Detailed research summary

Introduction

The renin-angiotensin-aldosterone system (RAAS) plays a crucial role in the fluid and salt homeostasis. One of the key biochemical steps within the RAAS is the conversion of the inactive angiotensin I decapeptide (AngI) to the active angiotensin II (AngII) octapeptide by the angiotensin converting enzyme (ACE). ACE was first identified in 1956 by Skeggs et al [1], and ACE inhibitors were subsequently introduced into the clinical practice. They represent a first line therapy for a wide range of cardiovascular maladies, such as hypertension [2, 3] and heart failure [4].

The molecular properties of the successful ACE inhibitors generally show low lipophilicity (with the exception of fosinopril) [5]. This indicates that the primary target of these drugs is the water-soluble (circulating) form of the enzyme. Accordingly, the factors affecting circulating ACE activity were implicated in the pathomechanism of cardiovascular disease. The prime example of these factors is that the circulating ACE concentration is controlled by a genetic polymorphism affecting the ACE gene (an insertion/deletion polymorphism) [6]. This polymorphism was implicated in systolic heart failure [7].

According to a widely accepted consensus, ACE is expressed primarily by endothelial cells, particularly those of the lung [8]. However, the human heart also expresses ACE [9], suggesting that the lung is probably not the only organ contributing to circulating ACE in human. Moreover, levels of ACE expressions in the kidneys and in the small intestines were found to be comparable to that in the lung [10]. These facts suggest a complex regulation of tissue ACE expression, ultimately determining the circulating ACE levels, which is the prime target of ACE inhibitory medication in heart failure.

Another important finding was the identification of an endogenous inhibitor for circulating ACE [11], which was identified as the serum albumin [12]. Serum albumin almost fully inhibited circulating ACE activity at the physiological concentrations [12], implicating the existence of an additional mechanism for endogenous ACE regulation, besides to the genetical control. According to that, circulating ACE activity is not only determined by its genotype (ACE I/D) dependent expression, but also by interacting (inhibitory) proteins. This raises an intriguing interplay between tissue ACE expression and circulating ACE regulation, which can contribute to the cardiovascular disease development.

The outbreak of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) epidemics in 2019 resulted in a never seen global health emergency (COVID-19). SARS-CoV-2 is distinguished by high virulence and elderly patients show devastatingly high mortality rates. Considerable efforts were made to correlate the significant vulnerability of elderly (suffering frequently in cardiovascular diseases) to the mechanism of the SARS-CoV-2 infection.

Cell entry of the coronavirus depends on binding of the viral spike (S) proteins to the angiotensin converting enzyme 2 (ACE2) protein [13, 14]. ACE2 is a member of the RAS, being targeted by various first choice drugs in a wide range of cardiovascular diseases, such as hypertension and heart failure. It was proposed that ACE2 levels are affected by RAS inhibitory medication, resulting in an increase in circulating ACE2 levels, representing increased expression of ACE2 in the lungs, providing a target for coronavirus cellular entry [15]. Concordantly, ACE2 is elevated in a wide range of cardiovascular diseases. In particular, our previous studies showed that ACE2 inversely correlates with left ventricular ejection fraction (a clinical marker of left ventricular systolic function) [16]. Moreover, we reported that circulating ACE2 activity is elevated in hypertension (without significant systolic dysfunction), and thus implicated that circulating ACE2 correlates with cardiovascular disease development [17]. Taken together, it appears that circulating ACE2 elevates with the progression of cardiovascular disease.

It was a hot new twist in the field that it was proposed by *Lei Fang et al [18],* Murray and Danielle Eshler [19] and Ying-Ying Zheng et al [20] in the middle of March, 2020, that RAS inhibitors are worsening the coronavirus outcome. These proposals were made on the basis of animal experiments, showing that angiotensin receptor blockers (ARBs) are elevating circulating ACE2 levels. These authors postulated that if it is also true in human, then the elevated circulating ACE2 levels are representing the elevated lung tissue ACE2 expression, providing a target reach environment for SARS-CoV-2 infection. These concerns were taken seriously by the media, forcing both the relevant European(ESC) [21] and US scientific societies (HFSA/ACC/AHA) [22] to immediately issue public statements regarding RAS inhibitory medication in COVID-19. All professional societies supported continuing medication with RAS inhibitors in cardiovascular patients, and referred to insufficient clinical data supporting the above mentioned hypotheses.

