Dysregulated Platelet Function and Thrombosis in Patients with Post-Acute Sequelae of COVID-19

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Running Title: Decreased thrombosis in long COVID-19

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Abstract

Objective: Post-acute sequelae of COVID-19 (PASC, also referred as Long-COVID) sometimes follows COVID-19, a disease caused by SARS-CoV-2. While SARS-CoV-2 is well-known to promote a prothrombotic state, and especially to activate platelets acutely, less is known about the thrombosis risk in PASC.

Approach and Results: PASC patients and age-matched healthy controls were enrolled in the study on average 15 months after documented SARS-CoV-2 infection. Platelet activation was evaluated by Light Transmission Aggregometry (LTA) and flow cytometry in response to platelet surface receptor agonists. Thrombosis in platelet-deplete plasma was evaluated by Factor Xa activity. A microfluidics system assessed thrombosis in whole blood under venous shear stress conditions. While only a mild increase in platelet aggregation in PASC patients through the thromboxane receptor was observed platelet activation through the glycoprotein VI (GPVI) receptor was markedly decreased in PASC patients compared to age- and sex-matched healthy controls. Thrombosis under venous shear conditions as well as Factor Xa activity were reduced in PASC patients. Plasma from PASC patients was an extremely potent activator of washed, healthy platelets – a phenomenon not observed using age- and sex-matched platelets from healthy individuals.

Conclusions: PASC patients demonstrate dysregulated responses in platelets and coagulation in plasma, likely caused by a circulating plasma-derived molecule that promotes thrombosis. A hitherto undescribed protective response appears to exists in PASC patients to counterbalance ongoing thrombosis that is common to SARS-CoV-2 infection.

56 **Introduction:**

57 COVID-19, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-
58 CoV-2), may have residual effects following recovery. One third of infected patients are
59 asymptomatic, while others experience pulmonary and cardiovascular symptoms
60 leading to morbidity and mortality [1]. The risk of both severe and persistent symptoms of
61 SARS-CoV-2 is increased in males, in the advanced-age patient population, in
62 individuals requiring immunosuppression, and in patients with chronic kidney disease [2].
63 The risk of severe and persistent symptoms of SARS-CoV-2 remains in spite of
64 widespread vaccination [3,4].

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66 Post-acute sequelae of COVID-19 (PASC, also referred as Long COVID) is an
67 emerging multi-systemic condition characterized by persistent symptoms four weeks
68 beyond the initial SARS-CoV-2 infection. While acute respiratory distress syndrome
69 (ARDS) is a feared consequence of severe COVID-19, many patients in intensive care
70 units are reported to have thromboembolic complications including deep vein
71 thrombosis (DVT) and pulmonary embolism (PE). The incidence of venous
72 thromboembolic (VTE) may be as high as 40% [5,6]. Observational data suggests that
73 PASC patients continue to develop or have persistent symptoms long after recovery
74 that were initially labeled Long COVID by the Centers for Disease Control (CDC) and
75 the National Institutes of Health (NIH). The National Institute for Health and Care
76 Excellence (NICE) defines PASC as symptoms 4-12 weeks post-infection [7-9]. Patients
77 blighted by the specter of PASC show structural and functional organ derangements
78 involving the respiratory, cardiovascular, neurological, genitourinary, gastrointestinal,

79 and musculoskeletal systems. PASC symptoms typically include fatigue, dyspnea,
80 chest pain/tightness, headache, cough, difficulty concentrating, and altered sensorium.
81 Poor memory and concentration are the most frequent symptoms reported by patients
82 with PASC, closely followed by cognitive impairment, sleep disturbance, and post-
83 traumatic stress disorder (PTSD) [9-13].
84
85 Elevated blood markers biomarker of a pro-thrombotic state including D-dimer, C-
86 reactive protein, and neutrophil extracellular traps formation have been detected in
87 acute SARS-CoV-2 infection. In addition, many PASC patients complain of dyspnea on
88 exertion, which leads to physical inactivity and may further increase thromboembolic
89 risk. Virus-induced endothelial injury of severe COVID-19 initiates dysregulated
90 coagulation in patients. Increased levels of von Willebrand Factor (VWF), likely from
91 both endothelial cell and platelet activation, lead to hypercoagulability and thrombosis in
92 COVID-19 patients [7,8,14]. Platelets have an important and established hemostatic
93 function by preventing excessive bleeding (hemostasis) as well as an important immune
94 response to invading viral and microbial infections, especially SARS-CoV-2 [15].
95
96 In a large observational study of COVID-19 patients who never experienced thrombosis,
97 no additional risk for VTE after hospitalization with acute SARS-CoV-2 infection was
98 noted after hospital discharge[16]. Contrasting this, the MICHELLE study suggested a
99 beneficial effect of an oral Factor Xa inhibitor on discharged patients by protecting
100 against DVT if the patient was were previously hospitalized, even without an initial
101 thrombotic event [17]. These findings raise the possibility that following SARS-CoV-2

recovery, patients may have ongoing functional impairment in the coagulation cascade or abnormal platelet function. The Aim of this study was to evaluate both platelet function and thrombotic potential in whole blood, in platelet-deplete plasma, and in isolated platelets from patients following acute SARS-CoV-2 recovery but with clear PASC symptoms.

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127 **Methods**

128 **Human Subjects:** 20 patients with Post-Acute Sequelae of SARS-CoV-2 infection
129 (PASC) were enrolled on the basis of positive Covid-19 diagnosis and with persistent
130 symptoms, including shortness of breath, fatigue, brain fog, dyspnea on exertion, mood
131 disturbance, weakness, and memory impairment. PASC patients were compared to 13
132 matched healthy controls. Patients and healthy subjects were recruited only after
133 informed consent by a coordinator not involved in clinical care. All study procedures
134 were conducted in accordance with the Declaration of Helsinki and our approved local
135 Institutional Review Board protocol.

