# Dysregulated Platelet Function 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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**Key words:** COVID-19, Post-acute sequelae of COVID-19 (PASC), Platelets, Thrombosis.

**Abstract**

**Background:** 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, less is known about the thrombosis risk in PASC.

**Aim:** Our objective was to evaluate the platelet function and thrombotic potential in patients following recovery from SARS-CoV-2 with clear symptoms of PASC.

**Methods:** PASC patients and 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 shear stress conditions.

**Results:** A mild increase in platelet aggregation in PASC patients through the thromboxane receptor was observed and platelet activation through the glycoprotein VI (GPVI) receptor was decreased in PASC patients compared to age- and sex-matched healthy controls. Thrombosis under 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 when stimulating healthy platelets after incubation with plasma from healthy individuals.

**Conclusions:** PASC patients show dysregulated responses in platelets and coagulation in plasma, likely caused by a circulating molecule that promotes thrombosis. A hitherto

55 undescribed protective response appears to exists in PASC patients to counterbalance
56 ongoing thrombosis that is common to SARS-CoV-2 infection.
57
58 **Introduction:**
59 COVID-19, caused by the severe acute respiratory syndrome coronavirus 2
60 (SARS-CoV-2), may have residual effects following recovery. One third of infected
61 patients are asymptomatic, while others experience pulmonary and cardiovascular
62 symptoms leading to morbidity and mortality (1). The risk of both severe and persistent
63 symptoms of SARS-CoV-2 is higher males, in the advanced-age patient population, in
64 patients requiring immunosuppression, or with chronic kidney disease (2). The risk of
65 severe and persistent symptoms of SARS-CoV-2 remains despite vaccination (3, 4).
66 Post-acute sequelae of COVID-19 (PASC) is an recognized multi-systemic
67 condition characterized by persistent symptoms four weeks beyond the initial SARS-CoV-
68 2 infection. While acute respiratory distress syndrome (ARDS) is a feared consequence
69 of severe COVID-19, many acutely ill patients are reported to have thromboembolic
70 complications including deep vein thrombosis (DVT) and pulmonary embolism (PE). The
71 incidence of venous thromboembolic (VTE) complications may be as high as 40% (5, 6).
72 Observational data suggests that PASC patients develop or have persistent
73 symptoms long after recovery that were initially labeled Long COVID by the Centers for
74 Disease Control (CDC) and the National Institutes of Health (NIH). The National Institute
75 for Health and Care Excellence (NICE) now defines PASC as post-infection symptoms
76 beyond 4-12 weeks (7-9). Patients blighted by the specter of PASC show structural and
77 functional organ derangements involving the respiratory, cardiovascular, neurological,

78 genitourinary, gastrointestinal, and musculoskeletal systems. PASC symptoms typically
79 include fatigue, dyspnea, chest pain/tightness, headache, cough, difficulty concentrating,
80 and altered sensorium. Poor memory and concentration are the most frequent symptoms
81 reported, closely followed by cognitive impairment, sleep disturbance, and post-traumatic
82 stress disorder (PTSD) (9-13).

83 Elevated pro-thrombotic markers including D-dimer, C-reactive protein, and
84 neutrophil extracellular trap (NET) formation have been detected in acute SARS-CoV-2
85 infection. In addition, many PASC patients complain of dyspnea on exertion, which leads
86 to physical inactivity and may further precipitate thromboembolic complications. Virus-
87 induced endothelial injury of severe COVID-19 patients initiates a state of dysregulated
88 coagulation. Increased blood von Willebrand Factor (VWF), likely from both endothelial
89 cell and platelet activation, promotes hypercoagulability and thrombosis in patients with
90 COVID-19 (7, 8, 14).

91 Platelets have an important hemostatic function to prevent excessive bleeding
92 (hemostasis) alongside an important role in the host immune response to invading viral
93 and microbial infections, especially SARS-CoV-2 (15). In a large observational study, no
94 additional risk for VTE after hospitalization with acute SARS-CoV-2 infection was noted
95 (16). Contrasting this, the MICHELLE study revealed a protective effect of an oral Factor
96 Xa inhibitor on discharged patients by protecting them against DVT if they were previously
97 hospitalized, even without an initial thrombotic event (17). These findings raise the
98 possibility that following SARS-CoV-2 recovery, patients may have ongoing functional
99 impairment in the coagulation cascade or abnormal platelet function. The Aim of this study
100 was to evaluate both platelet function and thrombotic potential in whole blood, in platelet-

101 deplete plasma, and in isolated platelets in patients following SARS-CoV-2 but with clear
102 PASC symptoms.
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124 **Methods**

125 **Human Subjects:** 26 patients with Post-Acute Sequelae of SARS-CoV-2 infection
126 (PASC) were enrolled based on a confirmed Covid-19 diagnosis and with persistent
127 symptoms, including shortness of breath, fatigue, brain fog, dyspnea on exertion, mood
128 disturbance, weakness, and memory impairment. PASC patients were compared to 19
129 matched healthy controls. Patients and healthy subjects were recruited only after
130 informed consent by a research coordinator not involved in clinical care. All study
131 procedures were conducted in accordance with the Declaration of Helsinki and approved
132 by a local Institutional Review Board (IRB) protocol. Human platelet physiological studies
133 must be conducted within 90 minutes in order for meaningful data to be obtained. These
134 studies are labor-intensive and, in their entirety, cannot be conducted simultaneously for
135 every patient within 90 minutes. The volume of blood required to conduct studies in whole
136 blood, in platelet-rich plasma (PRP), in washed platelets, and in platelet deplete plasma
137 is also substantial and exceeds the blood volume permitted to be drawn in the IRB
138 protocol. Therefore, it is impossible to conduct every experiment in every enrolled PASC
139 patient or healthy individual. Rather, the studies were conducted in a manner that was
140 practically required as patients as PASC were identified.