Results

1. Patenting of a method capable to measure the biochemical efficacy of ACE inhibitory medication

We have completed the patenting process for our method to measure the biochemical efficacy of ACE inhibition in human blood [23]. The European patent has been granted and the patent has been introduced into the national phase in most European countries. As a result, we do have the exclusive right to perform this type of measurement in Europe. We will further develop this method to improve its economic viability and impact.

2. ACE2 as a biomarker of cardiovascular disease.

In a clinical study we enrolled 45 healthy individuals, 239 hypertensive patients, 141 patients with heart failure with reduced ejection fraction and 47 patients with heart failure with preserved ejection fraction [17]. This resulted in the recognition that ACE2 activity is elevated in patients with hypertension and further elevated in patients with reduced ejection fraction, but not in patients with preserved ejection fraction (Fig. 1). Moreover, ACE2 levels were

unaffected by confounding factors, like diabetes, dyslipidaemia or atrial fibrillation, but still seems to be higher in males.

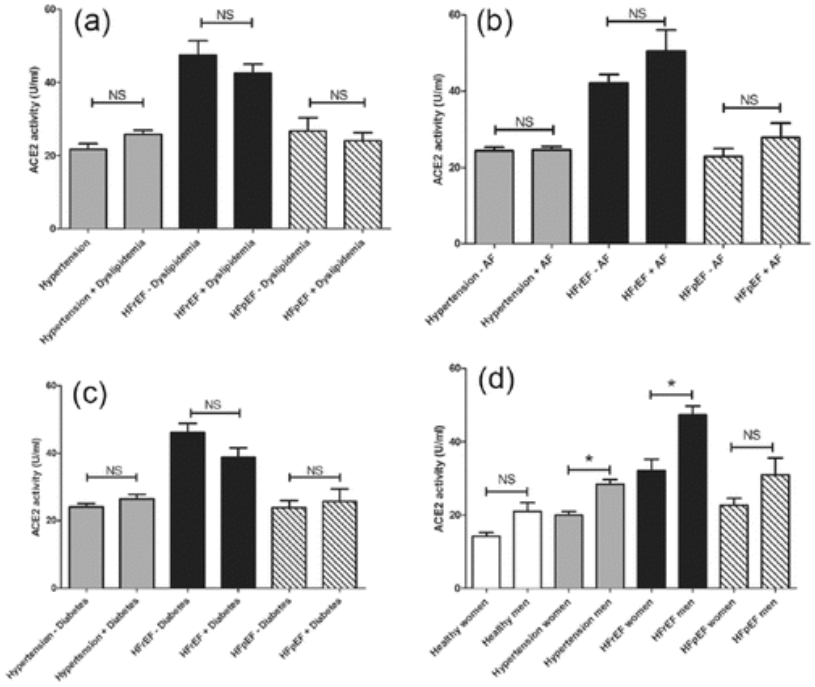

Figure 1. *Circulating ACE2 levels are elevated throughout the cardiovascular continuum.*

3. Circulating ACE activity as a biomarker of sarcoidosis

Another important translational aspect of our effort was that we introduced the methods developed to measure ACE activity into the diagnostic of sarcoidosis. Sarcoidosis is a disease characterized by the accumulation of macrophages in the lung. Its diagnosis is based on surgical sampling, which represent a high burden on the individuals as well as on the health care system. We aimed to develop a technique, which can be used to diagnose sarcoidosis by taking only a venous blood sample. We have established that ACE activity (corrected to the ACE I/D genotype) can be used for this purpose, being a biomarker of macrophage differentiation [24]. Later it was further improved by combining the circulating ACE activity by chitothirosidase [25]. Taking together, we have defined the most sensitive and selective blood test available for sarcoidosis (Fig. 2). It can be used to select patients undergoing invasive lung tissue sampling reducing their number by up to 90%.

