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Autoantibodies against ACE2 and angiotensin type-1 receptors increase severity of COVID-19

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Abstract

The renin-angiotensin system (RAS) plays a major role in COVID-19. Severity of several inflammation-related diseases has been associated with autoantibodies against RAS, particularly agonistic autoantibodies for angiotensin type-1 receptors (AA-AT1) and autoantibodies against ACE2 (AA-ACE2). Disease severity of COVID-19 patients was defined as mild, moderate or severe following the WHO Clinical Progression Scale and determined at medical discharge. Serum AA-AT1 and AA-ACE2 were measured in COVID-19 patients (n = 119) and non-infected controls (n = 23) using specific solid-phase, sandwich enzyme-linked immunosorbent assays. Serum LIGHT (TNFSF14; tumor necrosis factor ligand superfamily member 14) levels were measured with the corresponding assay kit. At diagnosis, AA-AT1 and AA-ACE2 levels were significantly higher in the COVID-19 group relative to controls, and we observed significant association between disease outcome and serum AA-AT1 and AA-ACE2 levels. Mild disease patients had significantly lower levels of AA-AT1 (p < 0.001) than moderate and severe patients. No significant differences were detected between males and females. The increase in autoantibodies was not related to comorbidities potentially affecting COVID-19 severity. There was significant positive correlation between serum levels of AA-AT1 and LIGHT (TNFSF14; r Pearson = 0.70, p < 0.001). Both AA-AT1 (by agonistic stimulation of AT1 receptors) and AA-ACE2 (by reducing conversion of Angiotensin II into Angiotensin 1-7) may lead to increase in AT1 receptor activity, enhance proinflammatory responses and severity of COVID-19 outcome. Patients with high levels of autoantibodies require more cautious control after diagnosis. Additionally, the results encourage further studies on the possible protective treatment with AT1 receptor blockers in COVID-19.

1. Introduction

Many recent studies have highlighted the major role of renin-angiotensin system (RAS) in severity of COVID-19 [1–3]. Angiotensin converting enzyme 2 (ACE2) plays a key role in the process and it is usually considered that ACE2 acts as a double-edged sword [4,5]. First, ACE2 transforms components of the pro-inflammatory RAS axis such as angiotensin I (Ang I) and particularly Ang II into components of the anti-inflammatory RAS axis such as Ang1-9 and particularly Ang 1–7. Ang II acts on angiotensin type 1 (AT1) receptors and activates the NADPH-oxidase complex producing superoxide and promoting cell pro-oxidative and pro-inflammatory responses [6,7], while Ang 1–7 acts on Mas and Mas-related receptors promoting cell antioxidative and anti-inflammatory responses. Therefore, an increase in ACE2 activity is essential to balance the RAS towards the anti-inflammatory response. Consistent with this, the protective effects of ACE2 and its product

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Ang1-7 against experimental lung injuries have previously been demonstrated [8]. Second, ACE2 is also the entry receptor for the virus [9], and upregulation of ACE2 expression may enhance cell infection. Third, both ACE2 functions interact each other, since SARS-CoV-2 binding to ACE2 decreases the levels of ACE2 at the cell surface [10, 11], thus shifting the RAS balance towards the pro-inflammatory Ang II/AT1 axis, which leads to inflammation, fibrosis and progression of disease severity.