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142 **Cross-activation study using Flow-cytometry:**

143 To check whether plasma from PASC patients would modify platelet activation from
144 healthy donors, we performed cross-activation tests. Platelet depleted plasma (20μl) from
145 the PACS patients (n=18) as well as age- and sex-matched healthy controls (n=20) was
146 added to the isolated washed platelets (80μl) from healthy controls (each performed in

147 triplicate) and incubated for 20 minutes at room temperature. Platelets were stimulated
148 with agonists: Thrombin Receptor Activator Peptide 6 (TRAP6; 5μM), Thromboxane $A_2$
149 ($TXA_2$) analogue U46619 (1μM), Adenosine diphosphate (ADP; 0.1μM), or Collagen
150 Related Peptide (CRP; 0.1μg/mL) for 15 minutes. Samples were next stained with
151 phycoerythrin (PE)-conjugated anti-CD62P (p-selectin) for 30 minutes at room
152 temperature in darkness to prevent fluorophore bleaching before fixing with 4%
153 paraformaldehyde. Fixed samples were read by flow cytometry (BD Accuri C6 Plus) to
154 acquire 20000 events and data was analyzed using FlowJo software.
155
156 **Antibody Profiling:**
157 Antibody profiling was performed by CDI labs using the HuProt 4.0 array. Briefly,
158 sequence-confirmed plasmids, which are used to make GST-purified recombinant
159 proteins in yeast. After purification, GST-tagged proteins are piezoelectrically printed on
160 glass slides in duplicate, along with control proteins (GST, BSA, Histones, IgG, etc.). The
161 slides are barcoded for tracking and archiving. Each microarray batch is routinely
162 evaluated by anti-GST staining to demonstrate quality of expression and printing. The
163 new HuProt v4.0 consists of unique human proteins, isoform variants, and protein
164 fragments of the 19,613 canonical human proteins described in the Human Protein Atlas
165 (18-21). We focused on protein exclusively expressed in platelets, highly expressed in
166 platelets, blood proteins known to be involved in the alterations in coagulation as noted.
167 Content includes major functional classes such as intracellular proteins, membrane
168 proteins, enzymes, secreted proteins, transcription factors, transporters, GPCRs,
169 cytokines, immune receptors, immune checkpoints, CD markers, ion channels, cytosolic

170 proteins, nuclear receptors. To measure antibody binding, slides are overlaid with a 1:100
171 dilution of serum, and stained with anti-IgG (red) and anti-IgM (green) secondary
172 antibodies. A heat map was generated to show each antibody concentration expressed
173 as standard deviations (SD) above or below the mean concentration found in healthy
174 individuals.
175
176 **Light Transmission Aggregometry (LTA):** Blood from healthy controls and PASC
177 patients was collected in sodium-citrate tubes after obtaining informed consent. Within 30
178 minutes to 1 hour of collection, anticoagulated whole blood was centrifuged at 200g for
179 15 minutes at room temperature (RT) to obtain platelet rich plasma (PRP). PRP was
180 collected in 15mL falcon tubes and Platelet poor plasma (PPP) was obtained from the
181 remaining sample by re-centrifugation at 2500 g for 10 minutes. PPP was used to initiate
182 baseline optical density. Platelet Aggregation was tested following stimulation with
183 agonists: ADP (1μM), TRAP-6 (5μM), U46619 (5μM) and CRP (1μg/mL). Optical density
184 changes were recorded after the addition of the agonists on Aggregometer (CHRONO-
185 LOG 700) for 6 minutes while stirring the sample at 1200 rpm at 37°C.
186
187 **Alpha granule exocytosis with Flow-cytometry:** Platelets were isolated from whole
188 blood collected from healthy controls and PASC patients in sodium-citrate tubes. Briefly,
189 whole blood was centrifuged at 200g for 15 minutes at RT and then PRP was collected
190 in the 15mL falcon tubes. Collected PRP was centrifuged again at 200g for 10 minutes to
191 remove remaining WBCs and RBCs. Platelets were washed using Tyrode's buffer in the
192 1:1 ratio with the addition of Prostaglandin I2 (PGI2; 10nM). Washed platelets were re-

193 suspended in 10mL of Tyrode's buffer. Washed platelets from PASC patients and healthy
194 controls were stimulated using different concentrations of agonist: TRAP-6 (1, 5, 10 and
195 20μM), U46619 (0.1, 0.5, 1.0, 10μM), ADP (0.01, 0.1, 1.0, 10μM) and CRP (0.05, 0.1,
196 0.5, 1.0μg/mL) for 15 minutes and then incubated with phycoerythrin (PE)-conjugated
197 anti-CD62P for 30 minutes at RT. Samples were fixed with 4% paraformaldehyde. P-
198 Selectin expressed on the surface on the platelets signifies an activated state. Samples
199 were read by flow cytometry (BD Accuri C6 Plus) to acquire 20000 events. Data were
200 analyzed using FloJo software.
201
202 **Total Thrombus formation Analysis System (T-TAS01)**: The T-TAS01 microfluidic
203 chamber system was used and the results were compared to healthy controls. T-TAS01
204 is a microchip flow-chamber system that measures thrombus formation under blood flow
205 conditions over time. Whole blood collected in the sodium-citrate tube was used for the
206 test. Briefly, before measurement 20μL of the CaCTI reagent (Corn trypsin inhibitor) was
207 added to 480μL of whole blood and then this blood was mixed gently with the pipette.
208 Then 450μL of mixed blood was loaded into the reservoir of the AR chip reservoir coated
209 with both collagen and thromboplastin (tissue factor) with a shear rate of 600 $s^{-1}$. As
210 coagulation and platelet activation progress, thrombosis is noted as pressure rises in the
211 channel following shear rate-mediated activation of blood in the microchip channel. These
212 pressure changes are monitored by a pressure sensor upstream of the chamber and are
213 defined as The Occlusion Start Time (OST), and the Occlusion Time (OT). The difference
214 between OT and OST was calculated for each subject. Furthermore, to check the pro-
215 thrombotic activity of the plasma of the PASC patients, 200μL plasma from the PASC

216 patients and healthy controls was incubated with 800μL whole blood from healthy control
217 for 30 minutes. Then this spiked whole blood with plasma was loaded into the reservoir
218 of the AR chip.
219
220 **Plasma Factor Xa activity:** Factor Xa (FXa) is the activated form of the coagulation factor
221 X. Factor X, a serine endopeptidase plays an important role in the coagulation pathway.
222 It converts prothrombin into active thrombin by complexing with activated co-factor V in
223 the prothrombinase complex. The Factor Xa Activity assay kit from Abcam (ab204711)
224 was used to measure the Factor Xa activity in the plasma of the PASC patients compared
225 to age- and sex-matched healthy controls. The assay was performed according to the
226 manufacturer's recommendations.
227
228 **Statistical Analysis:** All data were interrogated for normalcy using the Shapiro-Wilks
229 test. Normally distributed continuous variable group differences were assessed by the
230 student 2-tailed Student's t-test and skewed data assessed by the Mann-Whitney *U* test.
231 Analyses were conducted using GraphPad Prism 7 (GraphPad Software). For Gaussian-
232 distributed data in 3 or more groups, 1-way ANOVA then the Bonferroni multiple
233 comparisons test was used, otherwise the Kruskal–Wallis test followed by Dunn post-test
234 was used. Significance was accepted as a *P* value of less than 0.05.
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239 **Results**

240 *Baseline Study Population:* 26 patients with PASC (mean age 49.9) were recruited from
241 the pulmonary medicine clinic in Northeast Ohio and compared with 19 healthy volunteers
242 (mean age 41.4). Healthy recruits or PASC patients were comparable, showing no
243 significant difference between groups with respect to sex, BMI, and medical co-
244 morbidities of PASC patients who were more commonly smokers (**Table 1)**. Patients with
245 PASC presented on average 501 days ± 69 days after the last detection of a SARS-CoV-
246 2 amplicon by polymerase chain reaction (PCR). Of the 26 common PASC symptoms in
247 our patient population, most described fatigue (76.9%), brain fog (76.9%), shortness of
248 breath at rest (69.2%), and exertional dyspnea (69.2%) (**Supplemental Table 1**).