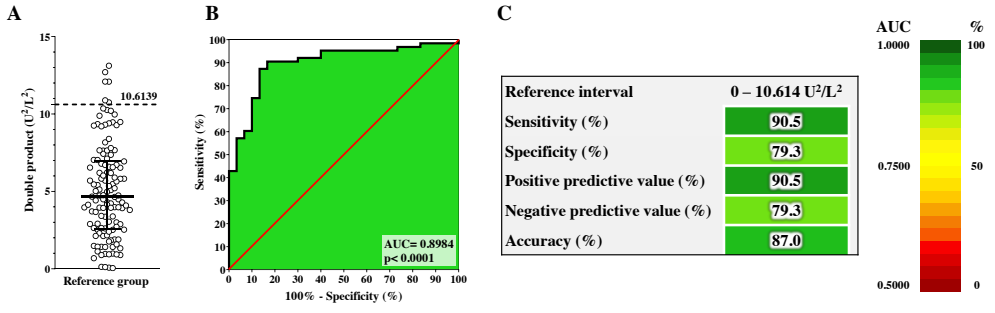

Figure 2. *Optimized diagnostic method to detect sarcoidosis from biomarkers in the blood.*

4. The SARS CoV-2 cell receptor ACE2 levels in cardiovascular patients: can it contribute to the mortality of COVID-19?

This become probably the most important topic of our investigations. To address the role of ACE2 in the high mortality in COVID-19 we have re-evaluated our data from previous clinical studies and performed a prospective analysis of lung tissue samples.

A retrospective single centre analysis was performed on samples originally collected to characterize ACE2 as a biomarker for cardiovascular diseases [16, 17]. We first tested whether circulating ACE2 activities are elevated in patients with RAS inhibitors. RAS inhibition with either ACE inhibitors (ACEi, 25±1 U, n=387, Fig. 3) or by angiotensin receptor blockers (ARB, 26±2 U, n=72, Fig. 3) resulted in an increase in circulating ACE2 activities when compared to patients with similar characteristics without RAS inhibitory medication (19±1 U, n=81, Fig. 3) or to healthy individuals (17±1 U, n=46, Fig. 3).

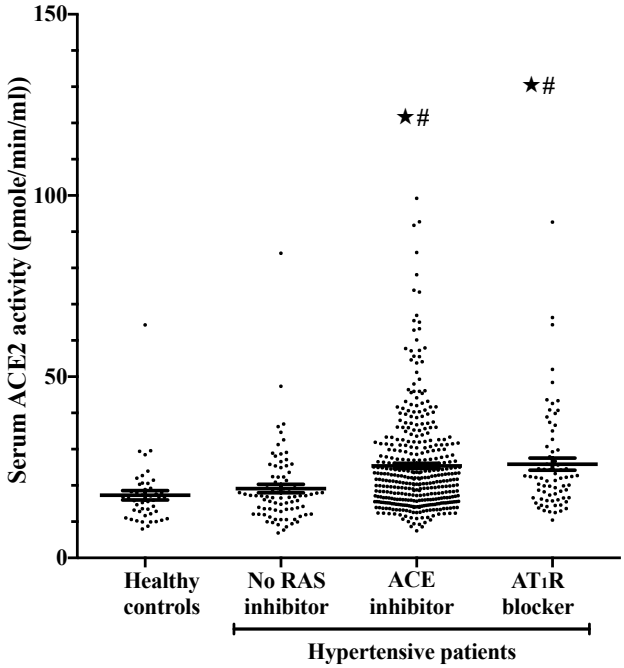

Figure 3. *RAS inhibitors elevate circulating ACE2 levels.*

Next, the class-effect was tested in the same population. Patients with ACE inhibitory medication uniformly had higher circulating ACE2 levels. In particular, patients treated with enalapril (26±2 U, n=59, Fig. 4), perindopril (26±1 U, n=167, Fig. 4) or Ramipril (26±1 U, n=113, Fig. 4) had all higher circulating ACE2 activities than patients without RAS inhibitors (19±1, n=81, Fig. 4).

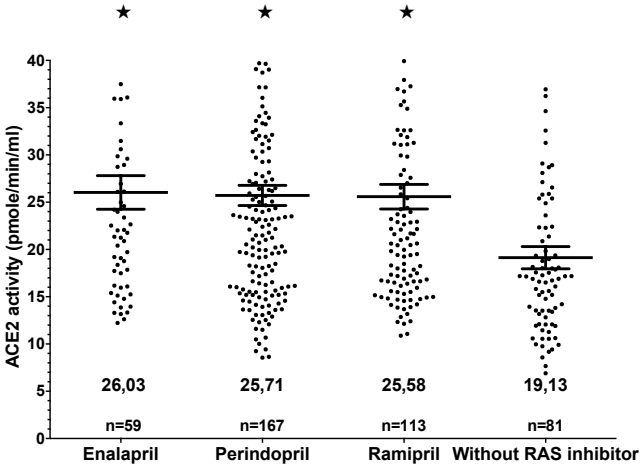

Figure 4. *ACE inhibitory medication elevates circulating ACE2 levels irrespectively to the chemical identity of the drug.*

The time course of the increase in circulating ACE2 activities was also tested. Circulating ACE2 activities were already higher at 12 months after initiation of the therapy (24±2 U, n=52, Fig. 5) when compared to patients without RAS inhibitory treatment (19±1 U, n=81, Fig. 5), and did not increase further in patients taking ACEi for a longer period (27±1 U, n=223, Fig. 5).

