Plasma angiotensin peptides as biomarkers of rheumatoid arthritis are correlated with anti-ACE2 auto-antibodies level and disease intensity

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Abstract
Background  This study aimed to explore a correlation between plasma angiotensin II/(1–7) (Ang II/Ang-(1–7)) ratio, anti-ACE2 autoantibodies level and disease activity in rheumatoid arthritis (RA) patients.
Methods  In a pilot study, the plasma level of Ang II, Ang-(1–7), and anti-ACE2 autoantibodies of twelve RA patients (five in active stage and seven in remission) were measured using an LC–MS/MS method and an ELISA kit, respectively.
Results  The Ang-(1–7) level was significantly higher in the remission group than in the active RA patients (7.63 ± 2.61 vs. 1.29 ± 0.81 ng/mL). On the contrary, the Ang II level was higher in those with active RA compared to the remission group (5.43 ± 1.82 vs. 0.87 ± 0.16 ng/mL). The mean ELISA score of anti-ACE2 autoantibodies in patients with active RA was significantly higher than patients in remission (1.41 ± 0.11 vs. 1.81 ± 0.11, p < 0.05).
Conclusion  This study result suggests that the angiotensin peptides concentration and anti-ACE2 autoantibodies levels can be used as biomarkers of RA. This will help clinicians evaluate better treatment success rates and disease prognosis to prevent long-term complications of RA.

Keywords  Renin Angiotensin system · Rheumatoid arthritis · Angiotensin-(1–7) · Angiotensin II · Anti-ACE2 autoantibodies

Introduction
Rheumatoid arthritis (RA) is an autoimmune disorder affecting about 1% of the world population (Firestein 2003). It is a chronic inflammatory autoimmune disease affecting many joints of the body, but primarily hands, wrists, and feet, causing pain, swelling, stiffness and disability, and systemic complexities that may lead to an early death (Choy 2012). The literature indicates that the renin–angiotensin (Ang) system (RAS) comprises different factors contributing to inflammatory reactions in various tissues. It is shown that many components of this system, including peptides (Phillips and Kagiyama 2002; Ruiz-Ortega et al. 2001) and enzymes (Goto et al. 1990), are involved in RA pathogenesis. The RAS (Fig. 1) consists of two opposing yet balanced arms, classical and protective, which act as a critical regulator of blood pressure, body fluid, and electrolyte homeostasis (Turner and Hooper 2002). However, this balance can be disturbed, and the plasma level of its components could be altered due to pathological inflammatory conditions such as RA (Moreira et al. 2021).

The classical RAS consists of Ang converting enzyme (ACE)/Ang II/Ang II type 1 receptor (AT1R), which is associated with a diverse range of biological effects through the AT1R activation by Ang II and initiation of intracellular activity cascades in different tissues. In addition to the conventional pathway of ACE, Ang II produces from its precursor Ang I through the catalytic action of chymase, a potent serine protease widely distributed in various human tissues (Urata et al. 1994). The outcome of such cascade activation is pro-inflammatory, proliferative, fibrotic, and vasoconstrictive effects (Dzau 2001). The RAS protective arm consists of ACE2/Ang-(1–7)/Mas receptor (MasR), which opposes...
the Ang II effects (Soro-Paavonen et al. 2012; Varagic et al. 2014). Ang-(1–7) is one of the active peptides of the RAS that can be directly formed from Ang I through the action of neutral endopeptidase (NEP) or prolyl endopeptidase (PEP) and mainly by the action of ACE2 on Ang II (Eriksen et al. 2002). Ang-(1–7) acts as the counterregulatory peptide within the RAS and presents with anti-inflammatory, anti-proliferative, anti-fibrotic, and vasodilatory effects and maintains hydro-electrolyte, blood pressure, and immune system balances (Souza dos Santos et al. 2001).

