

Full Title: Angiotensin II receptor I auto-antibodies following SARS-CoV-2 infection

Short Title: AT1R-Ab after SARS-CoV-2 infection

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1 **Abstract:**

2 BACKGROUND: Coronavirus disease 2019 (**COVID-19**) is associated with endothelial
3 activation and coagulopathy, which may be related to pre-existing or infection-induced pro-
4 thrombotic autoantibodies such as those targeting angiotensin II type I receptor (**AT1R-Ab**).
5 METHODS: We compared prevalence and levels of AT1R-Ab in COVID-19 cases with mild or
6 severe disease to age and sex matched negative controls. RESULTS: There were no significant
7 differences between cases and controls. However, there were trends toward a higher proportion
8 with AT1R-Ab positivity among severe cases versus controls (32% vs. 11%, $p=0.1$) and higher
9 levels in those with mild COVID-19 compared to controls (median 9.5U/mL vs. 5.9U/mL,
10 $p=0.06$). CONCLUSIONS: These findings suggest that AT1R-Ab are not consistently associated
11 with COVID-19 but do not exclude a contribution to endothelial pathology in a subset of people.

12 **Introduction:**

13 Coagulopathy is emblematic of severe disease from severe acute respiratory syndrome
14 coronavirus 2 (**SARS-CoV-2**) infection (1). Series from patients admitted to the intensive care
15 unit and autopsy series describe high rates of both pulmonary embolism and small vessel
16 inflammation and thrombosis in the lungs and other organs (2, 3). A number of mechanisms that
17 may mediate coronavirus disease 2019 (**COVID-19**)-associated coagulopathy have been
18 proposed including direct endovascular damage, altered platelet function, and pre-existing or
19 infection-induced pro-thrombotic auto-antibodies (1). Pro-thrombotic anti-phospholipid
20 antibodies have been described in some but not all patients with COVID-19 associated
21 coagulopathy (4), and recent research suggests the potential for cross-reactive auto-antibodies
22 following infection with as yet unidentified targets (5).

23 Amongst previously described anti-endothelial antibodies, those directed against the
24 angiotensin II type 1 receptor (**AT1R-Ab**) are associated with essential hypertension (6), pre-
25 eclampsia (7), and vascular rejection following renal transplant (8). AT1R is part of an
26 angiotensin II-driven signaling cascade that leads to increased blood pressure and inflammatory
27 cytokine production; the pathway may be inhibited by the activity of ACE2, which interestingly
28 also functions as the primary receptor for SARS-CoV-2 cell entry (9). In particular, AT1R-
29 activating auto-antibodies (**AT1R-AA**) directed against the second extracellular loop of AT1R
30 are associated with pathology (7, 8), including elevated pro-inflammatory TNF- α and IL-6
31 cytokine levels and increased disease severity in pre-eclampsia models (7). AT1R-AA are
32 reported to induce expression of tissue factor by vascular smooth muscle which may trigger
33 aberrant coagulation and clot formation (10). In renal transplant recipients, AT1R-AA are
34 associated with refractory rejection and malignant hypertension, as well as vasculopathy
35 including arterial inflammation, endothelial activation, tissue factor expression, and thrombosis
36 (8).

37 Multi-organ endothelial inflammation is strongly associated with disease severity and poor
38 outcome from COVID-19 (2, 3, 11). Such endothelial damage may allow the binding of or trigger
39 the development of anti-endothelial antibodies such as AT1R-Ab. Alternatively, structural
40 homology between epitopes of the SARS-CoV-2 Spike and the second extracellular loop of
41 AT1R might lead to the development of cross-reactive antibodies. These possibilities led us to
42 consider AT1R-Ab as a potential mediator of COVID-19-associated coagulopathy and disease.
43 If directed against the second extracellular domain of AT1R, such antibodies might trigger
44 hypertension, inflammation including cytokine storm, and pulmonary edema as seen in severe
45 COVID-19. In addition, these antibodies might contribute to further endothelial inflammation,
46 tissue factor expression, and hypercoagulability. We therefore tested the hypothesis that
47 COVID-19 infection is associated with a higher prevalence and levels of AT1R-Ab and assessed
48 whether SARS-CoV-2 negative individuals with AT1R-Ab demonstrated cross-reactivity against
49 SARS-CoV-2 Spike trimer.