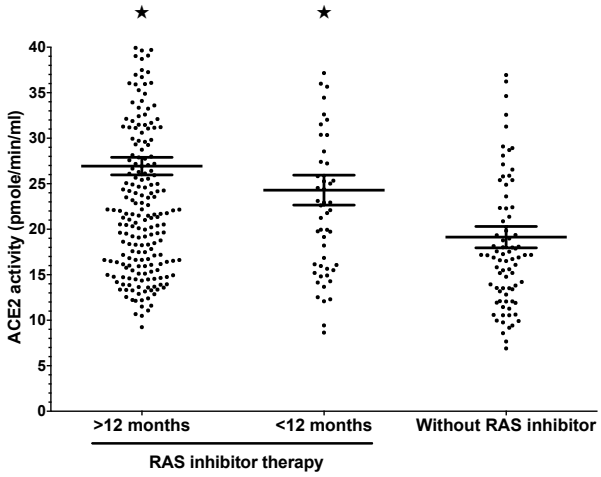

Figure 5. *ACE2 activities are elevated in an early phase of RAS inhibitory therapy.*

It is important to note, that circulating ACE2 activities can be increased by medical treatment or by the progression of the disease. In this respect an analysis was made to correlate circulating ACE2 activities to the biochemical efficacy of ACE inhibition. First, a method to measure biochemical efficacy of the ACEi medication was tested. Hypertensive patients without prescribed ACEi medication were used as a control group defining the levels of endogenous ACE inhibition by serum albumin [11, 12] under these conditions. The level of endogenous inhibition was 72.3±7.2% (mean±SD, n=153, Fig. 6A and 6B). The highest level of endogenous inhibition was 86.6%. According to these we postulated that patients successfully treated with ACEi drugs should have higher than 90% inhibition. Indeed, the majority of hypertensive patients had higher inhibitory values (93.5±7.8% (mean±SD), n=386, Fig. 6A and

6B). The biochemical values correlated with blood pressure. Systolic blood pressure was already reduced in patients with at least 90% inhibition, and the systolic blood pressure reducing effect of the ACEi medication reached the maximum (no more decrease) at 94% (Fig. 6C). Similarly, the diastolic blood pressure reducing effect of ACEi medication reached its maximum at 96% (Fig. 6D). In spite of this apparent correlation between the biochemical efficacy of ACEi medication and the clinical endpoint (blood pressure), circulating ACE2 levels were independent of the level of ACE inhibition (Fig. 6E).

