

# SARS-CoV-2 Receptors are Expressed on Human Platelets and the Effect of Aspirin on Clinical Outcomes in COVID-19 Patients

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## Research Article

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1 **SARS-CoV-2 Receptors are Expressed on Human Platelets and the Effect of Aspirin on Clinical**  
2 **Outcomes in COVID-19 Patients**

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9 **Running Title:** Platelets Express SARS-CoV-2 Receptors & Aspirin Does Not Protect in COVID-19

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26  
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31 **Key Words:** Platelets, SARS-CoV-2, COVID-19, Thrombosis, ACE2, TMPRSS2  
32

33 **Abstract**

34 Coronavirus disease-2019 (COVID-19) caused by SARS-CoV-2 is an ongoing viral pandemic marked by  
35 increased risk of thrombotic events. However, the role of platelets in the elevated observed thrombotic  
36 risk in COVID-19 and utility of anti-platelet agents in attenuating thrombosis is unknown. We aimed to  
37 determine if human platelets express the known SARS-CoV-2 receptor-protease axis on their cell surface  
38 and assess whether the anti-platelet effect of aspirin may mitigate risk of myocardial infarction (MI),  
39 cerebrovascular accident (CVA), and venous thromboembolism (VTE) in COVID-19. Expression of ACE2  
40 and TMPRSS2 on human platelets were detected by immunoblotting and confirmed by confocal  
41 microscopy. We evaluated 22,072 symptomatic patients tested for COVID-19. Propensity-matched  
42 analyses were performed to determine if treatment with aspirin or non-steroidal anti-inflammatory  
43 drugs (NSAIDs) affected thrombotic outcomes in COVID-19. Neither aspirin nor NSAIDs affected  
44 mortality in COVID-19. However, both aspirin and NSAID therapies were associated with increased risk  
45 of the combined thrombotic endpoint of (MI), (CVA), and (VTE). Thus, while platelets clearly express  
46 ACE2-TMPRSS2 receptor-protease axis for SARS-CoV-2 infection, aspirin does not prevent thrombosis  
47 and death in COVID-19. The mechanisms of thrombosis in COVID-19, therefore, appears distinct and the  
48 role of platelets as direct mediators of SARS-CoV-2-mediated thrombosis warrants further investigation.

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57 **Introduction**

58 COVID-19 is caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and curiously  
59 displays a propensity for thrombosis in multiple vascular beds. COVID-19-related thrombosis may  
60 contribute to severe organ injury and death. The incidence of thrombotic events was as high as 31% in  
61 one cohort<sup>1</sup>. Clinical and autopsy studies of COVID-19 patients suggest an increased risk of  
62 microthrombi, venous thromboembolism (VTE), and ischemic stroke<sup>2,3</sup>. Activated platelets are  
63 circulating mediators of thrombosis and, therefore, may serve as a logical therapeutic target in COVID-  
64 19. Two registered clinical trials (NCT04363840 and NCT04365309) will prospectively evaluate patient  
65 outcomes following low dose aspirin in the context of SARS-CoV-2 infection.

66

67 SARS-CoV-2 utilizes an spike glycoprotein to bind to the host transmembrane angiotensin-converting  
68 enzyme 2 (ACE2) and is then cleaved by the serine protease TMPRSS2 to coordinate entry into the host  
69 cell<sup>4,5</sup>. Therefore, co-expression of ACE2 and TMPRSS2 may be important for host cell entry and  
70 infectivity of SARS-CoV-2. Importantly, human tissue distribution of ACE2 and TMPRSS2 mirrors organ  
71 system involvement in COVID-19 and includes the lungs<sup>6-11</sup>, vascular endothelium<sup>9-12</sup>, heart<sup>11,13,14</sup>,  
72 kidneys<sup>8,10,13</sup>, liver<sup>8,10</sup>, digestive tract<sup>8,10,11,15</sup>, nasal epithelium<sup>7,10,11</sup> and central nervous system<sup>10,14</sup>.

73 Single-stranded RNA (ssRNA) viruses, including influenza, are engulfed by platelets and may contribute  
74 to immuno-thrombosis indirectly through developing neutrophil extracellular traps (NETs) by engaging  
75 the platelet toll-like receptor 7 (TLR7)<sup>16</sup>. SARS-CoV-2, another ssRNA virus, utilizes platelets to modulate  
76 immunologic responses including the development of neutrophil extracellular traps (NETs) that are  
77 emerging as pro-thrombotic responses in patients with COVID-19<sup>17</sup>. Further, elevation of soluble P-  
78 selectin and sCD40L in blood from patients with COVID-19 compared to controls provides indirect  
79 evidence of platelet activation in COVID-19 coagulopathy<sup>18</sup>. SARS-CoV-2 is a ssRNA virus, and therefore  
80 may directly augment platelet activation causing myocardial infarction (MI), stroke, and VTE.

81  
82 A recent report demonstrated that COVID-19 patients have a divergent platelet transcriptome from  
83 healthy individuals, and aspirin suppresses COVID-19 platelet activation *in vitro*<sup>19</sup>. The platelet surface  
84 receptor for SARS-CoV-2 was not clarified in this study, while a similar investigation by another group  
85 identified mRNA for SARS-CoV-2 in human platelets<sup>20</sup>. Thus, our goal was to determine if platelets  
86 express known SARS-CoV-2 receptor proteins and, as with influenza previously, contribute to thrombotic  
87 events in patients. In the absence of clinical trial data, we sought to evaluate the potential benefit in  
88 mitigating thrombotic responses *in vivo* with use of aspirin or other NSAID antiplatelet therapies by  
89 propensity matching patients using real-world data.

90

## 91 **Methods**

### 92 **Platelet Isolation**

93 Healthy volunteers without any known medical history or on antiplatelet therapy donated blood  
94 specimens in accordance with and approved by the Cleveland Clinic Foundation Institutional Review  
95 Board (IRB) approval. For each subject, venous blood was drawn by a medical professional into citrate  
96 plasma tubes, then centrifuged in a tabletop centrifuge at 1100 RPM for 15 minutes. The platelet rich  
97 plasma (PRP), collected well above the buffy coat, was decanted and the platelets were centrifuged at  
98 2600 RPM for an additional 5 minutes. These washed platelets were then used in immunoblotting and  
99 fluorescence-activated cell sorting (FACS) analyses.

100

### 101 **Immunoblotting**

102 Washed platelets from healthy subjects or patients with coronary artery disease (CAD) enrolled at the  
103 Cleveland Clinic main campus in Ohio were isolated and proteins separated by SDS-PAGE as we have  
104 previously documented<sup>21,22</sup> and in accordance with IRB protocols (#19-1451 for patients and #20-413

105 for healthy volunteers). We utilized human brain lysate, human placenta, and engineered human heart  
106 tissue as positive controls for TMPRSS2 and ACE2. Human brain lysate is commercially available (Novus  
107 #NB820-59177). Human placenta lysate was prepared as follows: placental villous tissue was collected  
108 immediately upon uncomplicated, full-term (37–42 weeks' gestation), elective C-section deliveries at  
109 MetroHealth Hospital in Cleveland, Ohio and approved by the Cleveland Clinic and MetroHealth IRB  
110 (#16–1311 and #16–00335, respectively). This tissue was normally discarded placentas with intact fetal  
111 membranes, and following inclusion in the study no protected health information, identifiers, or clinical  
112 data were collected. A waiver of consent was approved by the Cleveland Clinic Foundation IRB as  
113 the placentas were collected anonymously. Engineered human heart tissue was obtained as follows:  
114 human-induced pluripotent stem cells (generated by the California Institute of Regenerative Medicine)  
115 were differentiated into beating ventricular-like cardiomyocytes (iCMs) and grown in a monolayer. To  
116 enhance maturation, iCMs were subsequently grown as engineered heart tissues as we have previously  
117 described<sup>23</sup>. Immunoblotting was conducted using anti-TMPRSS2 (abcam #92323), anti-ACE2 (Abcam  
118 #15348), anti-tubulin (CST #3873S), and anti-GAPDH (CST #5174) antibodies. The mean ratio of  
119 TMPRSS2 or ACE2 to loading control  $\pm$  SEM is documented, unless stated otherwise. Primary antibody  
120 was used as in a 1:10000 titer overnight at 4°C in 3% bovine serum albumin/Tris-buffered saline-Tween  
121 20. Secondary antibody (GE Healthcare, Buckinghamshire, UK) was used in a 1:2000 titer in 5%  
122 milk/Tris-buffered saline-Tween for 1 hour at room temperature. Final autoradiographic films (Bioblot  
123 BXR, Laboratory Product Sales, Rochester, NY) were quantified by densitometry using ImageJ software  
124 (National Institutes of Health). All experiments were performed in accordance with relevant guidelines  
125 and regulations.