136 **Light Transmission Aggregometry (LTA):** Blood from healthy controls and PASC
137 patients was collected in sodium-citrate tubes after obtaining informed consent. Within
138 30 minutes to 1 hour of collection, anticoagulated whole blood was centrifuged at 200g
139 for 15 minutes at room temperature (RT) to obtain platelet rich plasma (PRP). PRP was
140 collected in 15mL falcon tubes and Platelet poor plasma (PPP) was obtained from the
141 remaining sample by re-centrifugation at 2500 g for 10 minutes. PPP was used to
142 initiate baseline optical density. Platelet Aggregation was tested following stimulation
143 with agonists: Adenosine diphosphate (ADP), Thrombin Receptor Activator Peptide 6
144 (TRAP-6), thromboxane A_2 (TXA_2) analogue U46619, and Collagen Related Peptide
145 (CRP). Optical density changes were recorded after the addition of agonists
146 (CHRONO-LOG 700 aggregometer) for 6 minutes while stirring the sample at 1200 rpm
147 at a temperature of 37°C.

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149 **Alpha granule exocytosis with Flow-cytometry:** Platelets were isolated from whole
150 blood collected from healthy controls and PASC patients in sodium-citrate tubes. Briefly,
151 whole blood was centrifuged at 200g for 15 minutes at RT and then PRP was collected
152 in the 15mL falcon tubes. Collected PRP was centrifuged again at 200g for 10 minutes
153 to remove remaining WBCs and RBCs. Platelets were washed using Tyrode's buffer in
154 the 1:1 ratio with the addition of Prostaglandin I2 (PGI2; 10nM). Washed platelets were
155 re-suspended in 10mL of Tyrode's buffer. Washed platelets from PASC patients and
156 healthy controls were stimulated using different concentrations of agonist: TRAP-6 (1, 5,
157 10 and 20μM), U46619 (0.1, 0.5, 1.0, 10μM), ADP (0.01, 0.1, 1.0, 10μM) and CRP
158 (0.05, 0.1, 0.5, 1.0μg/mL) for 15 minutes and then incubated with phycoerythrin (PE)-
159 conjugated anti-CD62P for 30 minutes at RT. Samples were fixed with 4%
160 paraformaldehyde. P-Selectin expressed on the surface on the platelets signifies an
161 activated state. Samples were read on the flow cytometer (BD Accuri C6 Plus) to
162 acquire 20000 events. Data were analyzed using FloJo software.

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164 **The Total Thrombus formation Analysis System (T-TAS01)**: The T-TAS01
165 microfluidic chamber system was use,d and the results were compared to healthy
166 controls. T-TAS01 is a microchip flow-chamber system that measures thrombus
167 formation under blood flow conditions over time. Whole blood collected in the sodium-
168 citrate tube was used for the test. Before measurement 20μL of the CaCTI reagent
169 (Corn trypsin inhibitor) was added to 480μL of whole blood and then this blood was
170 mixed gently with the pipette. Then 450μL of mixed blood was loaded into the reservoir

171 of the AR chip reservoir coated with both collagen and thromboplastin (tissue factor)
172 with a shear rate of 600 s^{-1}. As coagulation and platelet activation progress, thrombosis
173 is noted as increased pressure in the channel following shear rate-mediated activation
174 of blood in the microchip channel. These pressure changes are monitored by a pressure
175 sensor upstream of the chamber and are defined as The Occlusion Start Time (OST),
176 and the Occlusion Time (OT). The difference between OT and OST was calculated for
177 each subject. Furthermore, to check the pro-thrombotic activity of the plasma of the
178 PASC patients, 200µL plasma from the PASC patients and healthy controls was
179 incubated with 800µL whole blood from healthy control for 30 minutes. Then this spiked
180 whole blood with plasma was loaded into the reservoir of the AR chip.
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182 **Plasma Factor Xa activity:** Factor Xa (FXa) is the activated form of the coagulation
183 factor X. Factor X, a serine endopeptidase plays an important role in the coagulation
184 pathway. It covverts prothrombin into active thrombin by complexing with activated co-
185 factor V in the prothrombinase complex. Factor Xa Activity assay kit from Abcam
186 (ab204711) was used to measure the Factor Xa activity in the plasma of the PASC
187 patients compared to age- and sex-matched healthy controls. The assay was performed
188 according to the manufacturer's recommendations. The samples were read on the flow
189 cytometer (BD Accuri C6 Plus) to acquire 20000 events and data were analyzed using
190 FlowJo software. VICTOR Nivo multimode microplate reader (Pekin Elmer).
191
192 **Statistical Analysis:** All data were interrogated for normalcy using the Shapiro-Wilks
193 test. Normally distributed continuous variable group differences were assessed by the

194 student 2-tailed Student's t-test and skewed data assessed by the Mann-Whitney *U*
195 test. Analyses were conducted using GraphPad Prism 7 (GraphPad Software). For
196 Gaussian-distributed data in 3 or more groups, 1-way ANOVA then the Bonferroni
197 multiple comparisons test was used, otherwise the Kruskal–Wallis test followed by Dunn
198 post-test was used. Significance was accepted as a *P* value of less than 0.05.
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Results

Baseline Study Population: 20 patients with PASC (mean age 46.5 years) were recruited from a pulmonary medicine clinic in Northeast Ohio and compared with 13 healthy volunteers (mean age 52.3). Healthy recruits or PASC patients had comparable demographics and co-morbidities, showing no significant difference between groups as indicated in **Table 1**. Patients with PASC were studied on average 471 days ± 77 days after the last detected SARS-CoV-2 amplicon by polymerase chain reaction (PCR). Of the 26 common symptoms in our PASC patient population, the majority described fatigue (80%), brain fog (80%), shortness of breath at rest (70%), and exertional dyspnea (70%) (**Supplemental Table 1**).