Activation of the RAS with different etiologies results in a chronic imbalance between two arms of the RAS, which can potentially lead to the development of pathophysiological conditions in multiple tissues such as renal, cardiovascular (CV), central nervous system (CNS), or articular tissues. ACE2 is one of the main components of the RAS, which converts pro-inflammatory Ang II to tissue-protective Ang-(1–7). The urinary or plasma circulating level of ACE2 has been associated with different disease conditions such as renal (Soro-Paavonen et al. 2012), CV (Varagic et al. 2014), and metabolic disorders (Park et al. 2013). Many studies correlate plasma ACE2 levels with hypertension (Varagic et al. 2014), heart failure, microalbuminuria, and nephropathy in diabetic patients (Park et al. 2013). Despite the higher plasma level of ACE2, those patients presented with reduced ACE2 enzyme activity and had considerable circulating anti-ACE2 autoantibodies (Takahashi et al. 2010) (Fig. 2).

Herein, we investigated the production of anti-ACE2 autoantibodies in inflammatory conditions, impacting Ang peptides levels. Therefore, the measurement of Ang-(1–7) level as a product of ACE2 activity on Ang II and assessment of the Ang II/Ang-(1–7) ratio along with anti-ACE2 autoantibodies could offer reliable diagnostic and prognostic biomarkers for patients with different inflammatory diseases such as RA.

The present study sheds light on the relationship between the activated RAS and RA. The plasma levels of two main peptides of the RAS, Ang-(1–7) and Ang II, and anti-ACE2 autoantibodies ELISA scores were evaluated and compared in individuals with active or remission stage RA. We explored correlations between RAS biomarkers and RA status within each group, particularly the C-reactive protein (CRP) levels, and PAPID3 scores. With these findings, hypotheses can be made about the status of systemic RAS axes in RA and establishing reliable biomarkers for the diagnosis and prognosis of RA.

**Fig. 1** The schematic overview of the RAS and its opposing classical and protective arms. Ang; angiotensin, ACE; angiotensin-converting enzyme, ACE2; angiotensin-converting enzyme 2, MasR; Mas receptor, AT1R; angiotensin II type 1 receptor, AT2R; angiotensin II type 2 receptor, NEP; neutral endopeptidase, PEP; prolyl endopeptidase

**Fig. 2** An illustration of ACE2 inactivation by its specific autoantibodies. ACE2; angiotensin-converting enzyme 2

[Diagram showing the RAS and its opposing arms, including Angiotensinogen, Renin, Ang I, ACE, Ang II, ACE2, Ang-(1–9), Chymase, NEP, PEP, ACE, AT1R, AT2R, MasR, Proliferation, Vasoconstriction, Inflammation, Fibrosis, Hypertension, Classical Arm, Protective Arm.]

[Diagram showing the inactivation of ACE2 by anti-ACE2 autoantibodies, including Soluble ACE2 and anti-ACE2 autoantibody complex, ACE2 inactivation, Anti-ACE2 autoantibody, Soluble ACE2, Plasma, Shedding, ACE2, Cell membrane.

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Materials and methods

Materials

Ang-(1–7) and Ang II were purchased from AnaSpec (Fremont, CA, USA). [Asn¹ Val⁵]-Ang II was purchased from Sigma Aldrich (St. Louis, MO, USA) and used as LC–MS/MS assay internal standard (IS). Complete Mini™ protease inhibitor tablets were purchased from Sigma Aldrich (St. Louis, MO, USA). All solvents were LC–MS grade and were purchased from Sigma Chemical Co. The C₁₈ solid-phase extraction (SPE) cartridges (3 mL, 500 mg) were obtained from Waters Corporation (Milford, MA, USA). Human recombinant ACE2 was purchased from Abcam (ab151852, Cambridge, United Kingdom). Double-distilled deionized water was used in all experiments.

Study design

This investigation was an exploratory pilot study in which 12 patients with RA were enrolled using a convenience sampling approach at the time of the study. Patients were categorized into the “active” and “remission” groups based on the disease diagnosis criteria explained below. The Institutional Review Board approved this study through the IRB-FY2020-273 protocol. Written informed consent was obtained from all patients before admission to the study.