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129 **Confocal Microscopy**

130 Venous blood drawn into and separated as citrated plasma was lysed and fixed with BD FACS lysing  
131 solution (BD Biosciences, NJ, USA, cat# 349202) for 10 mins. The platelet pellet was washed with 1X  
132 PBS, centrifuged at 1500g for 7 mins, resuspended in HEPES-buffered Tyrode solution supplemented  
133 with 2% FBS and then stained for 1 hour with the following: CD41 to confirm platelets (ThermoFisher  
134 eBio cat #11-0419-42), ACE2 antibody (Novus cat#NBP2-72117AF647), TMPRSS2 antibody (SantaCruz  
135 cat#sc-515727 AF488) and DAPI to eliminate any DNA components. Mounted slides were resolved by  
136 fluorescent microscopy using a Scanning Disk Nikon A1 confocal microscope with 100x objective lens.  
137 All experiments were performed in accordance with relevant guidelines and regulations.

138

139 **Study Design**

140 Quality-assured clinical data from ambulatory and hospitalized Cleveland Clinic patients treated in  
141 Northeast Ohio and South Florida was used to appraise data on 22,072 symptomatic patients evaluated  
142 for COVID-19 with the goal of determining whether current aspirin use protects patients from death  
143 and/or the secondary composite outcome of MI, thrombotic stroke, and/or VTE. Positive testing for a  
144 SARS-CoV-2 amplicon by nasopharyngeal RT-PCR was used to determine infection status. The electronic  
145 medical record and hospital Medication Administration Record (MAR) was used to confirm new or  
146 ongoing administration of 81 mg aspirin or other NSAIDs for both outpatients and inpatients.

147

148 **Statistical Analysis**

149 Categorical factors are summarized using frequencies and percentages, while continuous factors are  
150 described using median and ranges. Initial descriptive analyses were performed. Comparisons were  
151 made between those with known death status and those with missing death information to identify if  
152 any differences exist in these cohorts. Then among those with known death status, differences in COVID

153 positive and COVID negative patients were assessed. Finally, after stratifying by COVID status,  
154 comparisons of those with and without aspirin use were performed. For all tables, continuous measures  
155 were compared using nonparametric Wilcoxon rank sum tests, while categorical factors were compared  
156 using Pearson chi-square tests or Fisher exact tests, for rare events.

157

158 Given the differences across many covariates, propensity score matching was performed to account for  
159 differences between those with and without aspirin use. This approach used two steps. First, multiple  
160 imputation was performed on all demographic and covariate measures within COVID status stratified  
161 datasets, using fully conditional specification methods. Ten imputed datasets were created. Then  
162 propensity score models were fit for each dataset, with aspirin use as the response and all other  
163 measures as predictors. Predicted probability of aspirin use from each model was calculated, and these  
164 probabilities were averaged across models for each patient. Greedy matching was then performed  
165 using a caliper of 0.2 standard deviations of the logit to create matched datasets for both COVID positive  
166 and negative patients. A small number of aspirin users could not be matched well and were excluded  
167 from the matched analysis. Comparisons of outcomes were performed using mixed effect logistic  
168 regression models to account for the matching process. Overlap weighting propensity score analyses  
169 were also performed<sup>24</sup> which data with the same conclusions. This analysis was repeated using NSAID  
170 groups. For significant effects, E-values<sup>25</sup> that represent the magnitude of the association between an  
171 unobserved covariate and both the medication group and outcome necessary to make the result non-  
172 significant was also calculated. Analyses were performed using SAS software (version 9.4; Cary, NC). A  
173 significance level of 0.05 was assumed for all tests.

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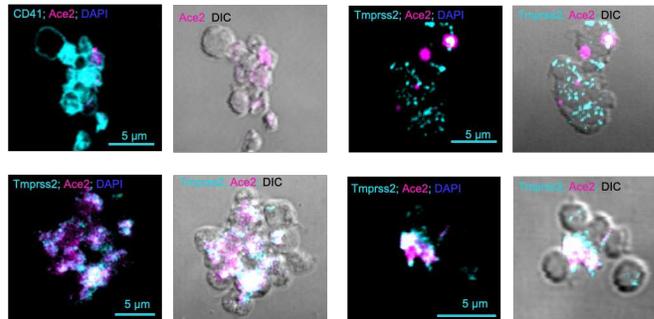
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177 **Results**

178 Expression of ACE2 (n=6) and TMPRSS2 (n=3) on the platelet surface was observed by confocal  
179 microscopy (Figure 1). Expression of TMPRSS2 in healthy subjects (mean age  $40.1 \pm 2.8$  years, n=20) was  
180 also confirmed by immunoblotting at the expected molecular weight of ~50 KDa.

181



182 **Figure 1. Expression of ACE2 and TMPRSS2 in Platelets by Confocal Microscopy.** Platelets isolated from venous  
183 blood of healthy individuals was stained for 1h with the following antibodies: CD41 (platelet-specific marker), ACE2,  
184 TMPRSS2, and DAPI to eliminate any DNA components. Mounted slides were resolved by confocal fluorescent  
185 microscopy using a 100x objective lens. Images are representative of n=6 donors for ACE2 and n=3 for TMPRSS2.  
186 Each image represents a different donor. The scale bar is noted.

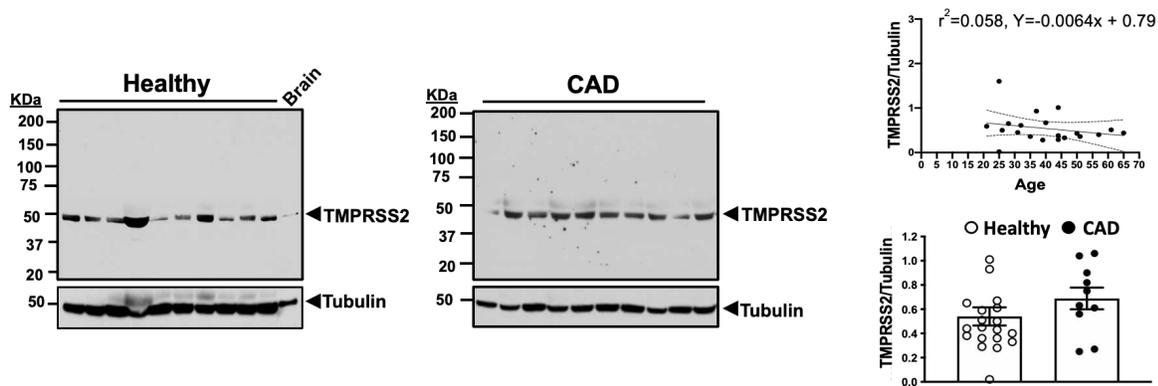
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183

184 Utilizing human brain as a positive control, TMPRSS2 expression was standardized to a loading control  
185 with no correlation between age and platelet TMPRSS2 expression (Figure 2A;  $r^2=0.058$ ,  $p=0.30$ ). Since  
186 ACE2 exists as multiple glycosylated proteins of variable molecular weight<sup>26-28</sup>, human brain<sup>29</sup>, human  
187 placenta<sup>30</sup>, and engineered heart tissue<sup>31</sup> were utilized as positive controls to confirm predominant  
188 migration at ~100 kDa as expected. Given that patients with confirmed CAD receive antiplatelet  
189 medications according to established guidelines, TMPRSS2 expression for healthy controls (n=20) was  
190 compared to patients with coronary artery disease (CAD, n=10) and, while numerically greater in CAD,  
191 was without a statistically significant difference (Figure 2A,  $p=0.15$ ).