249

250 *Platelet Reactivity in PASC patients*: When platelet-deplete plasma from healthy
251 individuals or PASC patients was added to healthy, twice washed platelets, there was a
252 surprisingly profound activation of platelets following stimulation with all platelet receptor
253 agonists tested caused by PASC patient plasma incubation (**Fig. 1**). Given that
254 immunothrombosis is common in COVID-19 and the complement system likely
255 contributes to these responses (22-24), we heated and inactivated plasma from PASC
256 donors at 55˚C and repeated the experiments. We observed that platelets were
257 persistently activated if spiked with room temperature plasma or heat-inactivated plasma
258 prior to agonist stimulation from PASC patients suggesting that complement activation is
259 unlikely to be a driver in the augmented platelet response observed (**Supplemental Fig.
260 S1**).

261 Platelet aggregation in PRP was evaluated by LTA after stimulating platelets with surface
262 receptor agonists for the $P2Y_{12}$ receptor (ADP), the thromboxane receptor (U46619), the
263 GPVI receptor (CRP), and PAR1 (TRAP-6). Overall, platelet aggregation by LTA in PASC
264 was similar to healthy individuals except through the thromboxane receptor which
265 demonstrated increased reactivity following stimulation with the agonist U46619 (87%±3
266 for PASC vs. 72%±4 for healthy subjects, p=0.03) (**Fig. 2 A-D**). To distinguish platelet
267 function that may be altered by mediators in PRP, these studies were repeated in a dose-
268 response manner after isolating and washing platelets twice, then assessing platelet
269 reactivity by alpha granule exocytosis by appearance of P-selectin on the surface of the
270 platelet as reported previously (25, 26). When washed platelets from healthy volunteers
271 and PASC patients were stimulated with platelet surface receptor agonists, we found
272 similar reactivity to healthy individuals through the following receptors: $P2Y_{12}$,
273 thromboxane, PAR 1 (**Fig. 3A-C**). Curiously, platelet reactivity through GPVI was
274 markedly decreased in PASC patients compared with healthy controls (**Fig. 3D**).
275
276 To test the hypothesis that changes in platelet function as observed by alpha granule
277 exocytosis augmented in PASC following platelet thromboxane receptor stimulation, **Fig.
278 2B**) or platelet aggregation (decreased in PASC following platelet GPVI stimulation, **Fig.
279 3D**) are a consequence of changes in platelet surface receptor expression, we performed
280 flow cytometry using receptors tagged with a fluorophore, ultimately finding no difference
281 (**Supplemental Fig. S2**).
282

283 *Whole blood and platelet-independent coagulation in PASC patients*: Whole blood was
284 drawn from patients who were healthy or with PASC, then passed through a 80 μm
285 channel in a microfluidics system at 600 dynes ($s^{-1}$) shear stress. This assay was
286 specifically chosen because thrombosis in most patients with SARS-CoV-2 occurs in the
287 venous vasculature, and the assay utilizes tissue factor and collagen to initiate
288 thrombosis, which simulates endothelial damage common to SARS-CoV-2. Overall, the
289 time to pressure increase in the microchannel (kPa), which is a signature of *ex vivo* blood
290 clotting as coagulated blood diminishes flow, was prolonged in PASC patients compared
291 with healthy conditions (occlusion time-occlusion start time, 94±8 vs. 73±8 seconds,
292 respectively, P=0.02) (**Fig. 4 A-B**). To test whether a mediator in plasma is responsible
293 for the difference in coagulation observed in PASC, whole blood from a healthy individual
294 was spiked with platelet-deplete plasma from a PASC patient was compared to spiking
295 platelet-deplete plasma from a healthy individual. This showed no difference between
296 occlusion time and occlusion start time (62±4 healthy plasma vs. 66±8 PASC plasma,
297 n=5 in each group, P=0.74). We next assessed Factor Xa activity in platelet-deplete
298 plasma which represents the point of convergence between the extrinsic and the intrinsic
299 arms of the coagulation cascade. We observed Factor Xa activity was reduced in PASC
300 vs. healthy individuals (23.6±5 vs. 50±8 ng/100μL, respectively; P=0.037) (**Fig. 4C**). While
301 complement, which can increase platelet reactivity, is well-known to be denatured at
302 55˚C, antibodies targeted against platelet proteins or the coagulation cascade may retain
303 some function (27-29). We therefore performed a limited antibody array using serum from
304 patients with PASC comparing this to healthy individuals for antibodies targeted against
305 platelet proteins that could alter platelet function of antibodies targeted against circulating

306 Factor II, Factor II Receptor, or Factor X. We did not find a significant difference in either
307 IgM or IgG against those protein targets comparing PASC patients to healthy controls
308 (**Supplemental Fig. S3**).
309
310 These experiments suggest there is a change in PASC plasma with dysregulated
311 coagulation compared with healthy conditions that does not involve complement or
312 circulating antibodies. Overall, the observation that washed platelets from PASC patients
313 are relatively unchanged or less reactive than healthy platelets, and that platelet-deplete
314 plasma in PASC is less likely to clot, suggests a potential adaptive response in the host
315 long after COVID-19 infection, possibly to prevent thrombosis that is common in patients
316 with acute COVID-19 infection.
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329 **Discussion**

330 SARS-CoV-2 is well-known to precipitate thrombotic events, especially DVT and PE (30,
331 31). Acute SARS-CoV-2 infection has also been shown to coincide with activated
332 platelets by multiple independent groups (32-35). Our primary objective was to assess
333 platelet function and thrombosis in patients with PASC given that rheologic manifestations
334 of this syndrome are an under-developed area of investigation. Our major finding in
335 patients more than one year following acute SARS-CoV-2 and with clear symptoms of
336 PASC is dysregulated platelet function, with slight platelet activation through the
337 thromboxane receptor, and attenuated activation through the GPVI receptor. We did not
338 observe a change in platelet receptor signaling through PAR1 or $P2Y_{12}$ in PASC using
339 two well-validated techniques to interrogate platelet reactivity.