Defining RA stage

The staging of RA activity was made using a Routine Assessment of Patient Index Data 3 (RAPID3) questionnaire (Pincus et al. 2008) in tandem with comparing the measured amount of CRP. RAPID3 questionnaire is a self-reporting index that includes the three patient-reported American College of Rheumatology (ACR) Core Data Set. RAPID3 measures RA based on physical function, pain, and the global patient status estimate (Pincus et al. 2008). Each of these criteria is scored 0 to 10, for a total of 0 to 30 scores. CRP is a protein made in the acute phase of RA and is the most common test used in clinical practice. CRP elevation can be a very early and sensitive response to most inflammation diseases and can be a valid marker for inflammation and infection. Furthermore, CRP is widely used as an index of disease activity in rheumatological and other connective tissue diseases, except for systemic Lupus Erythematosus (Schwenger et al. 1998).

In this study, a CRP level above 10 mg/L correlated with elevated RAPID3 measurements, confirming active RA disease in certain patients. Patients with less disease activity or disease remission had CRP levels below 10 mg/L and RAPID3 measurements in the “low” or “remission” levels.

Plasma sample collection

Two mL of blood was obtained from each participant and transferred to test tubes containing 100 μL of protease inhibitor cocktail solution containing 1.0 mM p-hydroxy mercury benzoate, 30 mM 1,10-phenanthroline, 1.0 mM phenylmethylsulphonyl fluoride, 1.0 mM Pepstatin A and 7.5% EDTA (all from Sigma Aldrich, St. Louis, MO, USA) to inactivate the peptidases based on the manufacturer’s brochure. No heparin was added as it interferes with the assay. Plasma was harvested after centrifugation for 10 min at 2500×g and 4 °C. Each plasma sample was aliquoted to avoid repeated freeze and thaw cycles and was stored at −80 °C until assayed.

Solid-phase extraction (SPE)

SPE was done based on a previously established method by Cui et al. with minor changes in the process (Cui et al. 2007). Briefly, to 200 μL of plasma samples, 50 μL of the [Asn¹ Val⁵] Ang II IS (20 ng/mL) was added and mixed. Then, formic acid was added to the final concentration of 0.5%, and after vortex mixing, samples were loaded onto the Waters C₁₈ SPE cartridges. The cartridges were preconditioned with 2 mL ethanol and 4 mL deionized water. Samples were then loaded onto the cartridge, followed by 3 mL of deionized water to wash it. A positive nitrogen flow was applied to dry the cartridges further. Then 2.5 mL of methanol containing 5% formic acid was added to elute the Ang peptides. The eluted solutions were collected and dried using a Savant 200 SpeedVac system (Thermo Fisher Scientific, Waltham, MA, USA). The dried samples were reconstituted in 100 μL of 16% acetoni tri le in water containing 0.1% formic acid, and 10 μL of samples were injected into the LC–MS/MS to quantify the Ang peptides concentrations.

LC–MS/MS analysis

For plasma sample analysis, a validated LC–MS/MS with multiple reaction mode (MRM) method (Cui et al. 2007) was used. The system was composed of liquid chromatography in tandem with mass spectrometry (Shimadzu, Columbia, MD, USA) with a controller (CBM-20A), two binary pumps (LC-30AD), an autosampler (SIL-30AC), and an AB SCIEX (Foster City, CA, USA) QTRAP 5500 quadrupole mass spectrometer in positive electrospray ionization mode (ESI). The chromatograms were monitored and integrated by the Analyst 1.6.3 software from AB Sciex.

LC separation was performed on an analytical reversed-phase column Kinetex-C₁₈ 100 × 2.1 mm (1.7 μm)
(Phenomenex, Torrance, CA) using a combination of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile as mobile phases at a flow rate of 0.2 mL/min. The mobile phase gradient started at 5% B and increased to 30% B in 5 min, kept at 30% B for 5 min. Subsequently, the gradient was returned to 5% B in 3 min and held at 5% B for 2 min before the next injection for column re-equilibrium.