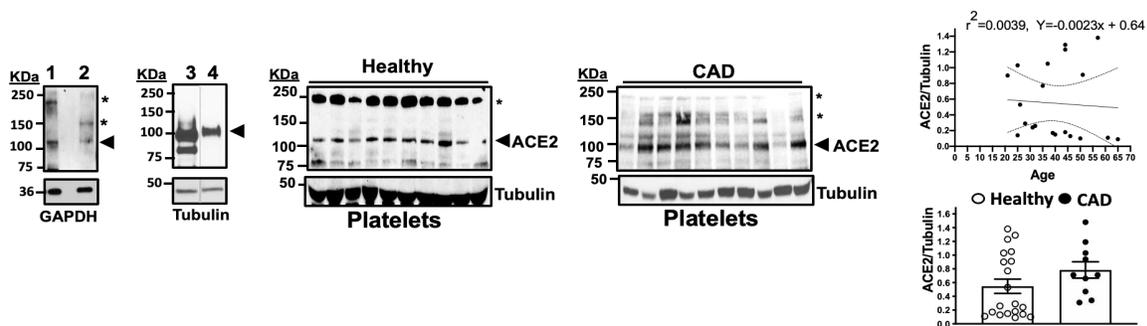
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193 Similarly, expression of ACE2 in healthy subjects (n=20) was confirmed by immunoblotting. ACE2  
 194 expression standardized to tubulin did not correlate with age (Figure 2B;  $r^2=0.0039$ ,  $p=0.79$ ).



**Figure 2A. Expression of TMPRSS2 in Platelets:** Washed platelets from healthy individuals (mean age  $40.1 \pm 2.8$  years,  $n=20$ ) were isolated and proteins separate by SDS-PAGE with molecular weight shown in KiloDaltons (KDa). Immunoblotting was conducted an using an anti-TMPRSS2 antibody or anti-tubulin immunoblotting as a loading control. The ratio of protein to loading control is expressed as a function of age and the correlation coefficient is noted ( $r \pm 95\% \text{ CI}$ ,  $P=0.30$ ). Human brain lysate served as a positive control for TMPRSS2 migrating at the expected molecular weight (~50 KDa). Data shown are representative of 20 healthy individuals (10 male and 10 female) and 10 patients with coronary artery disease (CAD). The mean ratio of TMPRSS2/Tubulin  $\pm$  SEM is noted,  $P=0.145$  between healthy and CAD by Mann Whitney  $U$ ). For clarity of presentation, the tubulin blot was cropped just above and below the 50 KDa marker line.

195  
 196 Platelet ACE2 in healthy subjects (n=20) was compared to patients with CAD (n=10) and, again, while  
 197 numerically higher in CAD, was without a statistical difference (Figure 2B,  $p=0.11$ ). Further, we did not  
 198 observe sex-specific differences in platelet expression of ACE2 or TMPRSS2 (20 men and 20 women in

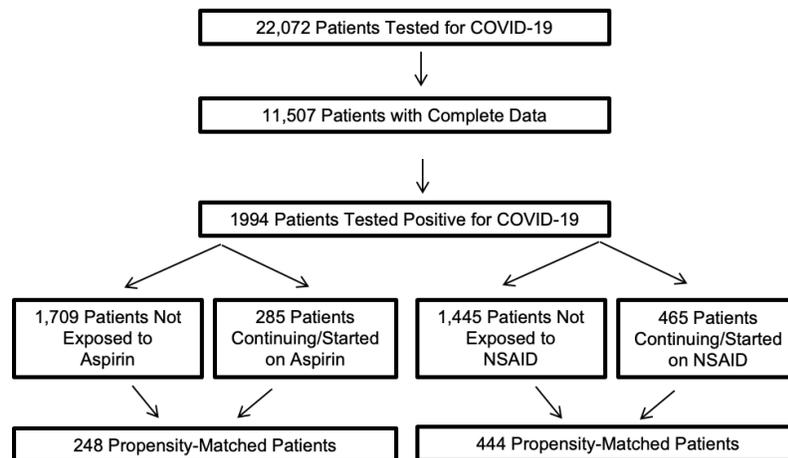


**Figure 2B. Expression of ACE2 in Platelets:** Washed platelets from healthy individuals (mean age  $40.1 \pm 2.8$  years,  $n=20$ ) were isolated and proteins separate by SDS-PAGE with molecular weight shown in KiloDaltons (KDa). Lane 1 is human platelet lysate, lane 2 is human brain lysate, lane 3 is human placenta lysate, lane 4 is lysate from engineered human heart tissue. Immunoblotting was conducted using an anti-ACE2 antibody. Anti-tubulin and anti-GAPDH are loading controls. ACE2 migrates at the expected molecular weight (~100 KDa) shown by an arrowhead with glycosylated forms indicated by \*. The ratio of ACE2 protein to loading control is expressed as a function of age and the correlation coefficient is noted ( $r \pm 95\% \text{ CI}$ ,  $P=0.79$ ). Data shown are representative of 20 healthy individuals (10 male and 10 female) and 10 patients with coronary artery disease (CAD). The mean ratio of ACE2/Tubulin  $\pm$  SEM is noted,  $P=0.112$  between healthy and CAD by Mann Whitney  $U$ ). For clarity of presentation, the tubulin and GAPDH blots are cropped just above and below the 50 KDa and 36 KDa marker lines, respectively and the ACE2 blot is cropped just below the 75 KDa marker. The grey partition line for ACE2 and tubulin are from the same blot separated by three lanes.

199 each group). Full size, uncropped immunoblots for ACE2, TMPRSS2, and loading controls are found in  
200 Supplemental Figure 1A-C.

201  
202 22,072 patients tested for COVID-19 at two Cleveland Clinic hospitals between March 13, 2020 to May  
203 13, 2020 were evaluated. Within this cohort, 11,507 patients had complete clinical data and 1,994  
204 tested positive for the SARS-CoV-2 amplicon by RT-PCR testing. Amongst these 1,994 patients, 1,709  
205 were not exposed and 285 patients were exposed to aspirin. In an attempt to differentiate an anti-  
206 platelet drug effect with aspirin from a more general NSAID class effect, we propensity-matched  
207 patients 1,445 patients not exposed and 465 patients exposed to NSAID therapy (Figure 3).

208



**Figure 3. Patients Testing Positive for SARS-CoV-2 taking Aspirin or NSAIDs.** Patients testing positive for a SARS-CoV-2 amplicon at two Cleveland Clinic hospitals were evaluated. Patients initiated with aspirin or NSAID therapy or continuing aspirin or NSAID if admitted to the hospital were included in this study. Clinical variables in each group were then re-evaluated following careful propensity matching

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213 Table 1 shows the unadjusted characteristics of each comparative cohort for aspirin.