50 **Materials & Methods:**

51 *Cohorts and samples*

52 Mild COVID-19 cases were from the Seattle Children's SARS-CoV-2 Recovered Cohort and the
53 Seattle Children's SARS-CoV-2 Prospective Cohort both approved by Seattle Children's
54 Hospital IRB, with study specimens from ≥ 14 days from COVID-19 symptom onset (**Table 1**).
55 Participants recovered at home, except for one participant who required hospitalization for
56 oxygen therapy but no additional support. Severe COVID-19 cases were participants in the
57 Swedish-Institute for Systems Biology Novel Coronavirus (**INCOV**) Biobank, approved by
58 Providence St. Joseph Health IRB, with samples from ≥ 7 days from symptom onset. The severe
59 COVID-19 cases had maximum WHO COVID-19 scores between 4 and 8 (median 5). Six of 19
60 participants with severe disease died from complications of COVID-19. Human leukocyte

61 antigen (**HLA**) typing by direct sequencing had previously been conducted for the INCOV
62 cohort. Cases were matched 1:1 to controls by age and sex. Healthy controls were derived from
63 the Children's SARS-CoV-2 Prospective Cohort. At the time of blood collection (March to July,
64 2020), controls reported no prior history of SARS-CoV-2-like illness, were nasal specimen PCR
65 negative for SARS-CoV-2, and were SARS-CoV-2 antibody negative on the SCoV-2 Detect™
66 IgG and IgM ELISAs (InBios, Seattle, WA). All participants provided written informed consent.

67 *Structural Modeling*

68 To assess the biologic plausibility of cross-reactive antibody binding sites, searches to find
69 potential regions of structural homology between AT1R and SARS-CoV-2 Spike protein were
70 performed. The UCSF Chimera MatchMaker tool was used, which aligns structures based on
71 pairwise sequence alignment followed by 3D structural alignment using residue C α positions.
72 Spike protein monomer [PDB: 6VXX] was used as the SARS-CoV2 reference structure
73 compared to human AT1R protein [PDB: 4YAY].

74 *Anti-AT1R Antibody Screening*

75 The concentration of AT1R-Ab in plasma was measured with a quantitative ELISA (Celltrend,
76 Luckenwalde, Germany) using the entire AT1R protein, with assays performed according to the
77 manufacturer's instructions. In brief, standards and diluted samples (1:100) were added to
78 AT1R-precoated microtiter plates and incubated for 2 h at 4°C. After washing, AT1R-Ab was
79 detected with HRP-labelled anti-human IgG antibody followed by enzymatic substrate reaction.
80 Samples were tested in duplicate. Optical densities (**OD**) were converted into concentrations by
81 comparison to a standard curve and mean concentration was used to define a sample as
82 positive when ≥ 17 U/mL, indeterminate when 10–16.9 U/mL, and negative when < 10 U/mL
83 according to the manufacturer's recommendation.

84 *Anti-SARS-CoV-2 Spike, Receptor Binding Domain, and Nucleocapsid Protein ELISAs*

85 In order to investigate potential cross-reactivity between AT1R-Ab and SARS-CoV-2 Spike, we
86 tested plasma from AT1R-Ab positive control subjects and randomly selected AT1R-Ab
87 negative controls for reactivity against SARS-CoV-2 Spike trimer (including both S1 and S2
88 domains), as previously published (12). Because we hypothesized that potential cross-reactive
89 antibodies might bind SARS-CoV-2 Spike with lower avidity, we started with an initial plasma
90 dilution of 1:10 followed by serial dilutions by a factor of two. To assess for potential cross-
91 reactivity induced by prior non-SARS-CoV-2 coronavirus infection, we additionally tested these
92 control subjects against the receptor binding domain (**RBD**) in S1 and the nucleocapsid protein
93 (**NP**) using a similar ELISA format. Endpoint titers were defined as the reciprocal of plasma
94 dilution at OD 0.1 at a wavelength of 450nm after the subtraction of plate background. Positive
95 antibody responses were defined at a titer greater than or equal to 1:50 for each antigen.

96 *Statistical Analysis*

97 AT1R-Ab prevalence and levels in all COVID-19 cases were first compared to those in the
98 entire set of controls. In secondary analysis, the subsets with mild and severe COVID-19 were
99 separately compared to their age and sex matched controls. The distribution of AT1R-Ab status
100 (positive versus intermediate/negative) was compared between groups using the Chi-square
101 test, and AT1R-Ab level was compared between groups using quantile regression on the
102 median. Distribution of HLA and SARS-CoV-2 antibody responses were compared with the Chi-
103 square test. Significance was defined as $p \leq 0.05$, whereas trend was defined as $p \leq 0.1$.

104 **Results:**

105 *Association between AT1R-Ab and SARS-CoV-2 infection*

106 Participants with mild versus severe COVID-19 were younger and more likely to be female
107 (**Table 1**), consistent with prior reports of older age and male sex as risk factors for severe
108 COVID-19.