The positive ion ESI mass spectrometric parameters were as follow: capillary voltage: 5.5 kV, temperature: 300 °C, declustering potential (DP): 100 V, and collision cell exit potential (CXP): 15 V. LC–MS/MS was performed with MRM transitions of m/z 300.6 → 371.2 (Ang-(1–7)), m/z 349.7 → 400.2 (Ang II), and m/z 516.5 → 769.4 (IS). Nitrogen was used as collision gas, and the collision energies were set at 20–30 eV. The calibration curves using peak height ratio (analyte over IS) were constructed over the range of 500 pg/mL to 10 ng/mL in plasma.

Detection of anti-ACE2 autoantibodies in plasma

Anti-ACE2 autoantibodies were detected using a previously-established method by Takahashi et al. (Takahashi et al. 2010). Briefly, an ELISA assay was performed using purified recombinant human ACE2. About 10 μg/mL of recombinant human ACE2 were first coated overnight onto a 96-well plate with bicarbonate buffer (pH 9.6) at 4 °C. The wells were then treated with a blocking buffer composed of 5% BSA in PBS and washed with a buffer consisting of 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20. The plasma samples of the patients was added to the plate and incubated for 1 h at room temperature. Bound anti-ACE2 autoantibodies were detected using horseradish peroxidase-conjugated anti-human IgG antibody (Novus Biologicals, Littleton, CO, USA). The optical density (OD) at 450 nm was measured after a 30-min incubation with SureBlue TMB microwell peroxidase substrate (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA). All samples were analyzed in triplicate.

Statistical analysis

All data are reported as means ± SEM. Data distribution was tested for their normality. The one-tailed Student’s t-test was used to analyze the demographics. The Mann–Whitney U and two-tailed Student’s t-test was used for the rest of the comparisons to determine significant differences at \( p < 0.05 \) using GraphPad Prism 8.0 software (San Diego, CA, USA). The Pearson correlation coefficient between variables was calculated using SAS Studio online version.

Results

Patients’ demographic and RA diagnosis

The demographics of patients having RA in remission \((n = 7)\) or active \((n = 5)\) groups are presented in Table 1. Each group had a similar distribution of males and females. There were no statistically significant differences in age, body mass index (BMI), medication use, or comorbidities. However, the score values for CRP and RAPID3 were significantly different between the two groups (Table 1).

RAPID3 is included by the American College of Rheumatology (ACR) among the indices used to measure RA disease activity (Saag et al. 2008). Based on the CRP levels and RAPID3 scores, five patients fall into the active RA category, and seven in the remission category.

Plasma Ang peptides quantification using LC–MS/MS

The mean ± SEM results of the levels of Ang-(1–7) and Ang II peptides are shown in Fig. 3A. The data indicate that Ang-(1–7) level in active RA (1.29 ± 0.81 ng/mL) is significantly lower than in remission RA (7.63 ± 2.61 ng/mL). In contrast to Ang-(1–7) levels, Ang II levels are significantly higher in active RA patients (5.43 ± 1.82 ng/mL) as in comparison to the remission group (0.87 ± 0.16 ng/mL).

The ratio of Ang-(1–7)/Ang II in remission and active group is shown in Fig. 3B. This ratio was significantly lower in the active (0.25 ± 0.12) than in the remission group (5.61 ± 0.67).

ELISA scores for plasma anti-ACE2 autoantibodies

Samples from active and remission patients showed an ELISA reactivity to ACE2. The OD values were above the baseline level, and it was determined as the mean ELISA

Table 1 Baseline demographic and clinical characteristics of RA patients

| Variable     | Remission group Mean ± SD | Active group Mean ± SD | \( p \)-value |
|--------------|---------------------------|------------------------|--------------|
| Age          | 68.29 ± 12.05             | 68.80 ± 15.22          | Ns           |
| BMI          | 28.57 ± 5.71              | 27.60 ± 5.46           | Ns           |
| Medication use | 7.43 ± 3.78              | 7.40 ± 2.07            | Ns           |
| Comorbidities | 1.71 ± 1.25              | 1.80 ± 0.84            | Ns           |
| CRP (mg/L)   | 1.59 ± 1.27               | 16.10 ± 10.97          | 0.0054*      |
| RAPID3 score | 8.29 ± 6.38               | 19.85 ± 7.64           | 0.0085*      |

Ns not significant

*One-tailed t-test was performed, \( p < 0.05 \)
Plasma angiotensin peptides as biomarkers of rheumatoid arthritis are correlated with anti-ACE2 autoantibodies ELISA score in those samples. The mean ELISA score was significantly higher in the active (1.81 ± 0.11) RA patients than in the remission (1.41 ± 0.11) RA patients (Fig. 4).