Platelet-dependent thrombosis in PASC: Platelet aggregation in PRP was evaluated by LTA after stimulating platelets with surface receptor agonists directed toward the $P2Y_{12}$ receptor (ADP), the thromboxane receptor (U46619), the GPVI receptor (CRP), and PAR1 (TRAP-6). Overall, platelet aggregation by LTA in PASC was similar to healthy individuals except through the thromboxane receptor, which demonstrated increased reactivity following stimulation with the platelet surface receptor agonist U46619 (87%±3 for PASC vs. 72%±4 for healthy subjects, p=0.03) (**Fig. 1 A-D**). To distinguish platelet function that may be altered by plasma mediators, these studies were repeated after washing platelets twice, then assessing platelet reactivity after dese-dependent agonist stimulation by alpha granule exocytosis and appearance of P-selectin on the surface of the platelet as reported previously[18,19] . When washed platelets from healthy volunteers

240 and PASC patients were stimulated with platelet surface receptor agonists, we found
241 similar reactivity to healthy individuals through the following receptors: $P2Y_{12}$,
242 thromboxane, PAR 1 (**Fig. 2A-C**). Curiously, platelet reactivity through GPVI was
243 markedly decreased in PASC patients compared with healthy controls (**Fig. 2D**).
244
245 *Platelet-independent thrombosis in PASC*: Whole blood was drawn from patients who
246 were healthy or with PASC, then passed through a 80 μm channel in a microfluidics
247 system at 600 dynes (s^{-1}) which approximates shear stress conditions in veins. This
248 assay was specifically chosen because thrombosis in most patients with SARS-CoV-2
249 occurs in the venous vasculature, and the assay utilizes tissue factor and collagen to
250 initiate thrombosis, which simulates endothelial damage common to SARS-CoV-2.
251 Overall, the time to pressure increase in the microchannel (kPa), which is a signature of
252 thrombosis, as coagulated blood demonstrates diminished flow, was prolonged in PASC
253 patients compared with healthy conditions (occlusion time-occlusion start time, 94±8 vs.
254 73±8 seconds, respectively, P=0.022) (**Fig. 3 A-B**). To test whether a mediator in
255 plasma is responsible for the difference in coagulation observed in PASC, whole blood
256 from a healthy individual was spiked with platelet-deplete plasma from a PASC patient
257 compared to spiking platelet-deplete plasma from a healthy individual. This experiment
258 showed no difference between occlusion time and occlusion start time (62±4 healthy
259 plasma vs. 66±8 PASC plasma, n=5 in each group, P=0.74). We next assessed
260 platelet-deplete plasma Factor Xa activity which represents the point of convergence
261 between the extrinsic and the intrinsic arms of the coagulation cascade. Factor Xa
262 activity was reduced in PASC patients compared to healthy individuals (23.6±5 vs. 50±8

ng/100μL, respectively, P=0.037). (**Fig. 3C**). These experiments suggest a change in PASC plasma resulting in dysregulated coagulation compared with healthy conditions.

A plasma mediator in PASC patients activates platelets: We demonstrated that washed platelets from PASC patients are relatively unchanged or less reactive than healthy platelets (**Fig. 2**) and that platelet-deplete plasma in PASC is less likely to clot *ex vivo* (**Fig. 3C**). These observations suggests a potential adaptive response long after COVID-19 infection, possibly to prevent thrombosis that is common in patients with acute COVID-19 infection. When platelet-deplete plasma from healthy individuals or PASC patients was added to healthy, twice washed platelets (n=20 plasma samples for PASC and n=18 plasma samples for healthy controls), there was a surprising and profound activation of healthy platelets through all platelet receptor agonists tested after PASC plasma inclubation (**Fig. 4**). Given that immunothrombosis is common in COVID-19 and the complement system likely contributes to these responses [20-22], we heated and therefore inactivated plasma from healthy donors or PASC patients at 55˚C and repeated these experiments. We observed that platelets were persistently activated if spiked with room temperature plasma or heat-inactivated plasma prior to agonist stimulation from PASC patients. This suggests complement activation is unlikely to be a key driver in the augmented platelet response observed by PASC plasma exposure to healthy platelets (**Fig. S2**).

286 **Discussion**

287 SARS-CoV-2 is well-known to precipitate thrombotic events, especially DVT and PE
288 [23,24]. Acute SARS-CoV-2 infection has been shown to coincide with activated platelets
289 by multiple independent groups [25-28]. Our primary objective was to assess platelet
290 function and thrombosis in human blood in patients with PASC after recovery from
291 COVID-19 given that rheologic manifestations of this syndrome are unknown. Our
292 major observation in patients more than one year following acute SARS-CoV-2 and with
293 clear symptoms of PASC is one of dysregulated platelet function, with slight selective
294 platelet activation through the thromboxane receptor, and attenuated activation through
295 the GPVI receptor. We did not observe a change in platelet receptor signaling through
296 PAR1 or $P2Y_{12}$ in PASC using two well-validated techniques to interrogate platelet
297 activation.

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299 This study may be the first to report a functional defect in the coagulation cascade given
300 our finding of reduced activated Factor Xa in platelet-deplete plasma in patients with
301 PASC and prolonged time to thrombosis of whole blood under conditions of
302 biomechanical shear under flow. [26]. Using radioligand binding technology or a similar
303 chromogenic substrate for Factor Xa activation as we employ, others have reported that
304 platelets and platelet-derived microparticles may be a source of Factor Xa activity in the
305 circulation [29-31]. Moreover, a putative signaling mechanism between the platelet GPVI
306 receptor and Factor Xa was previously reported [32]. In addition, the profoundly under-
307 reactive GPVI receptor identified by flow cytometry using CRP as the agonist in our
308 study complements the observation of prolonged time to thrombosis in human blood *ex*

309 *vivo* under conditions of venous shear stress since collagen is present in the
310 microfluidics channel.
311
312 The most significant finding of our study is that platelet-deplete plasma in PASC
313 patients 'primes' and markedly activates healthy, washed platelets through every
314 receptor signaling pathway we evaluated. We did not see this when platelet-deplete
315 plasma from healthy controls was incubated with washed platelets prior to platelet
316 receptor agonist stimulation. The hyper-reactive platelet phenotype persisted even after
317 heating plasma to 55 °C suggesting the complement cascade is unlikely to be
318 responsible for the augmented platelet activation observed. Therefore, we offer the
319 suggestion that a putative pro-thrombotic factor exists in the blood of patients with
320 PASC, and its presence leads to an adaptive response in the host in which platelet
321 reactivity and thrombosis are paradoxically limited, presumably as a protective response
322 from thrombosis.
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324 Our study in PASC patients differs from a recent research letter by Martins-Gonçalves
325 *et al.* in patients reporting at least one persistent respiratory symptom after acute
326 hospitalization for COVID-19 and with persistent platelet hyperreactivity[33]. Distinct
327 differences in the enrolled subjects is apparent. Firstly, while the average age of
328 patients enrolled by Martins-Gonçalves *et al.* was similar to our cohort, the represented
329 majority were men while 65% of our patients and 84% of our healthy controls were
330 women. Platelet reactivity has been shown to differ between men and women in health
331 and in cardiovascular disorders [19,29,34]. Secondly, the control group employed by