Factor	No Aspirin (N=1,709)		Aspirin Use (N=285)		p-value
	N	Statistics	N	Statistics	
<b>Medications</b>					
CLOPID	1,709	9 (0.53)	285	27 (9.5)	<0.001 <sup>c</sup>
Ticag	1,709	1 (0.06)	285	6 (2.1)	<0.001 <sup>d</sup>
Prasug	1,709	0 (0.00)	285	0 (0.00)	
Cangr	1,709	0 (0.00)	285	0 (0.00)	
Cilost	1,709	0 (0.00)	285	0 (0.00)	
Pentox	1,709	0 (0.00)	285	1 (0.35)	0.14 <sup>d</sup>
AntiPlt	1,709	10 (0.59)	285	285 (100.0)	<0.001 <sup>c</sup>
Multiple Therapy	1,709	0 (0.00)	285	34 (11.9)	<0.001 <sup>d</sup>
AC_therputic	1,709	94 (5.5)	285	56 (19.6)	<0.001 <sup>c</sup>
AC_prophylct	1,709	355 (20.8)	285	215 (75.4)	<0.001 <sup>c</sup>
NSAIDs	1,650	294 (17.8)	260	171 (65.8)	<0.001 <sup>c</sup>
<b>Covariates</b>					
Age	1,709	50.6 ± 17.5	285	70.0 ± 13.6	<0.001 <sup>a2</sup>
Platelets	689	217.4 ± 79.3	253	208.7 ± 85.3	0.14 <sup>a1</sup>
Gender	1,651		285		<0.001 <sup>c</sup>
Male		804 (48.7)		172 (60.4)	
Female		847 (51.3)		113 (39.6)	
Race	1,564		280		<0.001 <sup>c</sup>
White		948 (60.6)		144 (51.4)	
Black		506 (32.4)		124 (44.3)	
Other		110 (7.0)		12 (4.3)	
Ethnicity	1,480		277		<0.001 <sup>c</sup>
Hispanic		204 (13.8)		7 (2.5)	
Non-Hispanic		1,276 (86.2)		270 (97.5)	
Smoking	1,417		268		<0.001 <sup>c</sup>
No		924 (65.2)		123 (45.9)	
Former		362 (25.5)		124 (46.3)	
Current		131 (9.2)		21 (7.8)	
RespSuprt	1,709	191 (11.2)	285	117 (41.1)	<0.001 <sup>c</sup>
OnPressors	1,709	81 (4.7)	285	47 (16.5)	<0.001 <sup>c</sup>
HemodInstab	1,709	85 (5.0)	285	48 (16.8)	<0.001 <sup>c</sup>
COPD_emphysema	1,399	82 (5.9)	274	53 (19.3)	<0.001 <sup>c</sup>
Asthma	1,410	243 (17.2)	273	66 (24.2)	0.007 <sup>c</sup>
Diabetes	1,424	318 (22.3)	278	147 (52.9)	<0.001 <sup>c</sup>
Hypertension	1,447	659 (45.5)	281	244 (86.8)	<0.001 <sup>c</sup>
Coronary_artery_disease	1,405	116 (8.3)	275	100 (36.4)	<0.001 <sup>c</sup>
Heart_Failure	1,404	108 (7.7)	274	78 (28.5)	<0.001 <sup>c</sup>
Cancer	1,447	184 (12.7)	280	63 (22.5)	<0.001 <sup>c</sup>
On_immunosuppressive_treatment	1,456	144 (9.9)	277	36 (13.0)	0.12 <sup>c</sup>

Statistics presented as Mean ± SD, N (column %). p-values: a1=t test, a2=Satterthwaite t-test, c=Pearson's chi square test, d=Fisher's Exact test.

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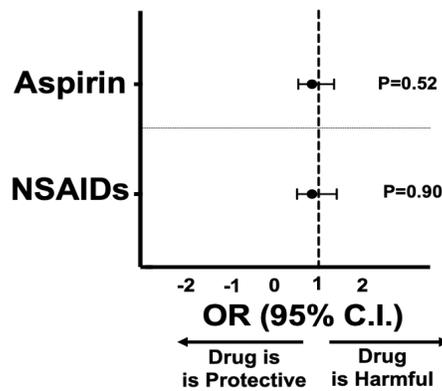
215 Table 2 shows the unadjusted characteristics of each comparative cohort for NSAIDs.

Factor	No NSAIDs (N=1,445)		NSAIDs (N=465)		p-value
	N	Statistics	N	Statistics	
<b>Medications</b>					
CLOPID	1,445	12 (0.83)	465	21 (4.5)	<0.001 <sup>c</sup>
Ticag	1,445	0 (0.00)	465	7 (1.5)	<0.001 <sup>d</sup>
Prasug	1,445	0 (0.00)	465	0 (0.00)	
Cangr	1,445	0 (0.00)	465	0 (0.00)	
Cilost	1,445	0 (0.00)	465	0 (0.00)	
Pentox	1,445	0 (0.00)	465	1 (0.22)	0.24 <sup>d</sup>
AntiPlt	1,445	96 (6.6)	465	174 (37.4)	<0.001 <sup>c</sup>
Multiple Therapy	1,445	5 (0.35)	465	26 (5.6)	<0.001 <sup>c</sup>
AC_therputic	1,445	95 (6.6)	465	45 (9.7)	0.026 <sup>c</sup>
AC_prophylct	1,445	328 (22.7)	465	203 (43.7)	<0.001 <sup>c</sup>
<b>Covariates</b>					
Age	1,445	51.5 ± 18.2	465	58.9 ± 17.1	<0.001 <sup>a</sup>
Platelets	574	213.1 ± 80.8	314	212.8 ± 78.0	0.97 <sup>a</sup>
Gender	1,390		462		0.36 <sup>c</sup>
Male		688 (49.5)		240 (51.9)	
Female		702 (50.5)		222 (48.1)	
Race	1,310		451		0.005 <sup>c</sup>
White		801 (61.1)		243 (53.9)	
Black		416 (31.8)		181 (40.1)	
Other		93 (7.1)		27 (6.0)	
Ethnicity	1,228		454		<0.001 <sup>c</sup>
Hispanic		178 (14.5)		31 (6.8)	
Non-Hispanic		1,050 (85.5)		423 (93.2)	
Smoking	1,162		452		<0.001 <sup>c</sup>
No		763 (65.7)		247 (54.6)	
Former		303 (26.1)		160 (35.4)	
Current		96 (8.3)		45 (10.0)	
RespSuprt	1,445	200 (13.8)	465	85 (18.3)	0.019 <sup>c</sup>
OnPressors	1,445	91 (6.3)	465	27 (5.8)	0.70 <sup>c</sup>
HemodInstab	1,445	94 (6.5)	465	29 (6.2)	0.84 <sup>c</sup>
COPD_emphysema	1,161	74 (6.4)	440	54 (12.3)	<0.001 <sup>c</sup>
Asthma	1,168	199 (17.0)	442	100 (22.6)	0.010 <sup>c</sup>
Diabetes	1,179	278 (23.6)	450	164 (36.4)	<0.001 <sup>c</sup>
Hypertension	1,198	571 (47.7)	453	292 (64.5)	<0.001 <sup>c</sup>
Coronary_artery_disease	1,162	107 (9.2)	445	98 (22.0)	<0.001 <sup>c</sup>
Heart_Failure	1,162	105 (9.0)	444	70 (15.8)	<0.001 <sup>c</sup>
Cancer	1,202	164 (13.6)	447	75 (16.8)	0.11 <sup>c</sup>
On_immunosuppressive_treatment	1,209	119 (9.8)	446	55 (12.3)	0.14 <sup>c</sup>
History_of_transplant	1,159	9 (0.78)	443	10 (2.3)	0.014 <sup>c</sup>

Statistics presented as Mean ± SD, N (column %).

p-values: a=t-test, c=Pearson's chi-square test, d=Fisher's Exact test.