340 This study may be the first to report a functional defect in the coagulation cascade
341 given our finding of reduced activated Factor Xa in platelet-deplete plasma in patients
342 with PASC (33). Curiously, using radioligand binding technology or a similar chromogenic
343 substrate for Factor Xa activation as we employ, others have reported that platelets and
344 platelet-derived microparticles may be a source of Factor Xa activity in the circulation (36-
345 38). Moreover, a putative signaling mechanism between the platelet GPVI receptor and
346 Factor Xa was previously reported (39). In addition, the profoundly under-reactive GPVI
347 receptor identified by flow cytometry for alpha granule exocytosis using CRP as the
348 agonist in our study complements the observation of prolonged time to thrombosis in
349 human blood *ex vivo* under conditions of venous shear stress since collagen is present
350 in the microfluidics channel which activates the GPVI receptor.

351 The most significant finding in our study is that platelet-deplete plasma in PASC
352 patients 'primes' and markedly activates healthy, washed platelets through every receptor
353 signaling pathway evaluated. We did not see this when platelet-deplete plasma from
354 healthy controls was incubated with washed platelets before agonist stimulation. The
355 hyper-reactive platelet effect caused by PASC plasma persisted even after heating
356 plasma to 55°C suggesting the complement cascade or circulating antibodies are likely
357 not responsible for augmented platelet activation. Using an antibody array, we could not
358 find antibodies directed against platelet protein targets of coagulation proteins in the blood
359 that could explain the observation in patients with PASC. Therefore, we offer the
360 suggestion that a putative pro-thrombotic factor exists in the blood of patients with PASC,
361 and its presence leads to an adaptive response in the host in which platelet reactivity and
362 thrombosis are paradoxically tipped in the opposite direction, presumably as a protective
363 response from thrombosis.

364 Our study in PASC patients differs from a recent letter by Martins-Gonçalves *et al.*
365 in patients reporting at least one persistent respiratory symptom after acute
366 hospitalization for COVID-19 and with persistent platelet hyperreactivity (40). Important
367 differences in the enrolled subjects were noted. Firstly, while the average age of patients
368 enrolled by Martins-Gonçalves *et al.* was similar to our cohort, the represented majority
369 were men while 73% of our patients and 79% of our healthy controls were women.
370 Platelet reactivity has been shown to differ between men and women in health and in
371 cardiovascular disorders (26, 36, 41). Secondly, the control group employed by Martins-
372 Gonçalves *et al*. tested negative for SARS-CoV-2 weekly for almost two years prior to
373 enrollment, while several of our control comparators reported SARS-CoV-2 infection in

374 the preceding two years but without residual symptoms at the time of enrollment and this
375 was the reason why they were selected as controls. Lastly, Martins-Gonçalves evaluated
376 platelet reactivity more in the convalescent setting 1-4 months after acute SARS-CoV-2
377 infection in 2021 whereas our PASC patients on average were evaluated >16 months
378 after the last documented acute SARS-CoV-2 infection in 2023. Clinical outcomes and
379 symptoms following SARS-Cov-2 infection appear to vary depending on vaccination
380 status and the viral strain of the infected individual (42). This means that interpretation of
381 data gained from patients evaluated during different waves of the COVID-19 pandemic
382 require careful attention. Given that SARS-CoV-2 was demonstrated to induce
383 morphological changes in platelets and promote apoptosis after viral internalization in an
384 ACE2-dendent and ACE2-independent receptor internalization pathway (15), platelet
385 reprogramming in patients with PASC may occur.
386
387 **Conclusion**
388 In summary, patients who have long recovered from acute SARS-CoV-2 but with
389 persistent symptoms under the constellation of PASC show evidence of dysregulated
390 platelet reactivity, alterations in normal coagulation of platelet-deplete plasma, and
391 diminished clotting in whole blood *ex vivo* under shear stress conditions. These events
392 are likely a consequence of adapting to a platelet-activating factor in plasma of patients
393 with PASC. The stimulus behind the exhausted platelet phenomenon, particularly
394 through GPVI, in PASC patients, should be investigated and identification of the putative
395 pro-thrombotic mediator should be prioritized given the implications for patients with
396 PASC being considered as donors of blood and plasma products in future.

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552 **Tables and Figures:**
553
554 **Table 1. Baseline Characteristics.** Patients with PASC (n=26, occurring at a mean
555 duration of 501 ± 69 days after a diagnosis of COVID-19) were recruited for the study and
556 compared with healthy volunteers (n=19). Data are reported as mean ± SEM. Differences
557 between groups was calculated by the student's t-test for continuous variables or Chi-
558 Squared for dichotomous variables. BMI=Body Mass Index. CVD=Cardiovascular
559 Disease. BP=Blood Pressure. NSAID=non-steroidal anti-inflammatory medication.
560
561 **Fig. 1. Activation of Platelets by PASC but not Healthy Plasma.** Isolated, washed
562 platelets were pre-incubated for 30 minutes with 20 µL of age- and sex-matched healthy
563 plasma or PASC plasma, then stimulated with TRAP-6, 5 µM (PAR1), U46619, 1 µM
564 (Thromboxane receptor), ADP, 0.1 µM (P2Y12 Receptor), or Collagen-Related Peptide,
565 CRP, 0.1 µg/mL (GPVI Receptor) for 15 minutes. Platelet reactivity was assessed by
566 FACS by surface CD62P (P-selectin) using a tagged antibody. Data are represented as
567 mean ± SEM. Group differences were assessed by the Mann-Whitney U test, n=20
568 plasma isolated for healthy individuals and n=18 plasma isolates for PASC patients.
569 Washed platelets from a healthy 25 year-old male.
570
571 **Fig. 2. Platelet Activation in PASC by Aggregometry**. **A-D:** Blood was drawn from
572 healthy individual or PASC patients and PRP was isolated prior to stimulation with
573 TRAP-6 (5µM), U466119 (5µM), ADP (1.0 µM), or CRP (1.0 µg/mL) and observed until
574 platelet aggregation was noted. Platelet aggregation was evaluated by LTA and data
575 reported as mean % maximum aggregation ± SEM. A representative aggregometry
576 tracing is shown. Group differences were assessed by the Mann Whitney U test, n=5 for
577 healthy individuals and n=5 for PASC or student's t-test, n=5 for healthy individuals and
578 n=5 for PASC.
579
580 **Fig. 3. Platelet Activation in PASC by Flow Cytometry. A-D**: Blood was drawn from
581 healthy individual or PASC patients and isolated, washed platelets were stimulated with
582 TRAP-6 (0-20µM), U466119 (0-10 µM), ADP (0-10 µM), or CRP (0-1.0 µg/mL) for 15
583 minutes, then reactivity was assessed by FACS by fold-change in surface CD62P (P-
584 Selectin) using a tagged antibody. Data are represented as mean fold-change ± SEM.
585 Group differences were assessed by the Kruskal-Wallis test, n=10 for healthy
586 individuals and n=9 for PASC. NS=not significant.
587
588 **Fig. 4. Whole Blood and Plasma Coagulation in PASC by Microfluidics and Factor**
589 **Xa Activity. A.** Blood was isolated and passed through a microfluidics chip using the
590 Total Thrombus formation Analysis System (T-TAS01) at roughly venous shear stress
591 (600 s-1). With clot generation, pressure in the microfluidics chamber (kPa) gradually
592 increases until cessation of blood flow. Data are represented as mean ± SEM. **B.** The
593 time from the start of occlusion to cessation of blood was calculated in seconds as
594 mean ± SEM. Group differences were determined by the student's t-test for PASC
595 patients (n=10) or or healthy volunteers (n=12). **C.** Citrate plasma devoid of platelets
596 was evaluated for Factor Xa activity using a chromogenic substrate in PASC patients
597 (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.