332 Martins-Gonçalves *et al*. tested negative for SARS-CoV-2 weekly for almost two years
333 prior to enrollment, while many of our control comparators reported SARS-CoV-2
334 infection in the preceding two years but without residual symptoms at the time of
335 enrollment. This was the major reason why they were selected as controls for our
336 study. Lastly, Martins-Gonçalves evaluated platelet reactivity more in the convalescent
337 setting 1-4 months after acute SARS-CoV-2 infection in 2021 whereas our PASC
338 patients on average were evaluated 15 months after the last documented acute SARS-
339 CoV-2 infection in 2023. Clinical outcomes and symptoms following SARS-Cov-2
340 infection appear to vary depending on vaccination status as well as the the viral strain of
341 the infected individual [35]. This means that interpretation of data gained from patients
342 evaluated during different waves of the COVID-19 pandemic require careful attention.
343 An important consideration is that different viral strains may manifest as different
344 symptoms in persons with PASC Given that SARS-CoV-2 was demonstrated to induce
345 morphological changes in platelets and promote apoptosis after viral internalization in
346 an ACE2-dendent and ACE2-independent receptor internalization pathway[15], platelet
347 reprogramming in patients with PASC may occur at the level of the megakaryocyte.
348
349 In summary, patients who since recovered from acute SARS-CoV-2 but with persistent
350 and long-standing symptoms under the constellation of PASC show evidence of
351 dysregulated platelet reactivity, alterations in normal coagulation in platelet-deplete
352 plasma, and diminished thrombosis in whole blood *ex vivo* under shear stress
353 conditions. These events are likely to be a consequence of adapting to a platelet-
354 activating factor in plasma. The stimulus behind the exhausted platelet phenomenon,

355 particularly through GPVI, in PASC patients, should be investigated and identification of
356 the putative pro-thrombotic mediator should be prioritized given the implications for
357 some patients with PASC being considered as donors of blood and plasma products in
358 future.
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Tables

Table 1. Demographics Enrolled PASC Patients and Healthy Individuals

Demographics	Healthy (n=13) N	%	PASC (n=20) N	%	P-Value
Characteristics					
Mean Age (± SEM)	46.5 ± 2.95		52.3 ± 4.03		0.26
28-40 years	3	23.1 %	6	30.0 %	0.660
41-60 years	8	61.5 %	3	15.0 %	*0.006*
61-82 years	2	15.4 %	9	45.0 %	0.08
Male	2	15.4 %	7	35.0 %	0.22
Female	11	84.6 %	13	65.0 %	
BMI (± SEM)	31.8 ± 1.37		29.4 ± 1.86		0.34
Smoker	1	7.7 %	7	35.0 %	0.07
Medical History					
Hypertension	4	30.8 %	4	20.0 %	0.48
Hyperlipidemia	2	15.4 %	4	20.0 %	0.73
Diabetes	1	7.7 %	1	5.0 %	0.75
History of CVD	1	7.7%	2	10.0 %	0.82
History of Thrombosis	1	7.7 %	1	5.0 %	0.75
History of Pulmonary Embolism	0	0.0 %	0	0.0 %	1.0
Medications					
Platelet inhibitors	1	7.7 %	4	20.0 %	0.34
Anticoagulants	1	0.0 %	3	15.0 %	0.52
Statins	2	15.4 %	4	20.0 %	0.73
NSAIDs	6	46.1 %	4	20.0 %	0.11
BP Medications	4	30.8 %	1	5.0 %	*0.04*

Table 1. Baseline Characteristics. Patients with PASC (n=20, occurring at a mean duration of 471 ± 77 days after a diagnosis of COVID-19) were recruited for the study and compared with healthy volunteers (n=13). Data are reported as mean ± SEM. Differences between groups was calculated by the student's t-test for continuous variables or Chi-Squared for dichotomous variables. BMI=Body Mass Index. CVD=Cardiovascular Disease. BP=Blood Pressure. NSAID=non-steroidal anti-inflammatory medication.

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536 **Supplemental Table 1.** Symptoms Experienced by PASC Patients

Symptoms	PASC (n=20) N	%
Mean Time After Infection (± SEM)	471.4 days ± 77.33	
Fatigue	16	80.0 %
Brain Fog	16	80.0 %
Shortness of Breath	14	70.0 %
Dyspnea on Exertion	14	70.0 %
Dizziness	13	65.0 %
Headaches	12	60.0 %
Changes in Mood	11	55.0 %
Insomnia	9	45.0 %
Palpitations	9	45.0 %
Chest Pain	8	40.0 %
Joint Pain	8	40.0 %
Cough	6	30.0 %
Parosmia/Dysgeusia	6	30.0 %
Diarrhea	1	15.0 %
Incontinence	3	15.0 %
Memory Deficits	3	15.0 %
Body Aches	2	10.0%
HF Symptoms	2	10.0 %
Nausea	2	10.0 %
Depression	1	5.0 %
Erectile Dysfunction	1	5.0 %
Fever	1	5.0 %
Neck Pain	1	5.0 %
Orthopnea	1	5.0 %
Limb Paresthesia	1	5.0 %
Syncope	1	5.0 %

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538 **Fig. S1. Persistent symptoms of enrolled PASC patients.**
539 Data are expressed as frequency and % of population and mean ± SEM.
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Figures

Fig. 1. Platelet Activation in PASC by Aggregometry. Blood was drawn from healthy individual or PASC patients and PRP was isolated prior to stimulation with **A.** TRAP-6, **B.** U46619, **C.** ADP, **D.** GPVI. Platelet aggregation was evaluated by LTA and data reported as mean % maximum aggregation ± SEM. A representative aggregometry tracing is shown. Group differences were assessed by the Mann Whitney *U* test, n=5 for healthy individuals and n=5 for PASC.