217 The 248 propensity-matched patients either treated with aspirin or not demonstrated no significant  
 218 group differences in demographics or clinical covariates. Aspirin therapy did not alter mortality (13.3%  
 219 vs 15.3%,  $p=0.53$ ). The 444 propensity-matched patients either exposed or not to NSAIDs demonstrated  
 220 no significant group differences in demographics or clinical covariates. NSAID therapy did not alter  
 221 mortality (7.0% vs 7.2%,  $p=0.90$ ). In propensity-matched patients treated with aspirin, the incidence of  
 222 MI (2.0% vs 0.81%,  $p=0.27$ ) and VTE (4.0% vs 1.6%,  $p=0.12$ ) were not significantly different, but aspirin  
 223 therapy was associated with an increased risk of thrombotic stroke (3.6% vs 0.40%,  $p=0.036$ ). In  
 224 propensity-matched patients treated with NSAIDs, the incidence of MI (0.68% vs 0.23%,  $p=0.34$ ), VTE  
 225 (2.0% vs 0.90%,  $p=0.17$ ), and thrombotic stroke (1.1% vs 0.45%,  $p=0.27$ ) was not affected. Using the  
 226 composite thrombotic endpoint of MI, VTE, and thrombotic stroke, both aspirin (9.3% aspirin vs 2.8% no  
 227 aspirin,  $p=0.005$ ) and NSAID therapy (3.8% NSAIDs vs 1.6% no NSAIDs,  $p=0.046$ ) were associated with  
 228 signals for thrombosis (Supplemental Figure 1). Overall, there was no change in mortality in COVID-19  
 229 for patient treated with either aspirin (OR 0.52, 95% CI: 0.51-1.41;  $p=0.52$ ) or NSAIDs (OR 0.97, 95% CI:  
 230 0.58-1.62;  $p=0.90$ ) (Figure 4).



**Figure 4. Mortality for Propensity-matched patients:** Propensity-matched data for patients testing positive for COVID-19 and outcomes taking either 81 mg aspirin ( $n=248$  in each group) or NSAIDs ( $n=444$  in each group) at the time of diagnosis. Forest plot representation of data as Odds Ratio (OR) with 95% confidence interval (C.I.) for the primary endpoint of death

231  
 232 However, both aspirin and NSAID use in COVID-19 show signals for harm with increased thrombotic risk

233 with aspirin (OR 3.52, 95% CI: 1.48-8.40; p=0.005) and NSAIDs (OR 2.49, 95% CI: 0.58-1.62; p=0.046) for  
234 the composite endpoint of MI, thrombotic stroke, and VTE (Supplemental Figure 2).

235

## 236 **Discussion**

237 In this study, we make the observation that both ACE2 and TMPRSS2 proteins which bind and ligate  
238 SARS-CoV-2 are expressed in healthy human platelets. The expression of these receptors in platelets  
239 does not vary significantly with age and, while numerically higher, are not strikingly different in patients  
240 with CAD compared to healthy controls. The presence of known SARS-CoV-2 receptors on platelets  
241 suggests the possibility that SARS-CoV-2 may directly activate platelets and contribute to thrombosis or  
242 promote thrombosis indirectly by mediators secreted from platelets .

243

244 A recent investigation revealed platelet reactivity is enhanced in COVID-19 patients<sup>20,32-34</sup> and appears to  
245 be suppressed by the presence of high dose aspirin *in vitro*<sup>33</sup>. In the absence of randomized controlled  
246 data for aspirin in patients with COVID-19, we conducted a propensity-matched analysis of patients  
247 showing aspirin has no mortality benefit in patients with COVID-19, and, in fact, displays a slightly  
248 increased signal for harm driven mostly by thrombotic stroke. Platelet reactivity data *in vitro* is often  
249 extrapolated to suggest a risk for harm, but it is important to acknowledge that the behavior of anti-  
250 platelet medications *in vivo* can be markedly different from *in vitro* studies. Our goal was to clarify this  
251 concern by using real-life data with both mortality and thrombotic end points.

252

253 The failure to show a protective effect of the antiplatelet medication aspirin in patients with COVID-19  
254 may be related to the dose administered, an insensitivity to aspirin's mechanism of platelet inhibition in  
255 COVID-19, or an altered platelet phenotype as was clearly demonstrated by Manne *et al.* comparing  
256 healthy platelets to platelets from patients with COVID-19<sup>33</sup>. Cameron *et al.* previously demonstrated a

257 divergent platelet phenotype in patients with chronic arterial disease and diabetes with resistance to  
258 aspirin and clopidogrel therapy in diseased but not healthy platelets<sup>21</sup>. Similarly, Liang *et al*  
259 demonstrated in platelets from patients with diabetes, surface P2Y<sub>12</sub> receptors are arranged in a  
260 different conformation and are impressively resistant to inhibition by clopidogrel<sup>35</sup>.

261

262 Elbadawi *et al.* reported the absolute neutrophil count and not D-dimer, a traditional biomarker  
263 associated with thrombosis, is an independent predictor of thrombotic events in patients with COVID-  
264 19<sup>36</sup>. The mortality benefit of dexamethasone, an immunosuppressant and anti-inflammatory  
265 medication, in hospitalized patients with COVID-19<sup>37</sup> and recent reports of immunothrombosis<sup>17,38-41</sup> and  
266 microvascular occlusion<sup>18,42-44</sup> by multiple independent groups suggest platelets may be indirect  
267 mediators of thrombosis and perhaps not the best direct targets for pharmacological intervention.

268 Contemporaneous with submission of this manuscript, a smaller, non-propensity matched study has  
269 shown aspirin treatment decreased mortality that was driven by reduced ICU level care and mechanical  
270 ventilatory needs but not thrombosis in patients with COVID-19. This report suggests a protective effect  
271 of aspirin that is distinct from altering end-organ thrombosis<sup>45</sup>, and possibly from immune-mediated  
272 acute respiratory distress syndrome (ARDS) as previously demonstrated<sup>46,47</sup>. By evaluating another anti-  
273 inflammatory mechanism using patients treated with NSAIDs in parallel with aspirin in the same hospital  
274 and locations in the U.S., we similarly show no effect on mortality, with all statistical models accounting  
275 for any contribution of prophylactic and therapeutic heparin use in hospitalized patients and subsequent  
276 outcomes.

277

278 The signal for increased composite thrombotic events in COVID-19 patients treated with aspirin was  
279 surprising and driven mostly by stroke. Recent observational studies show mixed results for COVID-19-  
280 related stroke risk with one small study suggesting an increased risk in younger patients<sup>48</sup>, one large

281 study showing an overall low risk<sup>49</sup>, and one very large study paradoxically showing that COVID-19  
282 infection is associated with a decreased risk of thrombotic cerebrovascular stroke<sup>50</sup>. A mechanistic  
283 explanation for our finding may be related to the known neuroprotective effect of interleukin-6 (IL-6)<sup>51</sup>  
284 which is greatly elevated in systemic SARS-CoV-2 infection<sup>52</sup> and reported to be reduced by aspirin<sup>53</sup>.

285  
286 We show quite clearly in our study with investigators working independently of each other in different  
287 regions of the U.S. that human platelets contain the SARS-CoV-2 receptors ACE2 and TMPRSS2. The  
288 inter-individual expression difference of platelet ACE2 and TMPRSS2 was striking. Our overall  
289 observation is consistent with the findings of Zaid *et al.* who identified SARS-CoV-2 mRNA in human  
290 platelets implying a mechanism of entry must exist, and then a report by Zhang *et al.* who identified  
291 ACE2 on human platelets<sup>20,54</sup>. Our data are at odds with Manne *et al.* who failed to detect ACE2 protein  
292 in platelets by immunoblotting using white blood cells (WBC) as a positive control<sup>33</sup>. Notably, Manne *et al.*  
293 *al.* employed a CD45 depletion step on isolated platelets to eliminate the possibility of WBC  
294 contamination prior to immunoblotting. CD45 is also present on platelets, and we previously  
295 demonstrated this step decreases the platelet yield available for immunoblotting<sup>22</sup>. Lastly, Nassa *et al.*  
296 have very elegantly shown that the platelet transcriptome and proteome are dynamic and often mRNA  
297 to protein concordance is not observed but, rather, dependent on external platelet cues<sup>55</sup>. Overall, our  
298 data are congruent with Koupenova *et al.* suggesting that the ssRNA virus SARS-CoV-2 may behave  
299 similarly to the ssRNA influenza virus by utilizing platelets to modulate immune function that ultimately  
300 may lead to immunothrombosis<sup>16</sup>.