It is known that differences in immunological responses in different patients play a major role in progression and severity of COVID-19. Progression and severity of several inflammation-related processes have been associated with the presence of autoantibodies against major components of the RAS. Particularly, the presence of autoantibodies for AT1 receptors (AA-AT1), which act as AT1 receptor agonists, enhanced the proinflammatory RAS activity in several tissues and processes [12]. In addition, the presence of autoantibodies against ACE2 (AA-ACE2) has also been detected in several processes, and AA-ACE2 have been involved in progression of inflammation [13]. ACE2 autoantibodies have been related to inhibition of ACE2 function [13], which decreases the anti-inflammatory arm activity and shift the balance towards the proinflammatory RAS. The mechanisms responsible for generation of the above-mentioned autoantibodies have not been totally clarified. However, the increase in levels of cytokines such as IL-6 and TNF-α [14, 15], and particularly TNFSF14 (tumor necrosis factor ligand superfamily member 14, LIGHT) [16] has been associated to the increase in generation of AA-AT1. An increase in levels of pro-inflammatory cytokines also plays a major role in severity of COVID-19 outcome. However, it is not known whether these autoantibodies (AA-AT1, AA-ACE2) are increased in COVID-19 patients and whether the levels of RAS autoantibodies could be associated to progression of COVID-19 severity. In the present study, we investigated the levels of AA-AT1 and AA-ACE2 antibodies at the time of diagnosis in COVID-19 patients relative to non-infected controls, and whether levels of these autoantibodies at diagnosis are associated with mild, moderate and severe COVID-19 outcome determined at medical discharge on the basis of WHO Clinical Progression Scale.

2. Methods

2.1. Study design

In the present study, a total of 23 adult non-infected controls and 119 adult patients testing positive for SARS-CoV-2 RT-PCR were included. Controls were recruited from users of a dental clinic at the Primary Health-Care Unit Fontiñas (Santiago de Compostela). COVID-19 patients were prospectively recruited from April 2020 to December 2020 at University Hospital Complex of Santiago de Compostela. The sample size per group was calculated assuming a minimum large effect size in f-test of 0.4 between any pair of groups (n = 4) [17] and accepting an alpha risk of 0.05 and a beta risk of 0.2 in a two-sided test. A minimum number of 20 subjects is necessary in each group to recognize statistically significant differences. It has been anticipated a missing data rate of 10% due to incomplete primary endpoints. This calculation was carried out using the PWR package [18] in R [19]. Patient’s requirements to be recruited were a positive RT-PCR for SARS-CoV-2; to be older than 18 years old and to sign an informed consent (designed for this purpose). At the time of recruitment, a blood sample was obtained, and serum was separated by centrifugation at 1500g for 20 min. Then, aliquots of serum samples were stored at – 80 °C until processed for quantification of AA-AT1, AA-ACE2 and LIGHT levels. Clinical outcome of patients was followed until medical discharge, and finally patients were divided into three groups according to WHO Clinical Progression Scale [20]: mild, moderate, or severe. Mild disease (scores 1–3; n = 31) patients were enrolled from the Emergency Room Department. Samples from moderate (scores 4–5; n = 68) and severe (scores 6–10; n = 20) hospitalized patients were initially obtained from the Emergency Room Department before hospitalization. Samples were also obtained from the University Hospital Complex of Santiago de Compostela Biobank and from Murcian Institute for Biosanitary Research Biobank. All patients were followed-up with electronic health information system until January 2021. Clinical data assessing demographics, comorbidities, symptoms, physical and radiological findings, disease stage, treatment and laboratory tests results were collected from electronic medical records. The study was approved by the Galician Drug Research Ethics Committee (CEIm-G), protocol 2020/212. The research was carried out in accordance with the principles of the Helsinki Declaration.

2.2. Serum samples and anti-AT1 and anti-ACE2 autoantibody measurements

Serum samples were stored at – 80 °C until the biochemical analysis. Serum AA-AT1 and AA-ACE2 were measured using two specific solid-phase, sandwich enzyme-linked immunosorbent assays (ELISAs) for quantitative determination of these autoantibodies (Catalog Number 12000 and 16000, respectively; Cell Trend; Luckenwalde, Germany). Manufacturer’s instructions were strictly followed. Absorbance was measured at 450/620 nm using an Infinite M200 multiwell plate reader (TECAN) and AA-AT1 and AA-ACE2 concentrations were quantified using specific standard curves from each one (4PL curve fit). In both cases, samples with values over the standard curve were diluted with assay buffer to get their absorbances within the standard curve.