Fig. 2. Platelet Activation in PASC by Flow Cytometry. Isolated, washed platelets were stimulated with **A.** TRAP-6, **B.** U46619, **C.** ADP, **D.** CRP for 15 minutes, then reactivity was assessed by FACS by fold-change in surface CD62 (P-Selectin) using a tagged antibody. Data are represented as mean ± SEM. Group differences were assessed by the Kruskal-Wallis test, n=10 for healthy individuals and n=9 for PASC. NS=not significant. CRP=C=reactive protein.

Fig. 3. Platelet-independent Thrombosis in PASC. A. Blood was isolated and passed through a microfluidics chip using the Total Thrombus formation Analysis System (T-TAS01) at roughly venous shear stress (600 s^{-1}). With thrombus generation, pressure in the microfluidics chamber (kPa) gradually increases until cessation of flow. Data are represented as mean ± SEM. **B.** The time from the start of occlusion to cessation of blood was calculated in seconds as mean ± SEM. Group differences were determined by the student's t-test. **C.** Citrate plasma devoid of platelets was evaluated for Factor Xa activity using a chromogenic substrate in PASC patients (n=7) or healthy volunteers (n=10). Data are represented as mean ± SEM. Group differences were assessed by the student's t-test, n=12 for healthy individuals and n=10 for PASC.

Fig. 4. Activation of Platelets by PASC but not Healthy Plasma. Isolated, washed platelets were pre-incubated for 30 minutes with 5 µL of age- and sex-matched healthy plasma or PASC plasma, then stimulated with TRAP-6, 5 µM (PAR1), U46619, 1 µM (Thromboxane receptor), ADP, 0.1 µM ($P2Y_{12}$Receptor), or Collagen-Related Peptide, CRP, 5 µg/mL (GPVI Receptor) for 15 minutes. Platelet reactivity was assessed by FACS by surface CD62P (P-selectin) using a tagged antibody. Data are represented as mean ± SEM. Group differences were assessed by the Mann-Whitney *U* test, n=20 plasma isolated for healthy individuals and n=18 plasma isolates for PASC patients. Washed platelets from a healthy 25 year-old male.

Supplementary Figures:

Fig. S2. PASC Symptoms of Enrolled Subjects. Platelet-deplete citrate plasma from PASC patients already demonstrated to activate healthy male platelets divided in half and then either heat-treated (55 ˚C, 15 minutes) or not and incubated for 30 minutes prior to stimulation with a surface receptor agonist TRAP-6, 5 μM (PAR1), U46619, 1 μM (Thromboxane receptor), ADP, 0.1 μM ($P2Y_{12}$Receptor), or Collagen-Related Peptide, CRP, 5 μg/mL (GPVI Receptor). Platelet reactivity was then assessed by FACS by surface CD62P (P-selectin) using a tagged antibody. Data are represented as mean ± SEM. Group differences were assessed by Mann-Whitney *U* test for n=10;

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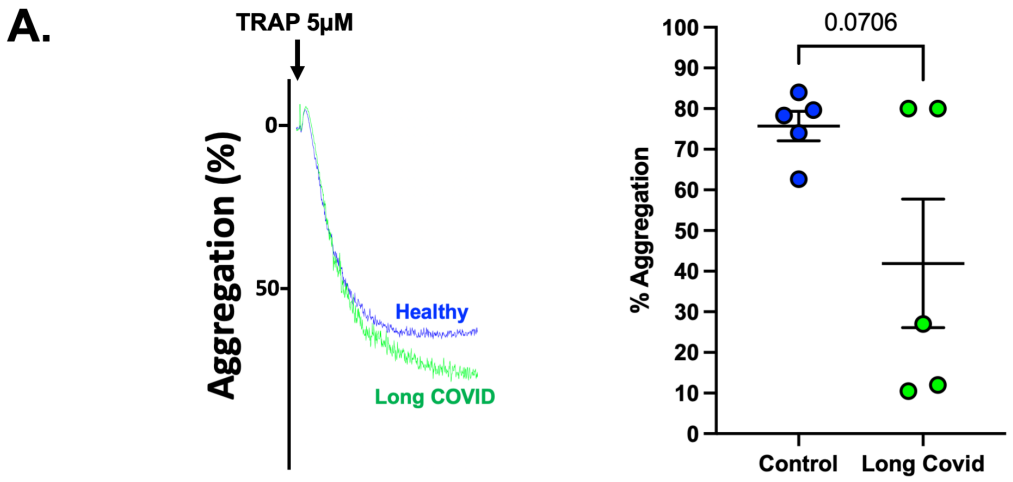

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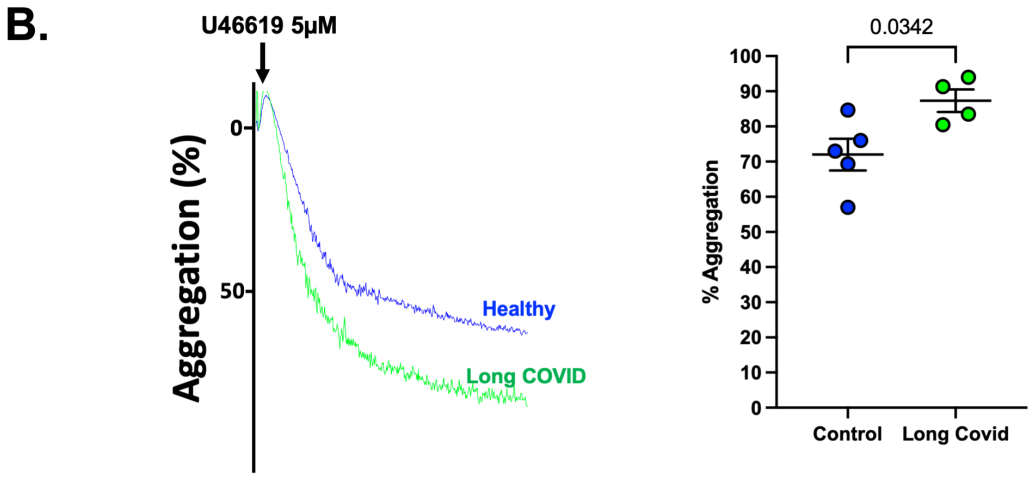


Fig. 1. Platelet Activation in PASC by Aggregometry. Blood was isolated from healthy individual or PASC patients and PRP was isolated prior to stimulation with ADP. Platelet aggregation was evaluated by LTA and data reported as mean % maximum aggregation ± SEM. A representative aggregometry tracing is shown. Group differences were assessed by the student's t-test, n=5 for healthy individuals and n=5 for PASC.