**Supplementary Tables and Figures:**

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

**Fig. S1. 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.

**Fig. S2. Plasma Antibody Analysis.** Blood was isolated from patients with PASC (n=66, blue) or matched controls (n=10, green) and antibody profiling was performed using the HuProt 4.0 array of unique human proteins known to be exclusively expressed in platelets: Platelet Factor 4 (PF4), Glycoprotein VI (GP6), integrin alpha2 gene (ITGA2). Other proteins highly expressed in platelets were also evaluated: von Willebrand Factor (vWF), Platelet And Endothelial Cell Adhesion Molecule 1 (PECAM1), E-selectin (SELE), P-selectin (SELP), Vascular Cell Adhesion Molecule 1 (VCAM1). Coagulation cascade protein or receptors were also evaluated: Factor II (F2), factor II receptor (FIIR), Factor X (F10). A heat map was generated to show each antibody concentration expressed as standard deviations (SD) above or below the mean concentration found in healthy individuals.

**Fig. S3. Platelet Surface Receptor Expression in PASC Patients.** Blood was drawn from healthy individuals (n=6) or PASC patients (n=6) and isolated, washed platelets were assessed by FACS for expression the following receptors tagged with fluorescent antibodies: $P2Y_{12}$, PAR, Thromboxane, GPVI. Data are represented as ± SEM. Group differences were assessed by t-test with p-values as noted.

644 **Table 1.** Demographics for PASC Patients and Healthy Controls

| Demographics | Healthy (n=19) N | % | PASC (n=26) N | % | P-Value |
|---|---|---|---|---|---|
| ***Characteristics*** | | | | | |
| Mean Age (± SEM) | 41.4 ± 2.89 | | 49.9 ± 3.61 | | 0.090 |
| 18-27 years | 4 | 21.0% | 3 | 11.5% | 0.30 |
| 28-40 years | 4 | 21.0 % | 7 | 26.9 % | 0.76 |
| 41-60 years | 9 | 47.3 % | 5 | 19.2 % | 0.51 |
| 61-82 years | 2 | 10.5 % | 11 | 42.3 % | 0.62 |
| Male | 4 | 21.0 % | 7 | 35.0 % | 0.73 |
| Female | 15 | 78.9 % | 19 | 73.0 % | |
| BMI (± SEM) | 29.5 ± 1.29 | | 30.9 ± 2.17 | | 0.61 |
| Smoker | 1 | 7.7 % | 13 | 50.0 % | *0.002* |
| ***Medical History*** | | | | | |
| Hypertension | 4 | 21.0 % | 6 | 23.0 % | 0.99 |
| Hyperlipidemia | 2 | 10.5 % | 5 | 19.2 % | 0.68 |
| History of CVD | 1 | 5.2 % | 3 | 11.5 % | 0.62 |
| Diabetes | 2 | 10.5 % | 1 | 3.8 % | 0.56 |
| History of Pulmonary Embolism | 0 | 0.0 % | 1 | 3.8 % | 0.99 |
| History of Thrombosis | 1 | 5.2 % | 0 | 0.0 % | 0.42 |
| ***Medications*** | | | | | |
| Platelet inhibitors | 1 | 5.2 % | 6 | 23.0 % | 0.21 |
| Aspirin | 1 | 5.2 % | 5 | 19.2% | |
| DAPT (Dual Antiplatelet Therapy) | | | 1 | 3.8% | |
| Anticoagulants | 0 | 0.0 % | 2 | 7.6 % | 0.50 |
| Warfarin | | | 1 | 3.8% | |
| Apixaban | | | 1 | 3.8% | |
| Statins | 2 | 15.4 % | 5 | 19.2 % | 0.68 |
| NSAIDs | 6 | 31.5 % | 4 | 15.38 % | 0.28 |
| BP Medications | 4 | 21.0 % | 1 | 3.8 % | 0.14 |

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

| **Symptoms** | PASC (n=26) N | % |
|---|---|---|
| Mean Time After Infection (± SEM) | 501 days ± 69 | |
| Fatigue | 20 | 76.9 % |
| Brain Fog | 20 | 76.9 % |
| Shortness of Breath | 18 | 69.2 % |
| Dyspnea on Exertion | 18 | 69.2 % |
| Dizziness | 17 | 65.3 % |
| Changes in Mood | 11 | 55.0 % |
| Insomnia | 14 | 53.8 % |
| Headaches | 13 | 50.0 % |
| Palpitations | 13 | 50.0 % |
| Chest Pain | 11 | 42.3 % |
| Joint Pain | 9 | 34.6 % |
| Cough | 7 | 26.9 % |
| Parosmia/Dysgeusia | 6 | 23.0 % |
| Memory Deficits | 6 | 23.0 % |
| Depression | 6 | 23.0 % |
| Syncope | 6 | 23.0 % |
| Nausea | 5 | 19.2 % |
| Body Aches | 4 | 15.3% |
| Incontinence | 3 | 11.5 % |
| Neck Pain | 3 | 11.5 % |
| HF Symptoms | 2 | 7.69 % |
| Diarrhea | 1 | 3.8 % |
| Erectile Dysfunction | 1 | 3.8 % |
| Fever | 1 | 3.8 % |
| Orthopnea | 1 | 3.8 % |
| Limb Paresthesia | 1 | 3.8 % |