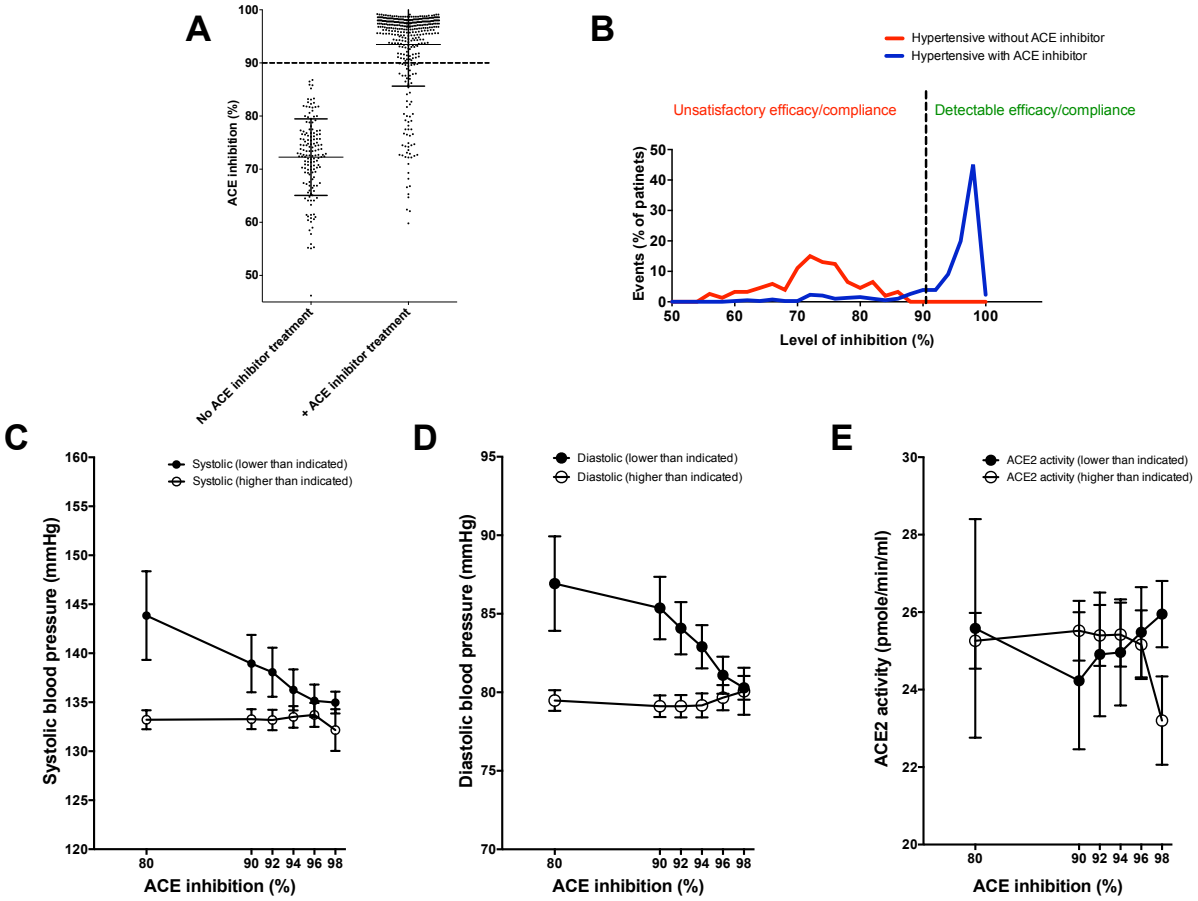

Figure 6. *The biochemical efficacy of ACE inhibitory medication correlates with the blood pressure, but not with the circulating ACE2 levels.*

Next, it was tested if the elevated circulating ACE2 levels are the result of the cardiovascular disease itself. We proposed earlier, that ACE2 activities are inversely correlated with the ejection fraction [17]. Here we tested this correlation in a wide patient population (including patients with hypertension, heart failure with preserved as well as with reduced ejection fraction) irrespectively to their medication. Left ventricular ejection fraction negatively correlated with the ejection fraction (Fig. 7).

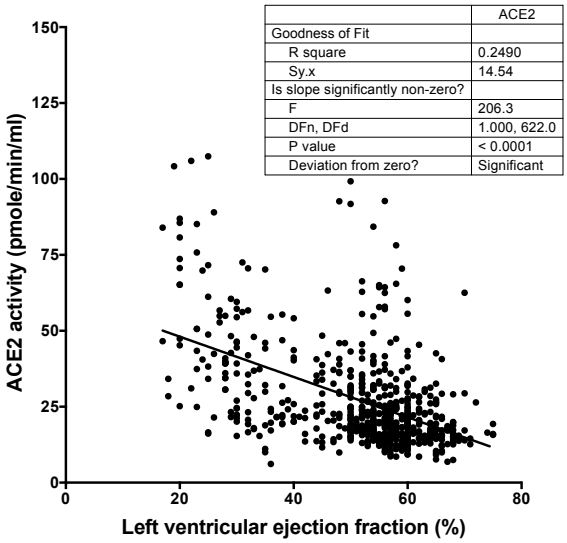

Figure 7. *Circulating ACE2 activity correlates with the left ventricular ejection fraction.*

Finally, the correlation between circulating and lung tissue ACE2 activities was tested. There was no correlation between lung tissue ACE2 activities and circulating ACE2 activities in the same patients (Fig. 8).