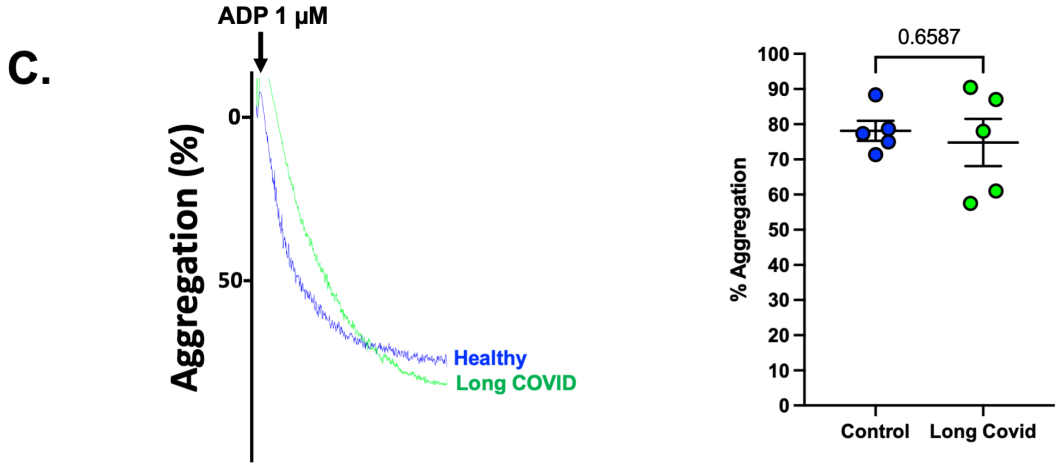
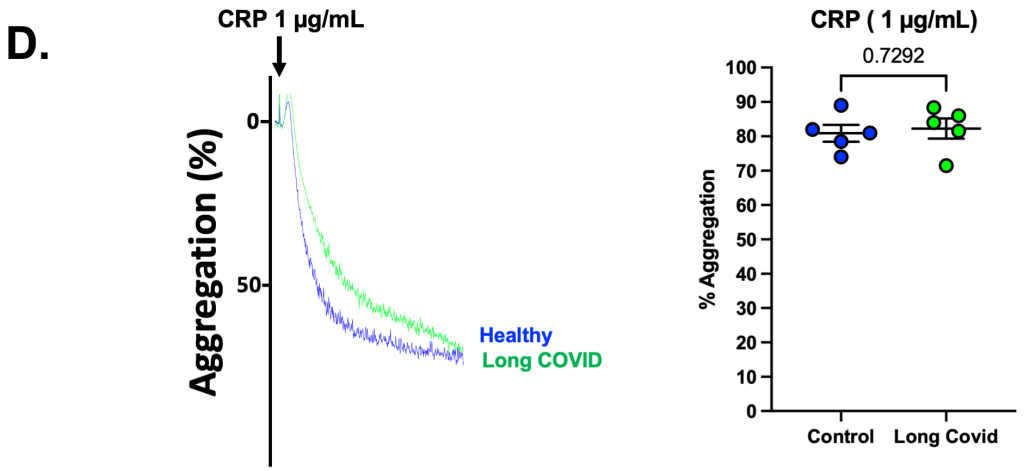


Fig. 1. Platelet Activation in PASC by Aggregometry. Blood was isolated from healthy individual or PASC patients and PRP was isolated prior to stimulation with CRP. Platelet aggregation was evaluated by LTA and data reported as mean % maximum aggregation ± SEM. A representative aggregometry tracing is shown. Group differences were assessed by the Mann Whitney *U* test, n=5 for healthy individuals and n=5 for PASC.



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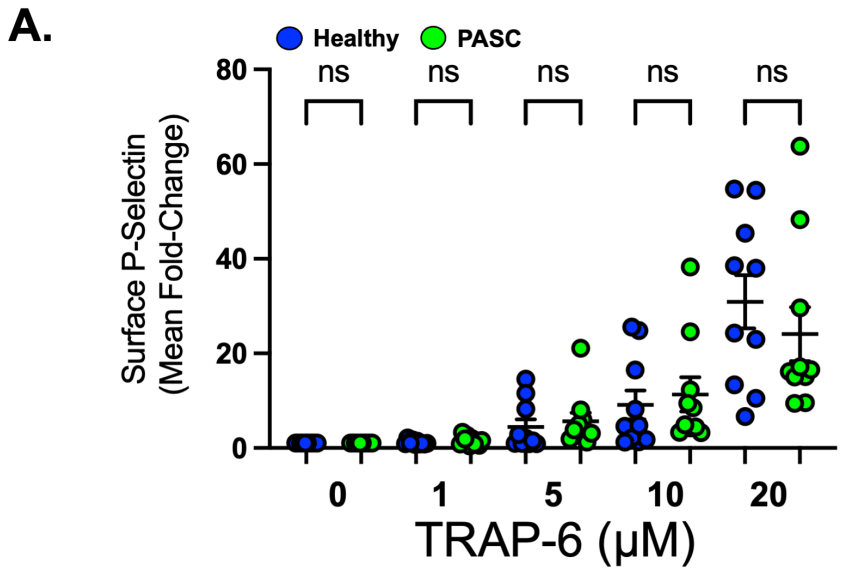