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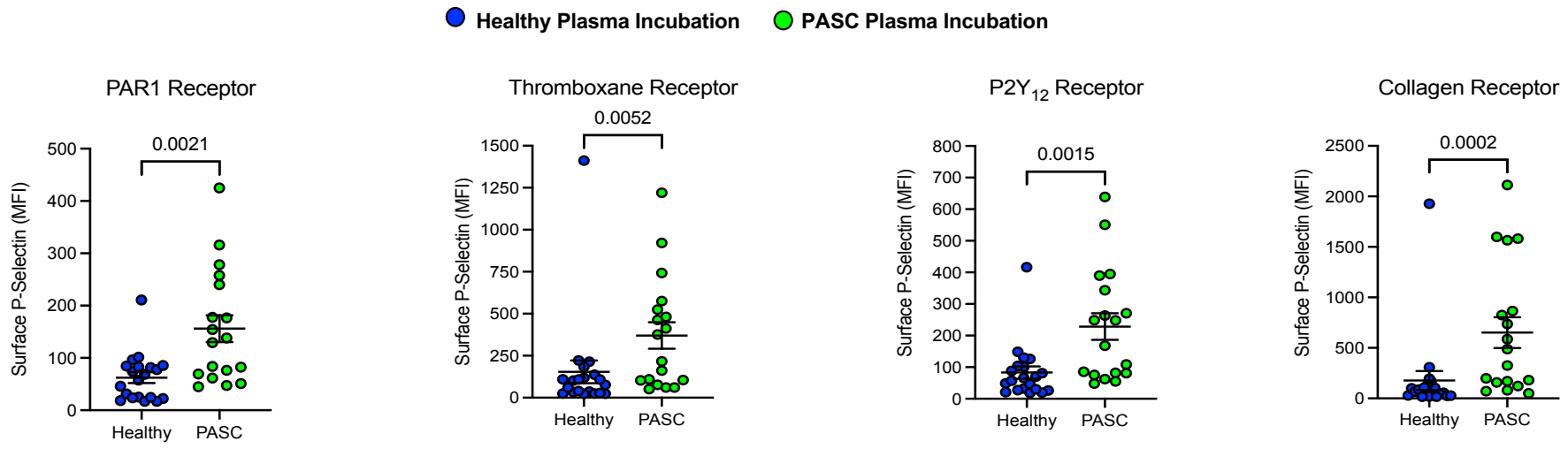


**Fig. 1. Activation of Platelets by PASC but not Healthy Plasma.** Isolated, washed platelets were pre-incubated for 30 minutes with 5 µL of age- and sex-matched healthy plasma or PASC plasma, then stimulated with TRAP-6, 5 µM (PAR1), U46619, 1 µM (Thromboxane receptor), ADP, 0.1 µM ($P2Y_{12}$ Receptor), 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.

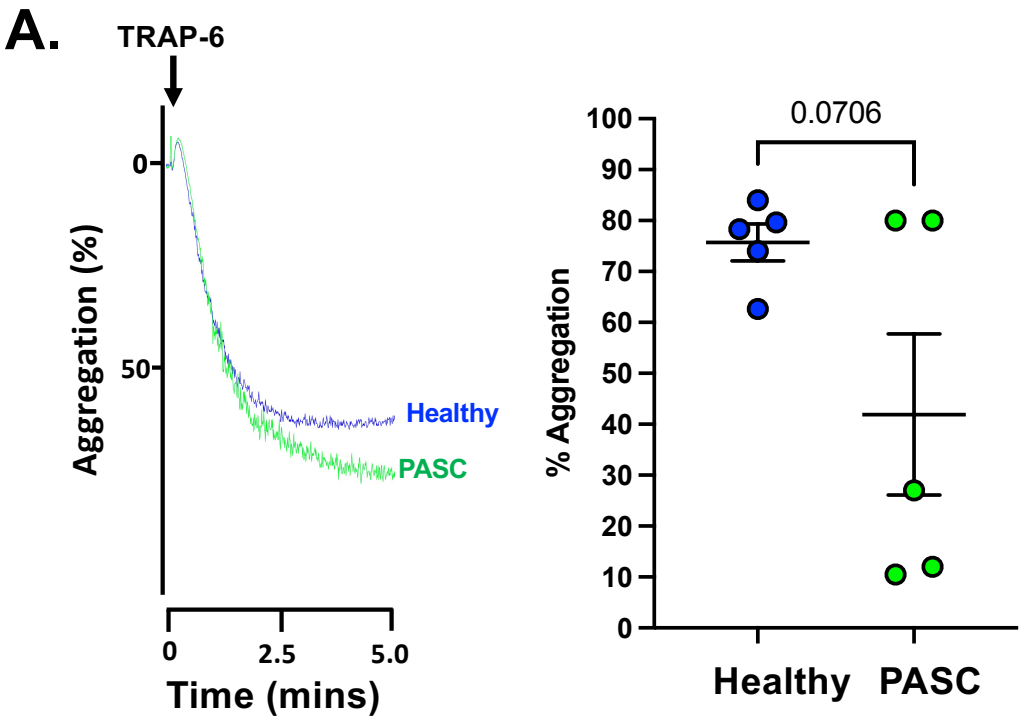


**Fig. 2A. Platelet Activation in PASC by Aggregometry.** Blood was drawn from healthy individual or PASC patients and PRP was isolated prior to stimulation with TRAP-6, 5µM until platelet aggregation was noted. Platelet aggregation was evaluated by LTA and data reported as mean % maximum aggregation ± 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.

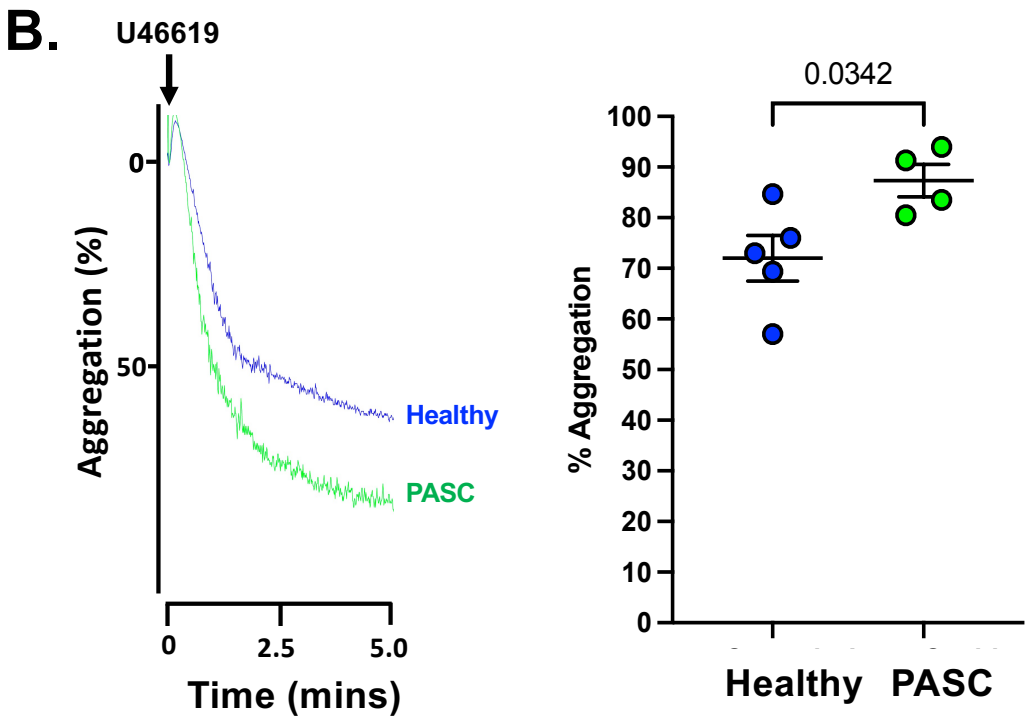


**Fig. 2B. Platelet Activation in PASC by Aggregometry.** Blood was isolated from healthy individual or PASC patients and PRP was isolated prior to stimulation with U466119, 5µM until platelet aggregation was noted. Platelet aggregation was evaluated by LTA and data reported as mean % maximum aggregation ± 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.