109

110

111 **Table 1. Cohort demographics by COVID-19 status**

Demographics	All COVID-19 Controls & Cases			Mild COVID-19 Controls & Cases			Severe COVID-19 Controls & Cases		
	Controls	Cases	p-value	Controls	Cases	p-value	Controls	Cases	p-value
Number	53	53		34	34		19	19	
Age, years, median [range]	46 [24-73]	45 [24-88]	0.8	41 [24-71]	40 [24-74]	1	62 [38-73]	64 [38-88]	0.6
Female, n (%)	34 (64%)	34 (64%)	1	27 (79%)	27 (79%)	1	7 (37%)	7 (37%)	1
Days from 1st symptom, median [range]		24 [7-82]			34 [14-82]			13 (7-37)	

112 There was no significant difference in the proportion of AT1R-Ab positive participants between
113 cases and controls (26% vs. 17%, $p=0.3$) or in the subset of mild cases versus controls (21%
114 vs. 21%, $p=1$) (**Figure 1A, 1B**). However, there was a trend toward higher proportion with
115 AT1R-Ab positivity among severe cases versus controls (32% vs. 11%, $p=0.1$) (**Figure 1C**).
116 Similarly, there was not a statistically significant difference in the median AT1R-Ab level
117 between any group, but a trend toward higher AT1R-Ab levels was observed in all cases versus
118 controls (median 9.0 vs. 6.0 U/mL, $p=0.1$), which was also found in the subset of mild COVID-19
119 cases and controls (median 9.5 vs. 5.9 U/mL, $p=0.06$), but not severe COVID-19 cases and
120 controls (both medians 6.7 U/mL, $p=1$) (**Figure 1**).

121 **Figure 1. Association between AT1R-Ab and COVID-19.** AT1R-Ab levels < 10U/mL were
122 defined as negative, 10-16.9 indeterminate, ≥ 17 positive (dotted lines). Solid lines indicate
123 medians. Open circles are cases with known thrombosis. (**A**) There was a trend toward higher
124 level of AT1R-Ab in all cases versus controls (median 9.0 vs 6.0, $p=0.1$) and (**B**) amongst the
125 subset of mild COVID-19 versus controls (median 9.5 vs 5.9, $p=0.06$). (**C**) There was a trend
126 toward higher proportion positive for AT1R-Ab amongst severe cases versus controls (32%
127 versus 11%, $p=0.1$).

128

129 *Association between AT1R-Ab, thrombotic events, and HLA-type amongst severe COVID*

130 Within the severe COVID-19 group, we tallied six participants with thrombotic events. These
131 included one participant with a myocardial infarction and one with a pulmonary embolism, both
132 with high levels of AT1R-Ab (33 and 18 U/mL respectively), and four participants with deep
133 venous thrombosis, with two having indeterminant levels of AT1R-Ab (11 and 14 U/mL). The
134 proportions of AT1R-Ab positive participants were not different in those with and without

135 thrombosis (33% vs 31%, $p=0.9$). While the median level of AT1R-Ab was higher in those with
136 thrombosis, this was not statistically significant (12.5 vs. 5.7 U/mL, $p=0.3$).

137 AT1R-Ab have previously been associated with the HLA DR1*04 group (13). A higher
138 proportion of AT1R-Ab positive versus negative participants carried a DRB1*04 allele; however,
139 this was not statistically significant (50% vs. 18%, $p=0.2$) (**Supplementary Table 1**).

140 *Structural homology between AT1R and SARS-CoV-2 Spike and assessment of antibody cross-*
141 *reactivity*

142 Potential cross-reactivity of antibodies between SARS-CoV-2 Spike and AT1R was predicted
143 based on our finding of structural homology between the aligned proteins in the S2 domain of
144 the SARS-CoV-2 Spike and the six-membered alpha-helical bundle of AT1R, including the
145 second extracellular domain targeted by AT1R-AA (**Figure 2**). To test the hypothesis that AT1R-
146 Ab cross-reacts against SARS-CoV-2 Spike, in particular the S2 domain, the nine AT1R-Ab
147 positive SARS-CoV-2 uninfected control participants and six randomly selected AT1R-Ab
148 negative SARS-CoV-2 uninfected control participants were tested for reactivity against SARS-
149 CoV-2 Spike trimer by ELISA. AT1R-Ab status was not associated with reactivity to SARS-CoV-
150 2 Spike trimer (17% AT1R-Ab negative vs. 22% positive control participants, $p=0.8$). In addition,
151 some AT1R-Ab positive control participants demonstrated low level reactivity against RBD (0%
152 AT1R-Ab negative vs. 22% positive participants, $p=0.2$) and NP (17% AT1R-Ab negative vs.
153 44% positive participants, $p=0.3$) (**Figure 3**).

