.

## ACE2 and pregnancy

The placenta is an organ with major Ang(1-7) and ACE2 expression<sup>69</sup> (Figure 3), suggesting that ACE2 may be involved in mother/fetus interactions, which is interesting regarding a potential role of ACE2 in fetal programming and pregnancy. In pregnant rats renal expression of ACE2 is increased compared to virgin controls<sup>16</sup>. A recent study showed no differences in ACE2 expression between normotensive and pre-eclamptic placentas in the third trimester<sup>69</sup>. However, pre-eclampsia is determined early in pregnancy, thus the role of ACE2 in pre-eclampsia should be further studied for example in animal models.

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Human:      30  DKFNHEAEDLFY  41
Mf:         -----
Felis:      E-----E-S-
Rn:         N---Q-----S-
Mumu:       NN--Q-----S-

Human:      82  MYPLQEIQNLTV  93
Mf:         -----
Felis:      T---A--H-T--
Rn:         NFS-----A-I
Mumu:       SFS-----TPII

Human:      353  KGDFR  357
Mf:         -----
Felis:      -----
Rn:         H-----
Mumu:       H-----

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**Figure 5.** Alignment of amino acid sequences of ACE2 from human, macaque (mf), cat (felis), rat (Rn) and mouse (Mumu) critical to SARS-CoV spike protein interactions.

## ACE2 and lung disease

There is abundant expression of RAS components in the lung including ACE and ACE2. Activation of the intrapulmonary RAS could influence the pathogenesis of lung injury<sup>70</sup>. Indeed, increased levels of ACE have been associated with pulmonary hypertension<sup>71,72</sup>, sarcoidosis<sup>73,74</sup>, idiopathic pulmonary fibrosis<sup>75</sup> and the acute respiratory distress syndrome<sup>76,77</sup>. The alleged role for ACE2 as a counter-regulatory mechanism of ACE may therefore be crucial in the lung. ACE2 is present in type I and type II alveolar epithelial cells and to a lesser extent in bronchiolar epithelial cells (Figure 3)<sup>6,78</sup>. Furthermore, as in other organs, ACE2 is present in endothelial cells and in arterial smooth muscle cells. With respect to the role of ACE2 in pulmonary hypertension, it can be envisaged that in particular the presence and function in smooth muscle cells of small arterioles is of relevance, although the actual role in this disease is unknown<sup>79</sup>.



Ace2 KO mice do not have lung abnormalities when compared to their wild type littermates<sup>30</sup>. However, it was recently shown that loss of ACE2 expression precipitates severe acute lung failure<sup>78</sup>. Moreover, injection of recombinant human ACE2 attenuates acute lung failure in ACE2 KO as well as in wild type mice<sup>78</sup>. With respect to the possible role of ACE2 in the lung in relation to acute lung injury in particular, the relation to the ACE2 expression at the alveolar capillary interface is of interest. It is tempting to speculate that increased ACE2 may play a role in reducing the initial leakage over the alveolar capillary interface. This would then slow down the vicious circle that often occurs after a damaging effect to this interface and leads to the clinical pathological picture of diffuse alveolar damage with intra-alveolar oedema and fibrin deposits. These data support a critical role for the intrapulmonary RAS in the pathogenesis of acute lung injury and show that ACE2 is a key molecule involved in the development and progression of acute lung failure.