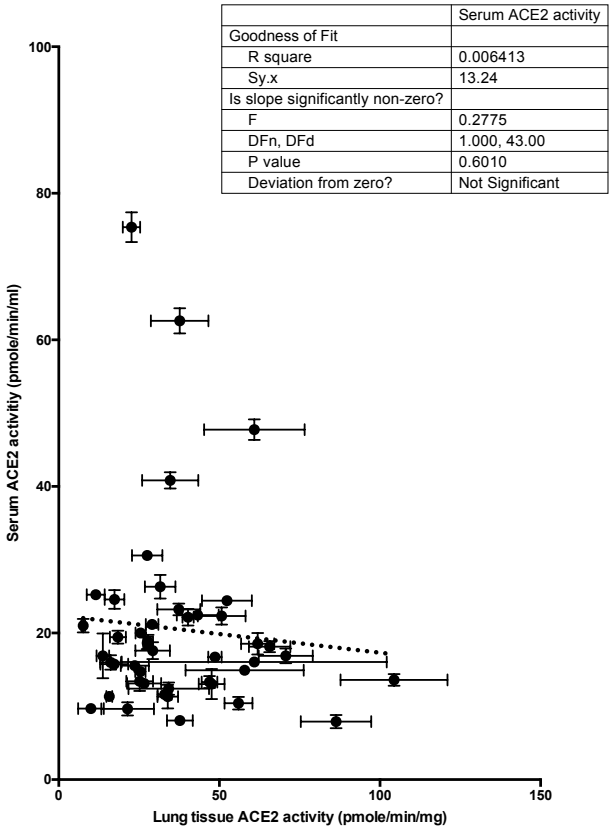

Figure 8. *No correlation between lung tissue ACE2 activity and circulating ACE2 activity.*

In summary, we found that circulating ACE2 levels are increased in patients, treated with RAS inhibitors. However, this increase appears to be elevated as a result of the progression of the

cardiovascular disease. In addition, the level of the ACE2 activity in the circulation does not appear to correlate with its expression in the lungs. These can be translated to the clinical message that RAS inhibitors are safe to take in COVID-19.

5. ACE expression is increased in activated monocytes.

The results related to the sarcoidosis mentioned above highlighted a role for ACE in the macrophages. Indeed, our unpublished data suggested that monocytes have very low (albeit detectable) ACE expression, which increases by 30-fold upon differentiation to monocytes, which further increases by about 10-fold upon activation. These data suggest that ACE expression may play a role in the macrophage dependent immunological processes. This is being studied now. Preliminary data suggests that tuberculosis (BCG vaccine treatment) does not affect ACE levels. However, viral infections (simulating SARS) increases TNF expression (a pro-inflammatory cytokine), which is partially blocked by the ACE inhibitor Lisinopril.
Taken together, the observed correlation between macrophage ACE levels and the inflammatory function may link the efficacy of ACE inhibition to reduced inflammation, explaining the particular efficacy of these drugs in cardiovascular diseases.

Discussion

We made successful efforts to translate some of our basic research findings into the clinical diagnostic practice. We proposed an improved diagnostic method for sarcoidosis. We also managed to pinpoint the circulating ACE2 as a biomarker of cardiovascular disease development. This latter research direction become a prime interest with the emerging COVID-19 epidemics. It was proposed that patients taking inhibitors of the renin angiotensin aldosterone system (RAS) have higher mortality as a result of their increased expression of ACE2, the cell receptor for SARS-2 CoV-2 entry. This correlation was proposed based on animal models, for which our previous clinical data provide a clinical evidence. Our data confirmed that patients with RAS inhibition indeed have higher circulating ACE2 levels. However, these increased ACE2 levels were independent of the biochemical efficacy of the ACE inhibitory treatment, suggesting that this effect is a property of the guideline-based treatment of the same state of cardiovascular disease. With other words, ACE2 is elevated because of the disease, which is being treated by RAS inhibitors, providing a causal relationship. It was further strengthened by the finding, that circulating ACE2 levels are not related to the lung tissue ACE2 levels, suggesting that the circulating ACE2 levels are not a biomarker of the lung ACE2 expression. Finally, our most recent findings suggest that ACE may play a role in the differentiation and activation of the macrophages. The two orders of magnitude increase in ACE expression observed in these processes may contribute to the physiological function of macrophages and the RAS. Preliminary data suggest, that ACE inhibition results in a reduced level of inflammatory cytokine secretion by macrophages upon viral, but not in bacterial infection models. This may provide a link between the cardiovascular and inflammatory systems. In particular, the apparent success of RAS inhibitors over other blood pressure reducing medications may be explained by their anti-inflammatory effects.

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