301  
302  
303  
304

305 **Study Limitations**

306 The observational nature of this study from just two hospitals has intrinsic limitations, and the small  
307 patient sample to allow for propensity matching limits generalizability of our findings. A few patients  
308 testing positive for SARS-CoV-2 were ambulatory and we relied on physician prescriptions making it  
309 impossible to confirm compliance to aspirin therapy.

310

311 **Conclusions**

312 SARS-CoV-2 high affinity receptors are present in platelets from healthy individuals. This finding crucially  
313 suggests platelets may be involved in COVID-19 pathogenesis and the observed thrombotic phenotype.  
314 However, our real-world clinical data suggests regular intake of low dose aspirin does not protect  
315 against adverse thrombotic events or death in COVID-19 patients. Platelets are fastidious components  
316 of the circulatory system with a wide range of critical functions, including contributing to  
317 immunoinflammatory host responses. Thus, targeting platelet thrombotic function may alter its roles in  
318 other domains. The nuanced mechanisms of thrombosis in COVID-19 may be unique and deserves  
319 further investigation. The use of traditional antiplatelet agents may not protect against thrombotic  
320 events or mortality in COVID-19, but, in fact, cause harm. The awareness of this potential harm and role  
321 of randomized controlled drug trials in assessing the suitability of antiplatelet agents in COVID-19 is  
322 critical.

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329 **Contributors**

330 Study design (AS, SJC, RB, AE, AK, JF, LGS, MKC, HK, JPI, MK, SK, TM, KRM)

331 Data collection (AS, SJC, RB, MKC, HK, JPI, MG, MK, JEF, JRB, EH, AA)

332 Data analysis (AS, SJC, AE, MKC, HK, JPI, MK, JEF, JRB, EH, SK)

333 Data interpretation (AE, AK, JF, LGS, MKC, HK, JPI, MG, MK, JEF, JRB, EH, AA, SK)

334 Figures (AS, SJC, MG, MK, JEF, JRB, EH, AA, SK)

335 Literature (AS, SJC, RB, AE, AK, JF, LGS, MKC, HK, MK, JEF, JRB, EH, SK, TM, KRM)

336 Writing (AS, SJC, RB, AE, AK, JF, MKC, HK, JPI, MG, MK, JEF, SK, TM, KRM)

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343

344 **Declaration of Interests**

345 None of the authors have any relevant conflicting financial, personal, or professional relationships.

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501 **Figures**

502

503 **Figure 1. Expression of ACE2 and TMPRSS2 in Platelets by Confocal Microscopy.** Platelets isolated  
504 from venous blood of healthy individuals was stained for 1h with the following antibodies: CD41  
505 (platelet-specific marker), ACE2, TMPRSS2, and DAPI to eliminate any DNA components. Mounted slides  
506 were resolved by confocal fluorescent microscopy using a 100x objective lens. Images are  
507 representative of n=6 donors for ACE2 and n=3 for TMPRSS2. Each image represents a different donor.  
508 The scale bar is noted.

509

510 **Figure 2. A. Expression of TMPRSS2 in Platelets:** Washed platelets from healthy individuals (mean age  
511  $40.1 \pm 2.8$  years, n=20) were isolated and proteins separate by SDS-PAGE with molecular weight shown  
512 in KiloDaltons (KDa). Immunoblotting was conducted an using an anti-TMPRSS2 antibody or anti-tubulin  
513 immunoblotting as a loading control. The ratio of protein to loading control is expressed as a function of  
514 age and the correlation coefficient is noted ( $r \pm 95\% \text{ CI}$ ,  $P=0.30$ ). Human brain lysate served as a positive  
515 control for TMPRSS2 migrating at the expected molecular weight (~50 KDa). Data shown are  
516 representative of 20 healthy individuals (10 male and 10 female) and 10 patients with coronary artery  
517 disease (CAD). The mean ratio of TMPRSS2/Tubulin  $\pm$  SEM is noted,  $P=0.145$  between healthy and CAD  
518 by Mann Whitney *U*). **B. Expression of ACE2 in Platelets:** Washed platelets from healthy individuals  
519 (mean age  $40.1 \pm 2.8$  years, n=20) were isolated and proteins separate by SDS-PAGE with molecular  
520 weight shown in KiloDaltons (KDa). Lane 1 is human platelet lysate, lane 2 is human brain lysate, lane 3  
521 is human placenta lysate, lane 4 is lysate from engineered human heart tissue. Immunoblotting was  
522 conducted using an using anti-ACE2 antibody. Anti-tubulin and anti-GAPDH are loading controls. ACE2  
523 migrates at the expected molecular weight (~100 KDa) shown by an arrowhead with glycosylated forms  
524 indicated by \*. The ratio of ACE2 protein to loading control is expressed as a function of age and the  
525 correlation coefficient is noted ( $r \pm 95\% \text{ CI}$ ,  $P=0.79$ ). Data shown are representative of 20 healthy  
526 individuals (10 male and 10 female) and 10 patients with coronary artery disease (CAD). The mean ratio  
527 of ACE2/Tubulin  $\pm$  SEM is noted,  $P=0.112$  between healthy and CAD by Mann Whitney *U*).

528

529 **Figure 3. Patients Testing Positive for SARS-CoV-2 taking Aspirin or NSAIDs.** Patients testing positive  
530 for a SARS-CoV-2 amplicon at two Cleveland Clinic hospitals were evaluated. Patients initiated with  
531 aspirin or NSAID therapy or continuing aspirin or NSAID if admitted to the hospital were included in this  
532 study. Clinical variables in each group where then re-evaluated following careful propensity matching .

533

534 **Table 1. Characteristics of Population taking Aspirin Therapy:** Unadjusted data are for patients testing  
535 positive for SARS-CoV-2 not taking aspirin or with established aspirin therapy or initiated with low dose  
536 aspirin at the time of diagnosis.

537

538 **Table 2. Characteristics of Population taking NSAID Therapy:** Unadjusted data are for patients testing  
539 positive for SARS-CoV-2 not taking NSAID or with established NSAID therapy or initiated with NSAID at  
540 the time of diagnosis.