**Ang-(1–7), Ang II and anti-ACE2 autoantibodies correlations**

The observed correlations of the anti-ACE2 autoantibodies ELISA score, Ang II peptide level, and Ang-(1–7)/Ang II ratio with RAPID3 score and CRP level are shown in Fig. 5. Positive correlations of anti-ACE2 autoantibodies ELISA score with CRP level (r = 0.7251) and RAPID3 score (r = 0.6132) were detected (Fig. 5A). Similarly, Ang II level was positively correlated with anti-ACE2 autoantibodies (r = 0.6796), CRP levels (r = 0.7820), and Rapid 3 score (r = 0.2555), but it was not significant in the latter case. As shown in Fig. 5C, in the case of Ang-(1–7)/Ang II ratio, the sign of those significant correlations was negative.

**Discussion**

This study evaluated and compared Ang-(1–7), Ang II plasma levels, Ang-(1–7)/Ang II ratio, and anti-ACE2 autoantibodies ELISA scores between active and remission RA patients to determine whether the RAS systemic axes were unbalanced in RA. Results indicate that Ang-(1–7) levels were significantly lower in the active RA patients, whereas Ang II levels were markedly higher than patients in remission. Similarly, the mean ELISA score of anti-ACE2 autoantibodies was substantially higher in active RA patients. As the circulating soluble ACE2 is mainly responsible for the conversion of Ang II to Ang-(1–7), the lower Ang-(1–7)/Ang II ratio in active RA patients was attributed to the deactivation of ACE2 enzyme by higher anti-ACE2 autoantibodies levels. This is in concert with the observed higher Ang II plasma levels in the active RA patients and positively correlated with CRP levels and RAPID3 scores.

It is worth mentioning the observed results indicate that both arms of the RAS are affected by RA. Accordingly, the higher score of anti-ACE2 autoantibodies in the active RA than in remission patients emphasizes the contribution of
the classical and protective arm to the disease’s status and intensity. It was previously reported that the plasma anti-ACE2 autoantibodies in patients with inflammatory conditions such as pulmonary arterial hypertension (Takahashi et al. 2010) and COVID-19 (Arthur et al. 2021) were associated with the intensity of the disease and poor prognostic outcome. The reported evidence aligns with the theory that anti-ACE2 antibodies can decrease soluble ACE2 activity and lower the Ang-(1–7)/Ang II ratio in inflammatory diseases. This phenomenon shifts the balance between the RAS arms toward the pro-inflammatory state and triggers observed arthritis symptoms in RA and cytokine syndrome in COVID-19 patients. This study is the first to report the RAS peptides and anti-ACE2 autoantibodies ELISA analysis associated with RA disease activity.

Braz et al. (2021) compared plasma levels of the RAS components between healthy and RA patients and correlated them with CV risk association. The study falls short of categorizing the patients based on their disease status of being at the active stage or in remission and did not look at the plasma anti-ACE2 antibodies. In line with our study, Braz et al. reported an association between CV risks of RA with the imbalance of the RAS arms. They also noticed an early, subclinical atherosclerotic disease in the cohort of female patients with at least 6 months of RA. The authors reported higher ACE, Ang II, and Ang-(1–7) peptides in RA patients and observed positive control between Ang II levels and disease activity indices, such as Disease Activity Score in 28 joints (DAS28) and Clinical Disease Activity Index (CDAI). Similar to previously reported studies (Soro-Paavonen et al. 2012; Varagic et al. 2014; Park et al.