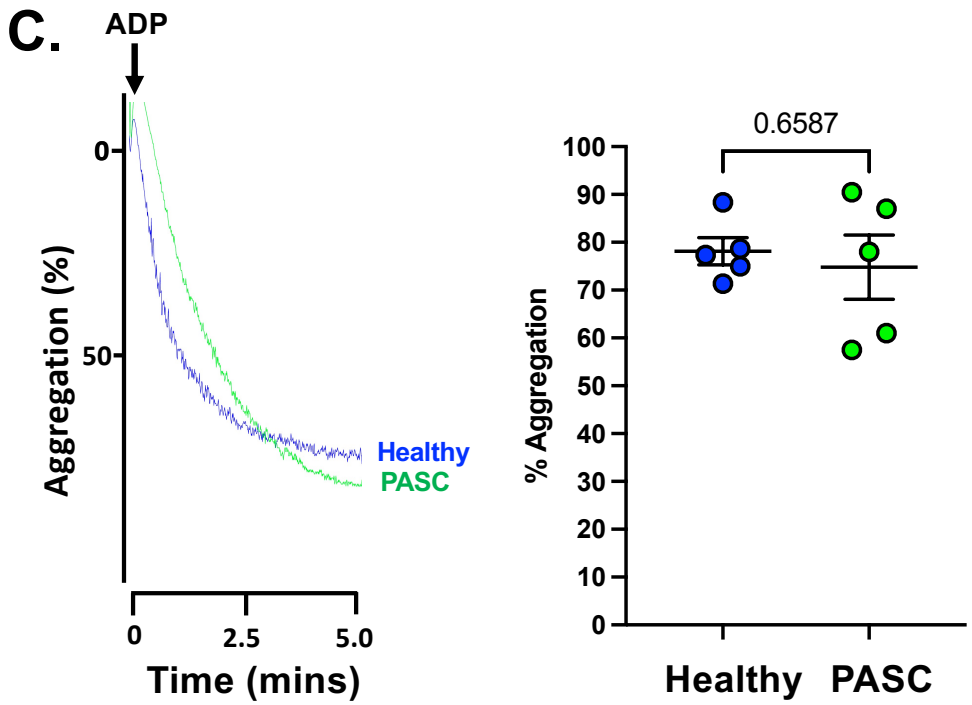


**Fig. 2C. Platelet Activation in PASC by Aggregometry.** Blood was isolated from healthy individual or PASC patients and PRP was isolated prior to stimulation with ADP, 1 µM until platelet aggregation was noted. Platelet aggregation was evaluated by LTA and data reported as mean % maximum aggregation ± 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.

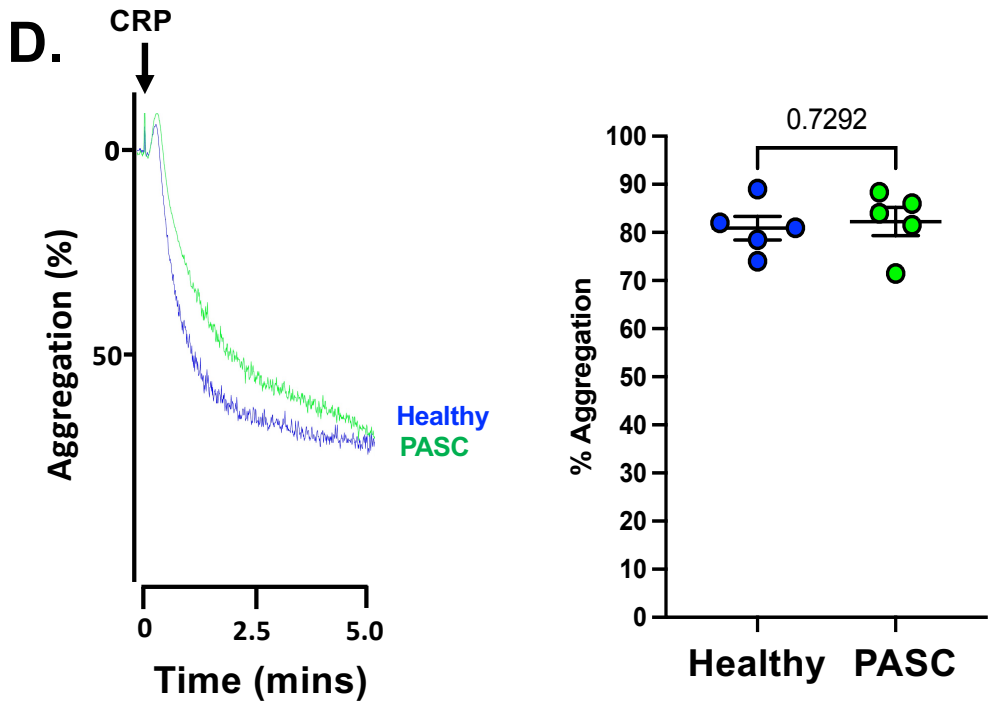


**Fig. 2D. Platelet Activation in PASC by Aggregometry.** Blood was isolated from healthy individual or PASC patients and PRP was isolated prior to stimulation with CRP, 1 µg/mL until platelet aggregation was noted. Platelet aggregation was evaluated by LTA and data reported as mean % maximum aggregation ± 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.