2.3. LIGHT/TNFSF14 determination

To analyse the serum concentration of TNFSF14, also known as LIGHT, a commercially available specific ELISA Kit was used (EH TNFSF14, Invitrogen) according to the manufacturer’s instructions. Protein levels were quantified using specific standard curve (4PL curve fit).

2.4. Statistical analysis

Data were expressed as median (interquartile range [IQR]). To test if the populations follow a normal distribution, Shapiro–Wilks test was used. Two group comparisons were carried out by two tailed t-test and multiple comparisons by one-way ANOVA followed by post-hoc t-tests. When normality assumption was violated, Wilcoxon test for two group comparisons and Kruskal-Wallis test followed by post-hoc Mann-Whitney U tests for multiple comparisons were used. Bonferroni correction for multiple comparisons was used on post-hoc p-values. When two response variables were present, two-way ANOVA were used if dataset passed the normality test and for nonparametric data, an Aligned Rank Transformation (ART) step was performed before the ANOVA test. Spearman’s correlation coefficient (r) was used to study the correlation between different parameters. Associations between categorical variables were tested by using the chi-square test or the Fisher’s exact test if expected value is less than 6 in a cell of the contingency table. P < 0.05 was considered significant for all the analyses. All the analyses were done using R software [19].

3. Results

3.1. Patient characteristics

A total of 23 controls (mean age: 66.13 years old ±6.77 SD; range 55–76 of which 14 were women and 9 men) and 119 COVID-19 patients were enrolled in the study. The patient group included 31 mild, 68 moderate and 20 severe patients, considering the clinical course of the disease at their medical discharge (Table 1). The severe group included 16 patients who required mechanical or non-invasive ventilation (scores 6–9) and 4 patients who died (score 10). Mean age of patients was 56.5 years old (56.5 ± 14.9 SD; range 20–93) and 48.3% were males. The
most relevant comorbidities in patient’s group included hypertension (33.8%), diabetes (12.8%), dyslipidemia (38.5%), obesity (35.3%), heart disease (7.6%), lung disease (8.2%) and renal disease (2.7%). Main clinical features are presented in Table 1. No significant differences in frequency of comorbidities were observed between control group and COVID-19 group. Relationships between COVID-19 severity and different comorbidities were studied using chi-square test or Fisher’s exact test (see Methods). A significant association ($X^2_{Pearson} (2) = 7.82; p = 0.002$) was found between severity and diabetes; severity and obesity ($X^2_{Pearson} (2) = 13.92; p = 0.001$) and severity and former smokers ($X^2_{Pearson} (2) = 6.56; p = 0.038$).

### 3.2. Serum anti-AT1 and anti-ACE2 autoantibodies

Blood samples from controls and COVID-19 patients were analysed to determine serum AA-AT1 and AA-ACE2. Median of AA-AT1 concentrations were 5.070 [Interquartile range (IQR) 3.775–8.170] U/mL for the control group and 8.209 [IQR 6.006–10.061] U/mL for COVID-19 patients. Median of AA-ACE2 concentrations were 4.691 [IQR 2.841–9.863] U/mL in the control group and 9.403 [IQR 4.485–21.552] U/mL for COVID-19 patients. Two-way ART-ANOVA analysis revealed significantly higher serum levels for AA-AT1 ($p = 0.002$) and for AA-ACE2 ($p = 0.013$) in COVID-19 group than in the control group. However, no significant differences were detected between males and females for serum levels of AA-AT1 ($p = 0.845$) and AA-ACE2 ($p = 0.342$) when sex differences were tested together with controls/patients, being the interaction terms not significant for both levels of autoantibodies. (Fig. 1A and B).