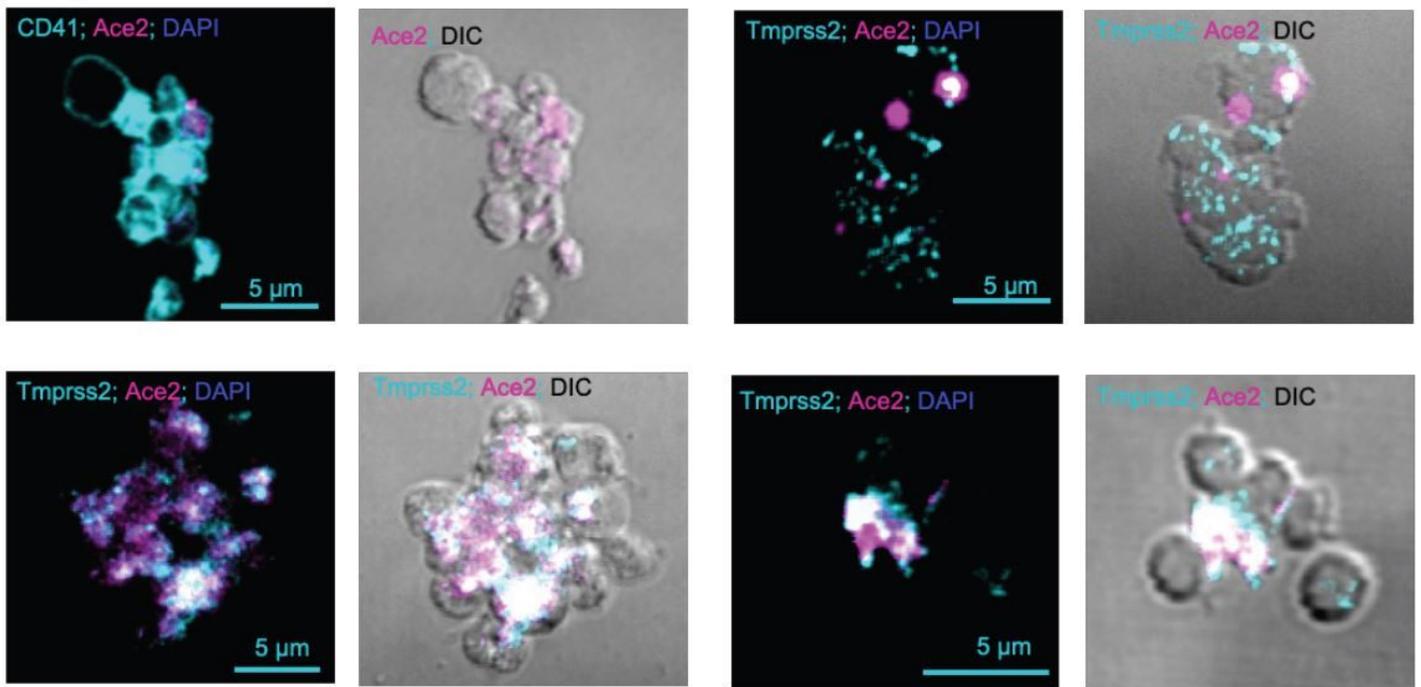
541

542 **Figure 4. Mortality for Propensity-matched patients:** Propensity-matched data for patients testing  
543 positive for COVID-19 and outcomes taking either 81 mg aspirin (n=248 in each group) or NSAIDs (n=444  
544 in each group) at the time of diagnosis. Forest plot representation of data as Odds Ratio (OR) with 95%  
545 confidence interval (C.I.) for the primary endpoint of death.