Fig. 5 Correlations between the Ang II concentration, anti-ACE2 autoantibodies ELISA, and RAPID3 scores (A), Ang II plasma concentration, CRP levels, and anti-ACE2 autoantibodies ELISA score (B). Ang-(1–7)/Ang II ratio, anti-ACE2 autoantibodies ELISA score, CRP levels, and RAPID3 scores (C)
2013), the authors observed a higher trend of ACE2 levels in RA patients. They also noted that Ang-(1–7) level was higher and Ang II/Ang-(1–7) ratio was lower in RA patients, which was interpreted as a compensatory mechanism of the RAS protective arm. The observed higher ACE/ACE2 ratio has been reported in animal models of arthritis. In contrast to Braz et al.’s findings, such imbalance resulted in an expected higher AngII/Ang-(1–7) ratio in plasma and heart tissues (Asghar et al. 2017). The discrepancy in Braz et al.’s study (a lower ratio of AngII/Ang-(1–7) in RA patients despite the higher ACE/ACE2 ratio) can arise from several reasons related to their study design. First, the composition of the patient cohort regarding the disease state (active vs. remission) is not clear. Our findings indicate that the RAS components levels and Ang-(1–7)/ Ang II ratio are significantly correlated with the intensity of the RA disease. Therefore, analysis of data without considering the disease stage could be misleading. It is known that inflammation activates the RAS and impacts it in the enzyme, peptide, and receptor levels in favor of its classical arm in different inflammatory conditions (such as RA, diabetes, cancer, etc.) (Moreira et al. 2021; Munro et al. 2017; Ribeiro-Oliveira Jr et al. 2008). This activation promotes disease progression by producing pro-inflammatory mediators through Ang II interaction with AT1R. Secondly, the use of ACE inhibitors (ACEi), Ang II receptor blockers (ARBs), nonsteroidal anti-inflammatory drugs (NSAIDs), and other anti-inflammatory medications increases ACE2 expression and Ang-(1–7) levels (Asghar et al. 2017; Ferrario et al. 2020). Therefore, such medications modulate the inflammation by restoring the disturbed balance of the RAS components, which could explain the Braz et al. reported-higher level of Ang-(1–7) beyond the componentry mechanisms. Third, despite the higher trend of ACE2 in RA patients, its activity could be compromised by developing anti-ACE2 autoantibodies, which have been observed in inflammatory diseases (Takahashi et al. 2010). Thus, if the patients in Braz et al.’s study were categorized based on the disease stage and their Ang II and Ang-(1–7) plasma levels were compared to their disease state instead of healthy vs. RA, a similar result of the current study could be observed.

The current pilot study suffers from some limitations. Although a statistical significance was observed for almost all correlations, there was a sign of clustered data rather than correlation in the case of CRP. Some level of caution should be taken in its interpretation. In addition, these results could be more solid and convincing if the sample size was larger and more individuals with RA were enrolled in the study. Including other disease index scores could also solidify the observed correlation of the RAS biomarkers with CRP and RAPID3 indices.

Conclusion

In conclusion, the findings support the hypothesis that the RAS classical arm is augmented and the protective arm is suppressed in RA. This study suggests that higher systemic and maybe local Ang-(1–7) levels could modulate and put the disease into remission and protect the patient from long-term consequences of RA. These hypotheses need future testing with more extensive, longitudinal follow-up studies and a more comprehensive assessment of the RAS components. As RA pathogenesis remains mainly unelaborated, it is essential to follow every lead that may help explain the disease.

These findings confirm that the RAS is one of the major players in different inflammatory disease pathophysiology, including RA. Therefore, utilizing the RAS components as the biomarkers of RA can serve as a reliable tool for early detection. This could also help clinicians evaluate the treatment success rate and determine disease prognosis to prevent long-term complications of RA.

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Data availability All datasets generated and analyzed during the current study are available from the corresponding author.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethics approval and consent to participate The Institutional Review Board approved this study through the IRB-FY2020-273 protocol. Written informed consent was obtained from all patients before admission to the study.

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