154 **Figure 2. Structural overlap between SARS-CoV-2 Spike and AT1R.** The structures of
155 SARS-CoV-2 Spike (S1 and S2) and AT1R were aligned to assess potential homology. Overlap
156 was identified between SARS-CoV-2 Spike (blue) in the S2 domain with the six-membered
157 alpha-helical bundle of AT1R (purple), including the second extracellular domain (green). The
158 root-mean square deviation over all 237 aligned residue pairs was 35.6 Angstroms.

159 **Figure 3. Assessment of SARS-CoV-2 protein immune reactivity by AT1R antibody**

160 **status.** ELISA for SARS-CoV-2 Spike trimer, RBD, and NP. Green: AT1R Ab negative, SARS-
161 CoV-2 negative controls. Red: AT1R Ab positive, SARS-CoV-2 negative controls. Black: SARS-
162 CoV-2 positive cases. Endpoint titers were defined as the reciprocal of plasma dilution at OD
163 0.1 read at 450nm after the subtraction of plate background. Positive threshold was defined as
164 1:50 and is indicated with dashed line. There was no clear relationship between AT1R antibody
165 status amongst SARS-CoV-2 negative subjects and immunoreactive pattern against SARS-
166 CoV-2 proteins.

167 **Discussion:**

168 Overall, AT1R-Ab levels were not significantly different between controls and cases with either
169 mild or severe COVID-19. However multiple trends were observed. Specifically, amongst severe
170 COVID-19 cases there was a trend towards an increase in the proportion of cases versus
171 controls who were AT1R-Ab positive and a trend towards higher levels of AT1R-Ab amongst
172 mild COVID-19 cases versus controls. Among severe cases, there was no significant
173 association with level or prevalence of AT1R-Ab and a thrombotic event. Although we did not
174 identify any significant associations, the trends we observed are consistent with a recent report
175 of an association between AT1R-Ab and worse outcomes in patients hospitalized for COVID-19,
176 suggesting they may play a role in endothelial activation during COVID-19 (14).

177 Amongst severe COVID-19 cases, where HLA typing was available, half of AT1R-Ab positive or
178 indeterminate participants carried a DRB1*04 allele (13). Of note, DRB1*04:01 and DRB1*04:05
179 are both associated with increased risk of autoimmune disease (15). These participants may
180 have had pre-existing AT1R-Ab not associated with their SARS-CoV-2 infection. Alternatively,
181 when infected with SARS-CoV-2, individuals with this allele may have had a higher risk of
182 developing AT1R-Ab in the setting of endothelial damage. We are not able to dissociate these
183 possibilities given the cross-sectional nature of our sampling at the time of COVID-19.

184 We identified a region of potential structural homology between AT1R and the S2 domain of
185 SARS-CoV-2 Spike; however, among controls without SARS-CoV-2 there was no clear
186 association between AT1R-Ab reactivity and low-level reactivity against SARS-CoV-2 Spike
187 trimer arguing against cross-reactivity as a driver of AT1R-Ab development. In addition, some
188 controls reacted against RBD and NP, suggesting that the reactivity we detected may represent
189 cross-reactive antibodies from prior endemic coronavirus infections (16). It is possible but less
190 likely that these participants had experienced an unrecognized SARS-CoV-2 infection given
191 their commercial antibody negative status and the time of enrollment when the prevalence of
192 infection remained low in the greater Seattle area.

193 Our study was limited by small sample size, particularly amongst our severe COVID-19 cases.
194 In addition, as we did not have samples from cases that pre-dated their SARS-CoV-2 infections
195 we could not determine whether AT1R-Ab were pre-existing or developed in the setting of
196 SARS-CoV-2 infection. Further, we were not able to determine whether AT1R-Ab positive
197 controls are likely to develop more severe COVID-19 if SARS-CoV-2 infected.

198 An improved understanding of the mechanisms driving COVID-19-associated coagulopathy
199 could point to therapies to lessen COVID-19 morbidity and mortality. We did not identify a
200 significant association between AT1R-Ab and COVID-19 severity in this small case-control
201 study, but the trends we observed support a possible association between AT1R-Ab and
202 COVID-19. Further research should explore whether other endothelial autoantibodies are
203 associated with hypercoagulability in COVID-19.

204

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212

213 **Conflict of Interest:**

214 J.D.G. declared research support from Gilead, Lilly, and Regeneron, and Monogram
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Figure 1