546

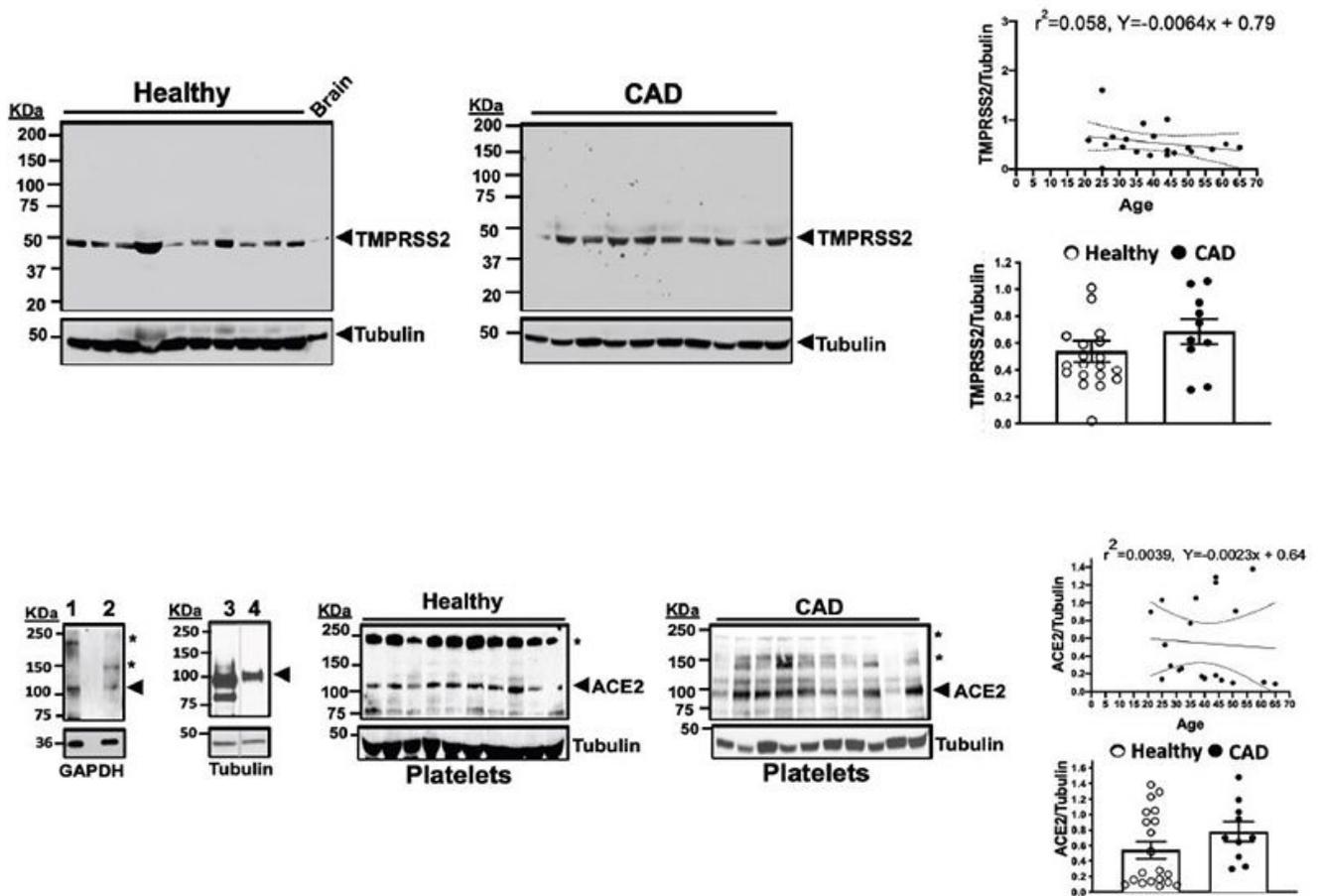
547

# Figures



**Figure 1**

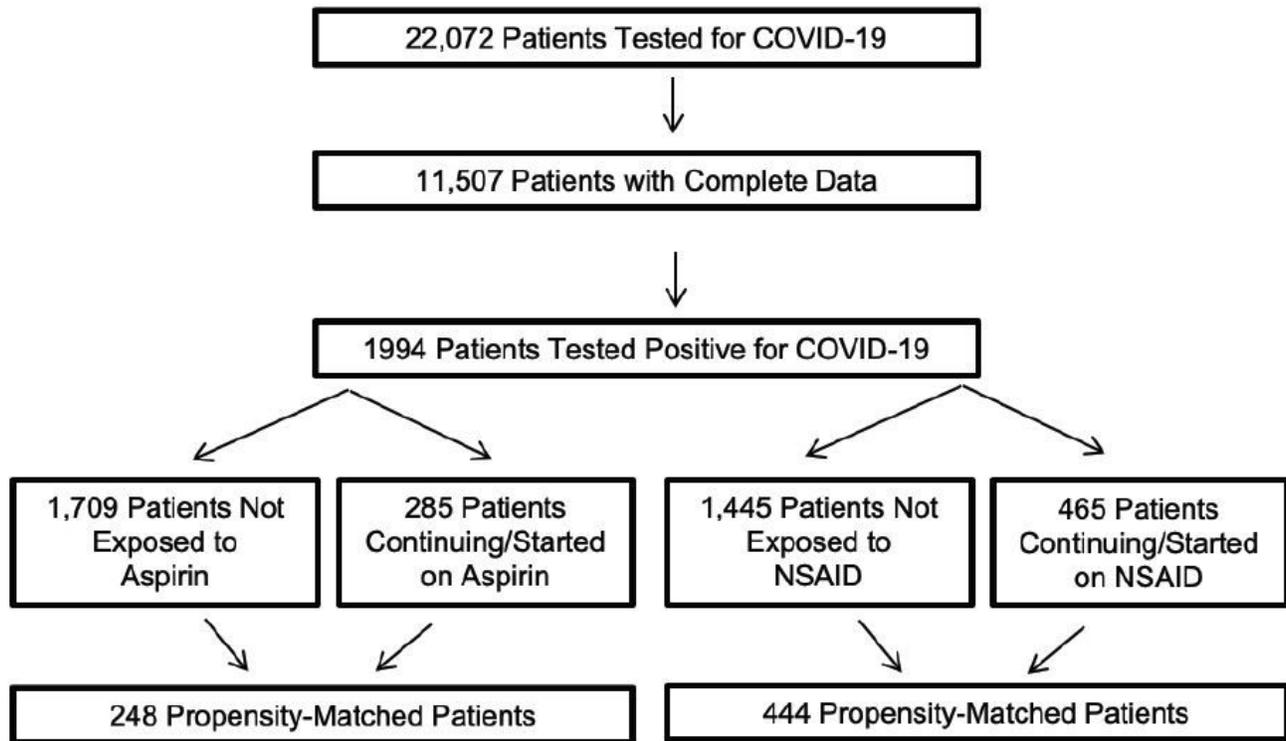
Expression of ACE2 and TMPRSS2 in Platelets by Confocal Microscopy. Platelets isolated from venous blood of healthy individuals was stained for 1h with the following antibodies: CD41 (platelet-specific marker), ACE2, TMPRSS2, and DAPI to eliminate any DNA components. Mounted slides were resolved by confocal fluorescent microscopy using a 100x objective lens. Images are representative of n=6 donors for ACE2 and n=3 for TMPRSS2. Each image represents a different donor. The scale bar is noted.



**Figure 2**

A. Expression of TMPRSS2 in Platelets: Washed platelets from healthy individuals (mean age  $40.1 \pm 2.8$  years,  $n=20$ ) were isolated and proteins separate by SDS-PAGE with molecular weight shown in KiloDaltons (KDa). Immunoblotting was conducted an using an anti-TMPRSS2 antibody or anti-tubulin immunoblotting as a loading control. The ratio of protein to loading control is expressed as a function of age and the correlation coefficient is noted ( $r \pm 95\% \text{ CI}, P=0.30$ ). Human brain lysate served as a positive control for TMPRSS2 migrating at the expected molecular weight ( $\sim 50$  KDa). Data shown are representative of 20 healthy individuals (10 male and 10 female) and 10 patients with coronary artery disease (CAD). The mean ratio of TMPRSS2/Tubulin  $\pm$  SEM is noted,  $P=0.145$  between healthy and CAD by Mann Whitney U). B. Expression of ACE2 in Platelets: Washed platelets from healthy individuals (mean age  $40.1 \pm 2.8$  years,  $n=20$ ) were isolated and proteins separate by SDS-PAGE with molecular weight shown in KiloDaltons (KDa). Lane 1 is human platelet lysate, lane 2 is human brain lysate, lane 3 is human placenta lysate, lane 4 is lysate from engineered human heart tissue. Immunoblotting was conducted using an using anti-ACE2 antibody. Anti-tubulin and anti-GAPDH are loading controls. ACE2 migrates at the expected molecular weight ( $\sim 100$  KDa) shown by an arrowhead with glycosylated forms indicated by \*. The ratio of ACE2 protein to loading control is expressed as a function of age and the

correlation coefficient is noted ( $r \pm 95\% \text{ CI}$ ,  $P=0.79$ ). Data shown are representative of 20 healthy individuals (10 male and 10 female) and 10 patients with coronary artery disease (CAD). The mean ratio of ACE2/Tubulin  $\pm$  SEM is noted,  $P=0.112$  between healthy and CAD by Mann Whitney U).



**Figure 3**

Patients Testing Positive for SARS-CoV-2 taking Aspirin or NSAIDs. Patients testing positive for a SARS-CoV-2 amplicon at two Cleveland Clinic hospitals were evaluated. Patients initiated with aspirin or NSAID therapy or continuing aspirin or NSAID if admitted to the hospital were included in this study. Clinical variables in each group were then re-evaluated following careful propensity matching.

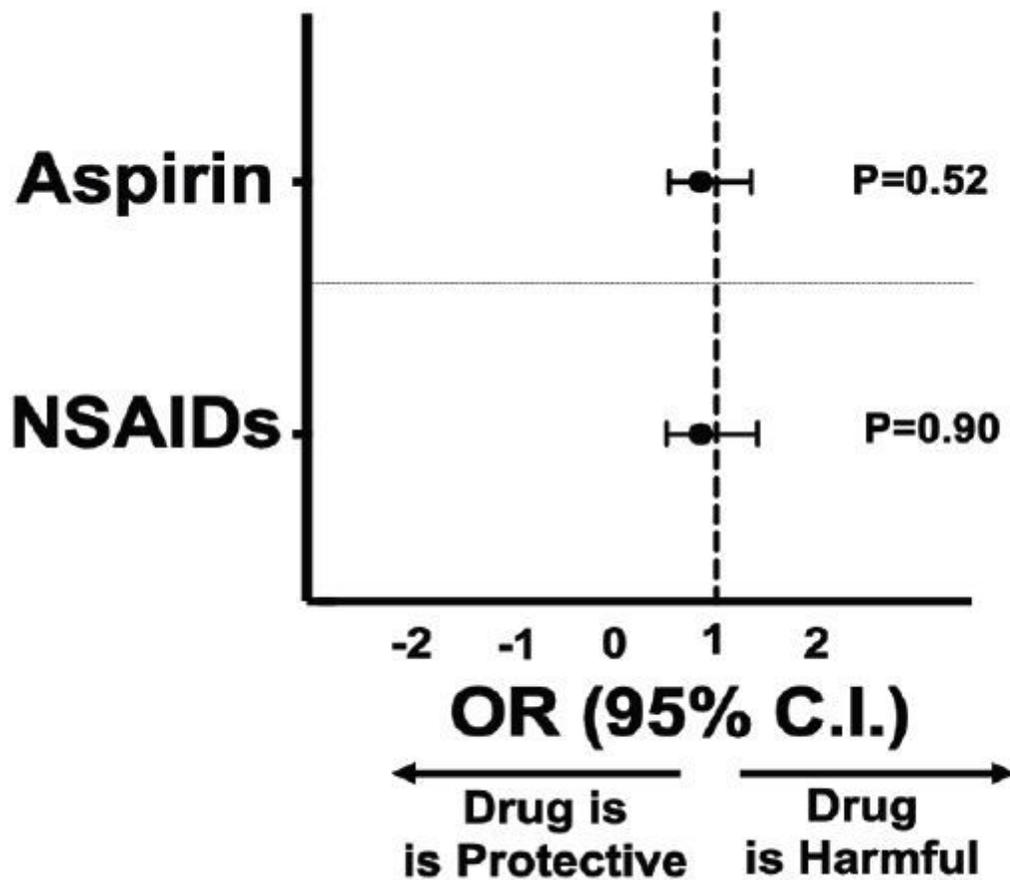


Figure 4

Mortality for Propensity-matched patients: Propensity-matched data for patients testing positive for COVID-19 and outcomes taking either 81 mg aspirin (n=248 in each group) or NSAIDs (n=444 in each group) at the time of diagnosis. Forest plot representation of data as Odds Ratio (OR) with 95% confidence interval (C.I.) for the primary endpoint of death.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalFile1221.20.pdf](#)