![Fig. 1](image-url)  
**Fig. 1.** Levels of AA-AT1 and AA-ACE2 in controls and COVID-19 patients. COVID-19 patients had significantly higher serum levels of AA-AT1 (A; two-way ART-ANOVA, $p = 0.002$) and AA-ACE2 (B; two-way ART-ANOVA, $p = 0.013$) than the control group. However, no significant differences were detected between males and females for serum levels of AA-AT1 (two-way ART-ANOVA, $p = 0.845$) and AA-ACE2 (two-way ART-ANOVA, $p = 0.342$). Interaction terms were not significant for both levels of antibodies. Data distribution is shown using a box plot with boxes representing the IQR and the median (black line) and whiskers representing ±1.5 IQR. IQR: Interquartile range.
ACE2 Median 19.597 [IQR 9.306–39.471] U/mL).

The correlation coefficient between AA-AT1 and AA-ACE2 was statistically significant; however, it is too low to consider that there is a relevant correlation between these variables and suggests that other major factors contribute to the increase observed in serum AA-ACE2 levels (see Discussion). Sex differences were studied together with severity (Fig. 2C and D; Supplementary Table 1). However, no significant differences were detected in our patients between males and females for serum levels of AA-AT1 (two-way ART-ANOVA coefficient, p = 0.95) and AA-ACE2 (two-way ART-ANOVA, p = 0.14).

### 3.3. RAS autoantibodies and comorbidities associated to COVID-19 patients

In order to know whether the increase in autoantibodies observed in our patients could be related to comorbidities potentially affecting COVID-19 severity, serum levels of AA-AT1 and AA-ACE2 were studied in different comorbidities (Wilcoxon-Mann-Whitney test). However, only AA-ACE2 levels were significantly higher in patients with diabetes (Median 16.630 [IQR 10.480–27.356] U/mL) than in non-diabetic patients (Median 7.957 [IQR 4.339–19.715] U/mL) (Table 2 and Fig. 3A). Moreover, we studied the possible relationship between COVID-19

Table 2
Statistical analysis for AA-AT1 and AA-ACE2 in different comorbidities.

| Comorbidity                  | AA-AT1 (Mann-Whitney test) |     |     |     | AA-ACE2 (Mann-Whitney test) |     |     |     |
|-----------------------------|----------------------------|-----|-----|-----|----------------------------|-----|-----|-----|
|                             | Statistic                  | p-value | Signif |     | Statistic                  | p-value | Signif |     |
| Obesity                     | 1707                       | 0.61871 | ns    |     | 1702                       | 0.63844 | ns    |     |
| Hypertension                | 2325                       | 0.76768 | ns    |     | 2623                       | 0.11398 | ns    |     |
| Diabetes mellitus           | 1535                       | 0.46662 | ns    |     | 1573                       | 0.04669 | <0.05*|     |
| Dyslipidemia                | 2345                       | 0.32846 | ns    |     | 2668                       | 0.7706  | ns    |     |
| Smoking                     | 579                        | 0.84562 | ns    |     | 626                        | 0.44267 | ns    |     |
| Cardiovascular disease      | 761                        | 0.8607  | ns    |     | 813                        | 0.5729  | ns    |     |
| Chronic Respiratory disease | 458                        | 0.20585 | ns    |     | 350                        | 0.9019  | ns    |     |
| Chronic kidney disease      | 332                        | 0.60699 | ns    |     | 369                        | 0.34116 | ns    |     |

Ns: not significant; Signif: Significance.
severity and diabetes using chi-square test. A significant association ($X_\text{Pearson}^2 = 7.82; p = 0.020$) was found between severity and diabetes. Then, we analysed whether the relationship of disease severity and autoantibodies was determined by diabetes. However, after controlling the possible confounding effect of diabetes by performing a two-way ART-ANOVA the relationship of antibodies and disease severity was still significant ($F(2) = 17.505, p < 0.001$).

Finally, we investigated the possible involvement of LIGHT (TNFSF14) in the increase in AA-AT1 observed in the present study. As commented below, considerable evidence supports the role for LIGHT in generation of AA-AT1 in other diseases. Consistent with this, a significant positive correlation between serum levels of AA-AT1 and LIGHT was observed ($r_\text{Pearson} = 0.70, p < 0.001$). Furthermore, a significant linear correlation was shown when applying a log transformation to the levels of AA-AT1 ($r_\text{Pearson} = 0.83, p < 0.001$) (Fig. 3B).