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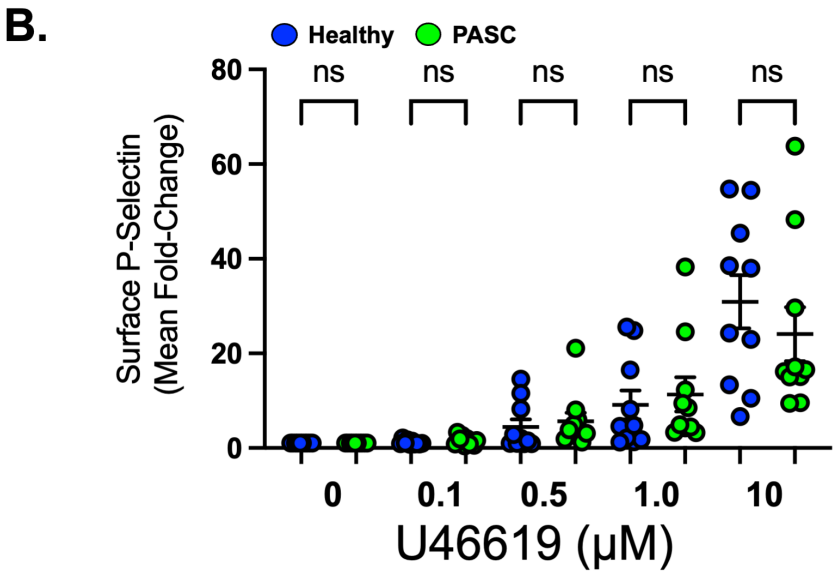
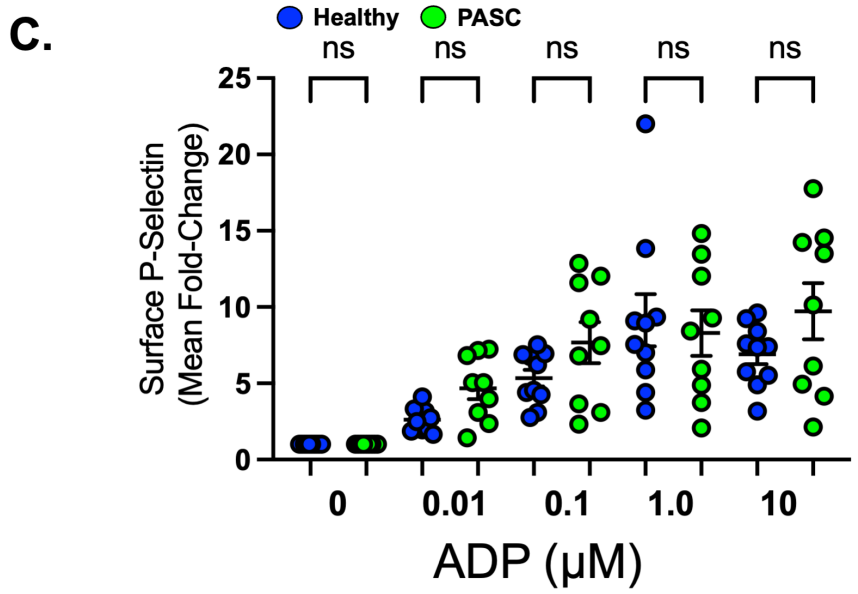


Fig. 2. Platelet Activation in PASC by Flow Cytometry. Isolated, washed platelets were stimulated with U46619 for 15 minutes, then reactivity was assessed by FACS by fold-change in surface CD62P (P-selectin) using a tagged antibody. Data are represented as mean ± SEM. Group differences were assessed by ANOVA, followed by the Bonferroni correction, n=10 for healthy individuals and n=10 for PASC.



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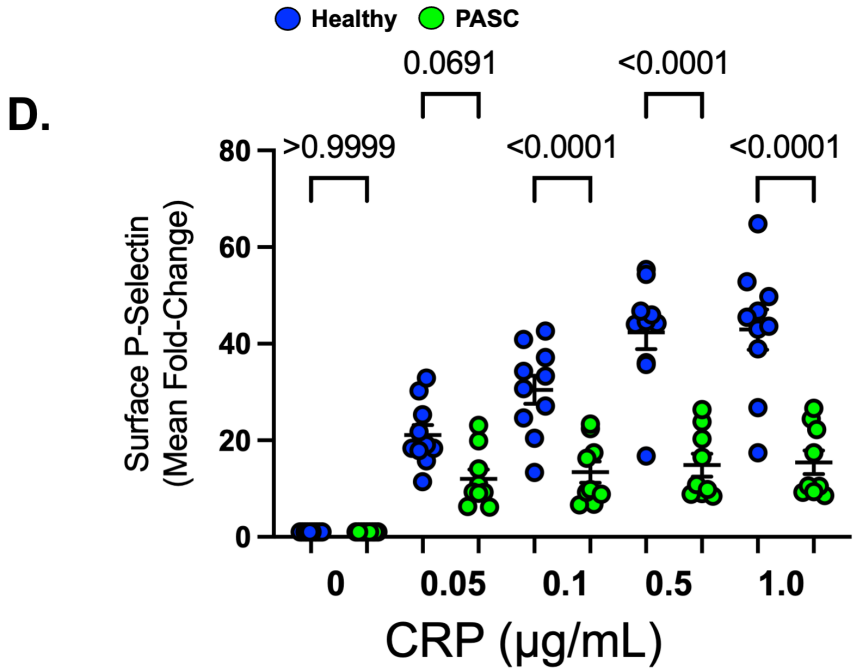
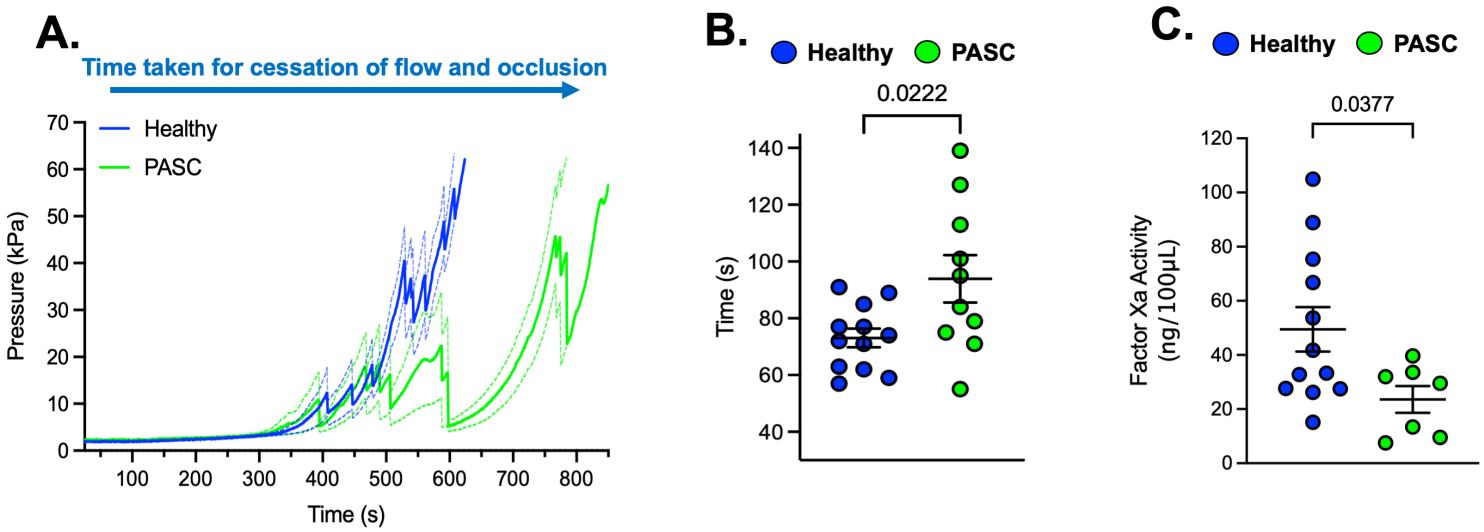