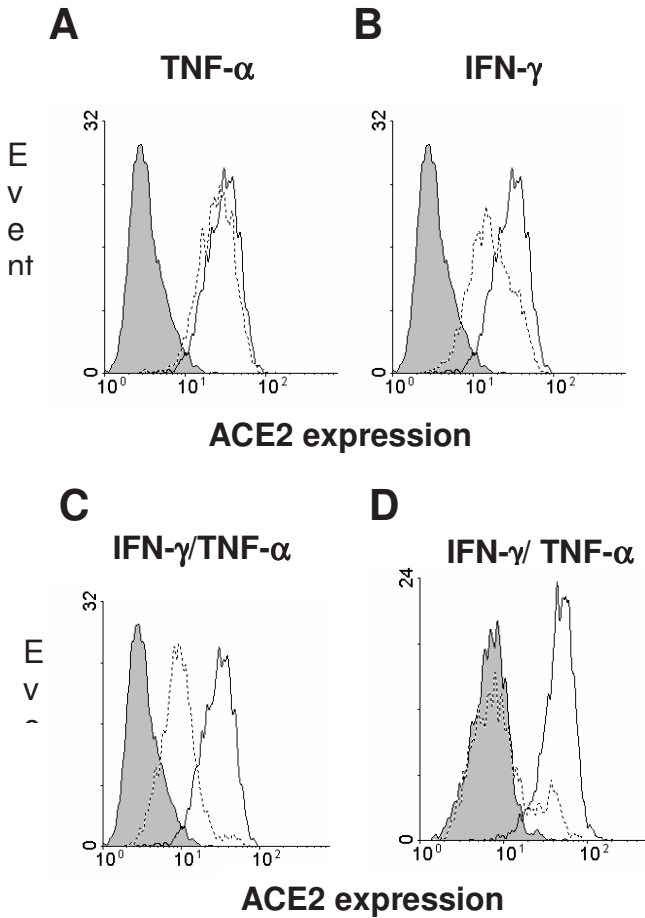
## ACE2 and human coronaviruses

ACE2 acts as a receptor for two coronaviruses (CoV), severe acute respiratory syndrome (SARS)-CoV and human CoV-NL63<sup>80,81</sup>, positive stranded RNA viruses with a “corona” like appearance<sup>82</sup>. Their genome is packaged together with several membrane proteins, the RNA binding nucleoprotein and the receptor binding spike protein that protrudes through the virion membrane. HCoV-NL63 infection causes clinical respiratory symptoms resembling those observed in children infected with common cold viruses. SARS-CoV causes severe lower respiratory tract disease including fever, non-productive cough, myalgia and dyspnea<sup>83</sup>. A specific region within the SARS-CoV spike protein (S1) interacts with ACE2<sup>81,84,85</sup>. The crystal structure at 2.9 angstrom resolution of this receptor binding domain bound with the peptidase domain of human ACE2 shows that it presents a gently concave surface, which cradles the N-terminal lobe of the peptidase<sup>84</sup>. After engagement with ACE2, SARS-CoV fuses with host cell membranes by which the conformational changes of the two heptad regions located in the S2 region, HR-1 and HR-2, cause the formation of an oligomeric structure, leading to fusion between the viral and target-cell membranes.

Using soluble ACE2 molecules, peptides derived thereof, and antibodies directed against ACE2 the SARS-CoV infection can be blocked<sup>81,86</sup>. Conversely, expression of ACE2 in refractory cell lines resulted in SARS-CoV replication<sup>87</sup>. Experiments in Ace2 KO mice revealed the importance of ACE2 as a receptor for SARS-CoV<sup>88</sup>. Moreover, autopsy specimens of patients who died of SARS revealed that ACE2-expressing cells are a direct target of SARS-CoV<sup>89</sup>.

Besides ACE2, lysosomal proteases such as cathepsin-L are required for productive SARS-CoV<sup>90,91</sup>, explaining discrepancies observed between ACE2 expression and absence of SARS-CoV replication in endothelial cells<sup>6,87</sup>. Members of the DC-SIGN family of proteins may enhance SARS-CoV infection, but are not sufficient to infect cells<sup>92</sup>.

Murine and rat ACE2 less efficiently bound the S1 domain of late phase SARS-CoV isolates and



**Figure 6.** IFN-gamma downregulates ACE2 expression in Vero E6 cells. ACE2 expression was determined by FACS analysis after treatment with TNF- $\alpha$  (A), IFN- $\gamma$  (B), IFN- $\gamma$  combined with TNF- $\alpha$  (C) all at 48 h or Vero E6 cells incubated for 96 h in the presence of IFN- $\gamma$  combined with TNF- $\alpha$  (D). Dotted lines represent cytokine-treated cells, while thick lines represent mock treated control cells. The shaded areas represent background staining. Modified from de Lang et al<sup>93</sup>.

supported less-efficient S protein-mediated infection<sup>94</sup>. In addition, spike proteins from isolates of palm civets utilized civet ACE2 more efficiently than human ACE2, whereas late phase isolates such as TOR2 utilized both receptors with equal efficiency<sup>95</sup>. Subsequent studies demonstrated that sequence variation in the spike protein binding sites of ACE2 hindered efficient binding<sup>95</sup> (Figure 5). The lower affinity of some of these S proteins could be complemented by altering specific residues within the S-protein-binding site of human ACE2 to those of civet ACE2, or by altering S-protein residues 479 and 487 to residues conserved during the 2002-2003 outbreak. This indicates that specific molecular interactions are important in the adaptation process of

SARS-CoV to human cells. Animal precursors of SARS-CoV are thus likely to be less pathogenic to humans, and exposure to such viruses may have led to limited clinical symptoms, but antigenic stimulation that results in a serological response. This was observed when SARS-CoV re-emerged in Guangdong in 2003, causing milder clinical disease<sup>96</sup>. Similarly, animal traders had high seroprevalence for human and animal SARS coronavirus, without having a history of SARS.

ACE2 is downregulated in the lungs of mice after acute lung injury, including SARS-CoV infection<sup>88</sup>. The cytokines IL-4 and IFN- $\gamma$  downregulated cell-surface expression of ACE2 (Figure 6), decreased ACE2 mRNA levels and also inhibited SARS-CoV replication in Vero E6 cells<sup>93</sup>. Furthermore, experiments *in vitro* indicated that ACE2 expression is dependent on the differentiation state of epithelia<sup>97</sup> and a role for the GATA family of transcription factors in regulating the expression of ACE2 has been suggested<sup>98</sup>. Downregulation of ACE2 expression may not only affect SARS-CoV entry, but also hamper angiotensin II cleavage causing pathological changes due to angiotensin II type 1a receptor activation<sup>88</sup>. Therefore, intervention strategies using soluble recombinant ACE2 proteins may neutralize SARS-CoV and dampen lung pathology. However, studies analysing the role of ACE2 gene polymorphisms in the progression of SARS have not found evidence that these affect outcome<sup>9,99</sup>.

## Conclusions and future perspectives

ACE2 is now implicated in a variety of (patho)physiological processes. Further understanding of its role in disease will hopefully lead to the exploration of novel therapeutic options. Functional studies using ACE2 inhibitors will be essential to elucidate the regulatory mechanisms of ACE2. Moreover, relatively little progress has been made on the development of such specific ACE2 inhibitors, largely because of lack of a clear therapeutic target. MLN4760 is the most potent and selective ACE2 inhibitor currently available<sup>100</sup>. Structure-based screening programmes have been applied to the identification of novel and selective ACE2 inhibitors with some success<sup>101,102</sup>. Since ACE2 opposes the vasoactive and proliferative actions of Ang II, upregulation of ACE2 expression or activity is a much more desirable characteristic. Modification of ACE2 levels by stimulating its expression or exogenous administration of recombinant ACE2 may have beneficial effects in several disease conditions and should be the scope of future studies.

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