4. Discussion

In the present study, we observed a significant increase in levels of AA-AT1 and AA-ACE2 in COVID-19 patients relative to non-infected controls. Furthermore, our results show a significant increase in levels of AA-AT1 and AA-ACE2 in moderate and severe COVID-19 patients relative to patients with mild outcome. Interestingly, levels of AA-AT1 showed a high correlation with levels of LIGHT. Levels of AA-AT1 showed a lower correlation with AA-ACE2. The results also suggest that increased levels of autoantibodies are related to differences in the response to SARS-CoV-2 infection rather than previous patient comorbidities. As could be expected, there is some degree of variablity in levels of antibodies between patients in the same group of severity, which is probably related to individual differences in the immunological response and effects of some previous comorbidities such as diabetes. However, we observed that the relationship of antibodies and disease severity was still significant in diabetic patients.

AA1 receptor autoantibodies were initially identified in preeclampsia [21]. More recently, an increase in circulating AA-AT1 has been related to several inflammatory diseases and progression of hypertension and cardiovascular processes [12,22]. Levels of AA-AT1 in our cohort of moderate and severe COVID-19 patients are similar to those reported by other authors in some autoimmune diseases such lupus nephritis [23], rheumatoid arthritis or Raynaud’s phenomenon [24]. However, higher levels of autoantibodies have been reported for systemic sclerosis and primary Sjögren’s syndrome [24]. The present results suggest that AA-AT1 contribute to COVID-19 severity, as AA-AT1 bind AT1 receptors and, together with Angiotensin II, increase the activity of the RAS pro-inflammatory axis (summarized in Fig. 4). It has been shown that Ang II enhances severity of several SARS experimental models, which is reduced by AT1 blockers [8,10]. In a recent study, we have observed that AT1 blockers (i.e. ARAII, ARBs, sartans) increased levels of transmembrane ACE2 in the lung while decreased internalization of viral spike protein by several mechanisms [10]. The present results suggest that patients with high levels of AA-AT1 are particularly suitable candidates to treatment with AT1 receptor blockers, which have been considered beneficial for COVID-19 outcome in several recent studies [25–27]. Increasing evidence indicates that treatments with AT1 receptor blockers should not be discontinued in patients with COVID-19 or at risk of COVID-19. Now, the question is if we should initiate treatment with AT1 blockers after COVID-19 diagnosis or in patients at risk of infection or risk of severity. Although the above-mentioned experimental and clinical studies suggest beneficial effects, additional clinical studies, specifically addressing this question, are necessary.

Both experimental [28,29] and clinical [30,31] studies have shown that the increase in levels of cytokines such as IL-6 and TNF-α in COVID-19 plays a major role in the severity of the disease. It has also been shown that infusion of IL-6 and TNF-α in animal models induces an increase in levels of AA-AT1 [14,15]. However, TNFSF14 (LIGHT) appears particularly interesting. Circulating LIGHT is mainly secreted by cells of the innate and adaptive immune system, although can also be produced by tissue structural cells [32,33]. LIGHT acts on structural and inflammatory cells to promote initiation of inflammatory and fibrotic responses and induces expression of several pro-inflammatory mediators, including the above-mentioned cytokines [34], so that LIGHT plays a major role in lung tissue remodeling, inflammation and fibrosis [35]. It is particularly remarkable that LIGHT, acting via tissue transglutaminase 2 (TG2), has been revealed as a major mechanism for generation of AA-AT1. TG2 modifies and stabilizes AT1 receptors [16,36], and the epitope sequence of AA-AT1 on the second extracellular loop of the receptor can be crosslinked to TG2 via glutamine residue Q187 [37]. TG2-modified AT1 receptors act as neoantigens that promote AA-AT1 generation [16] and AT1 receptor sensitization [38]. Consistent with this, we observed a significant correlation between AA-AT1 and LIGHT levels in COVID-19 patients.