Fig. 2. Platelet Activation in PASC by Flow Cytometry. Isolated, washed platelets were stimulated with collagen-related peptide (CRP) for 15 minutes, then reactivity was assessed by FACS by fold-change in surface CD62P (P-selectin) using a tagged antibody. Data are represented as mean ± SEM. Group differences were assessed by ANOVA, followed by the Bonferroni correction, n=10 for healthy individuals and n=9 for PASC.



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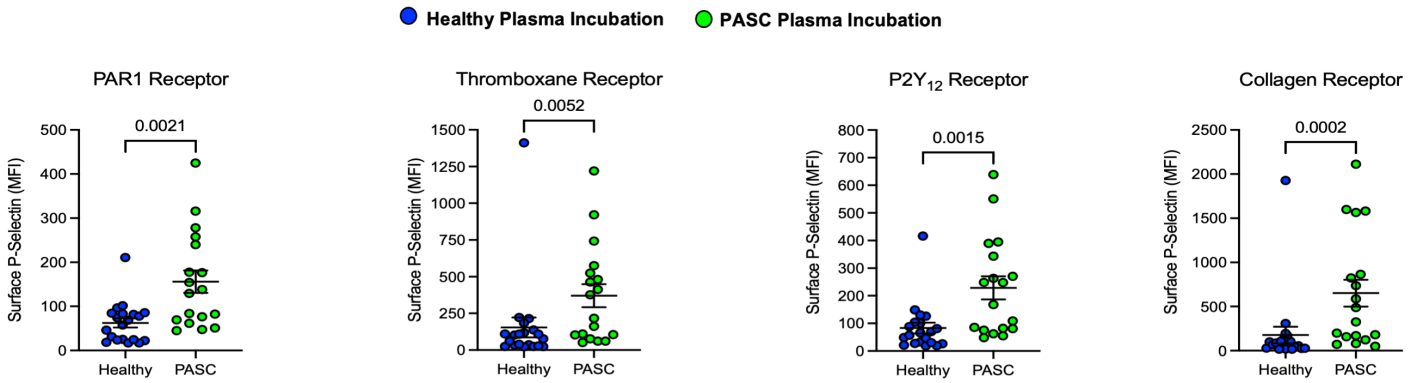

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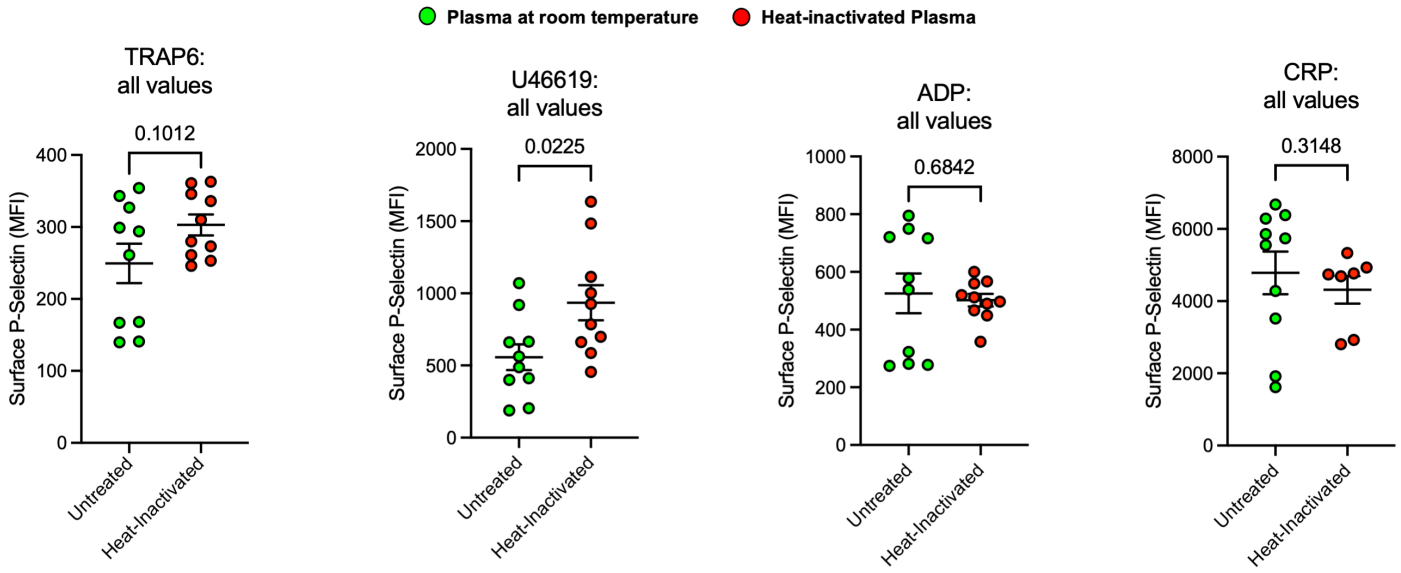

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