A reduction in ACE2 activity at the cell surface (i.e. transmembrane ACE2) shifts the RAS balance towards the pro-inflammatory axis, as ACE2 transforms Ang II into the anti-inflammatory Ang 1–7. The
The presence of SARS-CoV-2 infection increases the shift towards the RAS pro-inflammatory axis. It is known that binding of SARS-CoV-2 to ACE2 reduces levels of transmembrane ACE2 and induces virus internalization and shedding of ACE2 into a soluble/circulating form, as a result of activation of the proteases such ADAM17 (TACE, TNF-α-converting enzyme) and TMPRSS (transmembrane protease serine 2) [39,40]. Activation of the pro-inflammatory AT1 receptors also increases ADAM17 and TMPRSS activity, increasing viral entry and ACE2 shedding [29,41,42]. In the case of COVID-19, generation of AA-ACE2 may be enhanced by the presence of circulating immunocomplexes of ACE2-SARS-CoV-2 as a consequence of increased levels of circulating ACE2, due to virus-induced ACE2 shedding, and the presence of high levels of circulating viruses. The presence of high levels of AA-ACE2 may lead to further decrease in transmembrane ACE2 activity in the lung and other tissues. Therefore, both AA-AT1 (by agonistic stimulation of AT1) and AA-ACE2 (by a decrease in transmembrane ACE2 activity and a reduction of conversion of Ang II into Ang 1-7) lead to increase in AT1 receptor activity, enhancing the pro-inflammatory responses, and possibly viral internalization and ACE2 shedding [29,41,42], and a more severe outcome of the COVID-19 disease (Fig. 4).

Fig. 4. Proposed model for AA-AT1 and AA-ACE2 effects. SARS-CoV-2 infection induces increase in pro-inflammatory cytokines, particularly LIGHT, promoting AA-AT1, which act as AT1 receptor agonists and enhance the pro-inflammatory RAS axis. SARS-CoV-2 binds cell surface ACE2 leading to a decrease in this transmembrane ACE2 and an increase in levels of soluble/circulating ACE2. A decrease in transmembrane ACE2 further enhances the pro-inflammatory RAS axis and reduces anti-inflammatory axis activity. The increase in levels of circulating ACE2-SARS-CoV-2 complexes may increase levels of AA-ACE2, which further reduce transmembrane ACE2 activity and the anti-inflammatory RAS function. Green lines, beneficial effects; red lines, detrimental effects.

5. Conclusions

The present results suggest that both AA-AT1 and AA-ACE2 contribute to increase in severity of COVID-19 outcome and could be used as an index of probable progression of COVID-19 towards severity. Patients with high levels of autoantibodies require more cautious control after diagnosis of COVID-19. In addition, the results encourage further studies on the possible protective treatment with AT1 receptor blockers in COVID-19. Finally, the present results also reveal the need for further studies where the reported findings are associated with in-depth immunological investigations, characterizing adaptive and innate immune responses in different COVID-19 phases.

Author contributions

JLL-G, AIR-P and CML designed research and integrated the clinical and laboratory data; MC-A, PM-C and JA S-Q were responsible for subject selection, collected samples and clinical data; RV and MAP conducted the AA measurements; AIR-P, CML and JLL-G writing, review and critique; all authors edited the manuscript.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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AA-ACE2 autoantibodies against ACE2
AA-AT1 autoantibodies for angiotensin type-1 receptors
ACE2 Angiotensin converting enzyme 2
ADAM17, TACE, TNF-α converting enzyme; Ang, angiotensin
AT1 angiotensin type 1 receptors
ELISA enzyme-linked immunosorbent assay
IQF Interquartile range
RAS renin-angiotensin system
TG2 transglutaminase 2
TMPRSS transmembrane protease serine 2

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