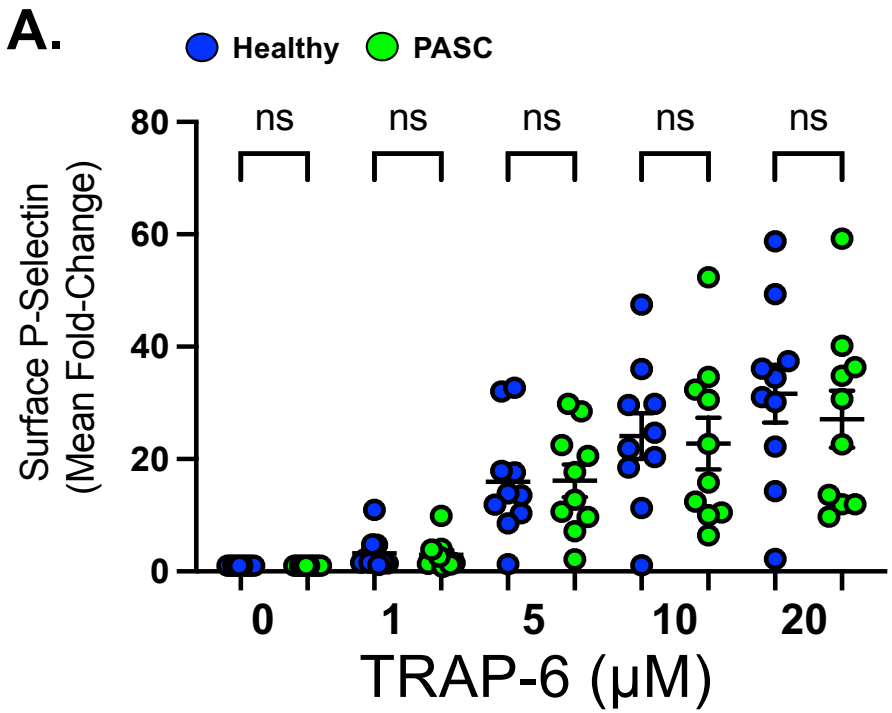


**Fig. 3A. Platelet Activation in PASC by Flow Cytometry.** Isolated, washed platelets were stimulated with TRAP-6 for 15 minutes, then reactivity was assessed by FACS by fold-change in surface CD62P (P-Selectin) using a tagged antibody. Data are represented as mean fold-change ± SEM. Group differences were assessed by the Kruskal-Wallis test, n=10 for healthy individuals and n=9 for PASC. NS=not significant.

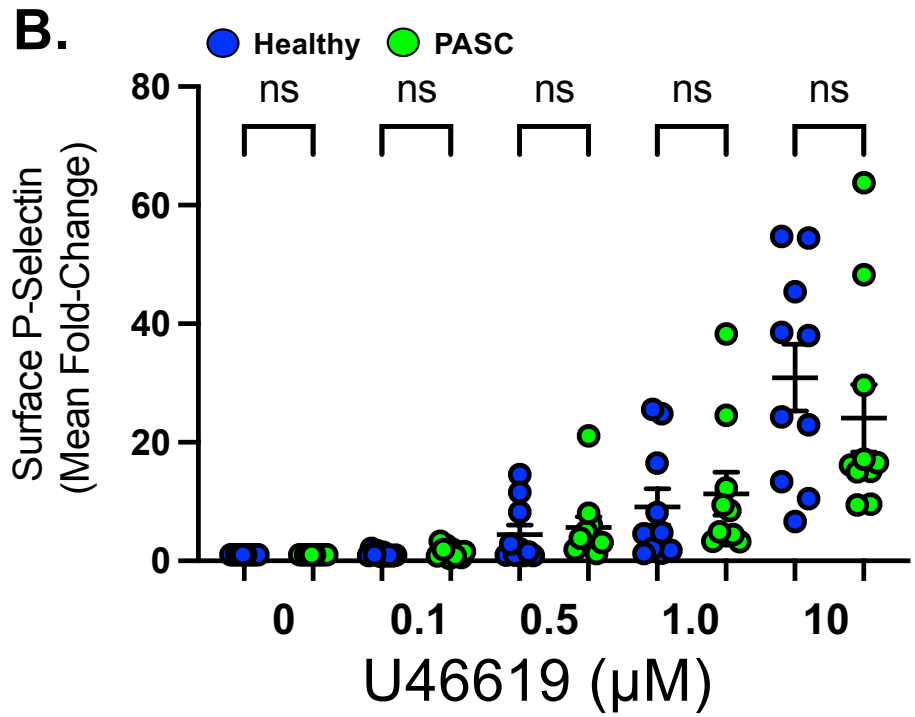


**Fig. 3B. Platelet Activation in PASC by Flow Cytometry.** Isolated, washed platelets were stimulated with U46619 for 15 minutes, then reactivity was assessed by FACS by fold-change in surface CD62P (P-selectin) using a tagged antibody. Data are represented as mean fold-change ± SEM. Group differences were assessed by ANOVA, followed by the Bonferroni correction, n=10 for healthy individuals and n=10 for PASC.

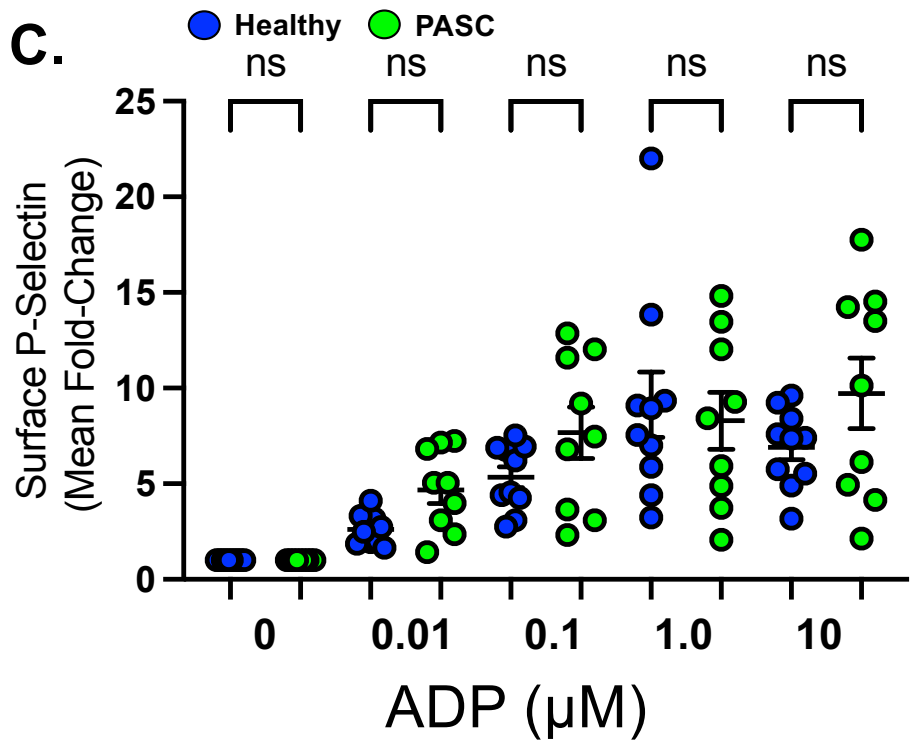


**Fig. 3C. Platelet Activation in PASC by Flow Cytometry.** Isolated, washed platelets were stimulated with ADP for 15 minutes, then reactivity was assessed by FACS by fold-change in surface CD62P (P-selectin) using a tagged antibody. Data are represented as mean fold-change ± SEM. Group differences were assessed by ANOVA, followed by the Bonferroni correction, n=10 for healthy individuals and n=9 for PASC.

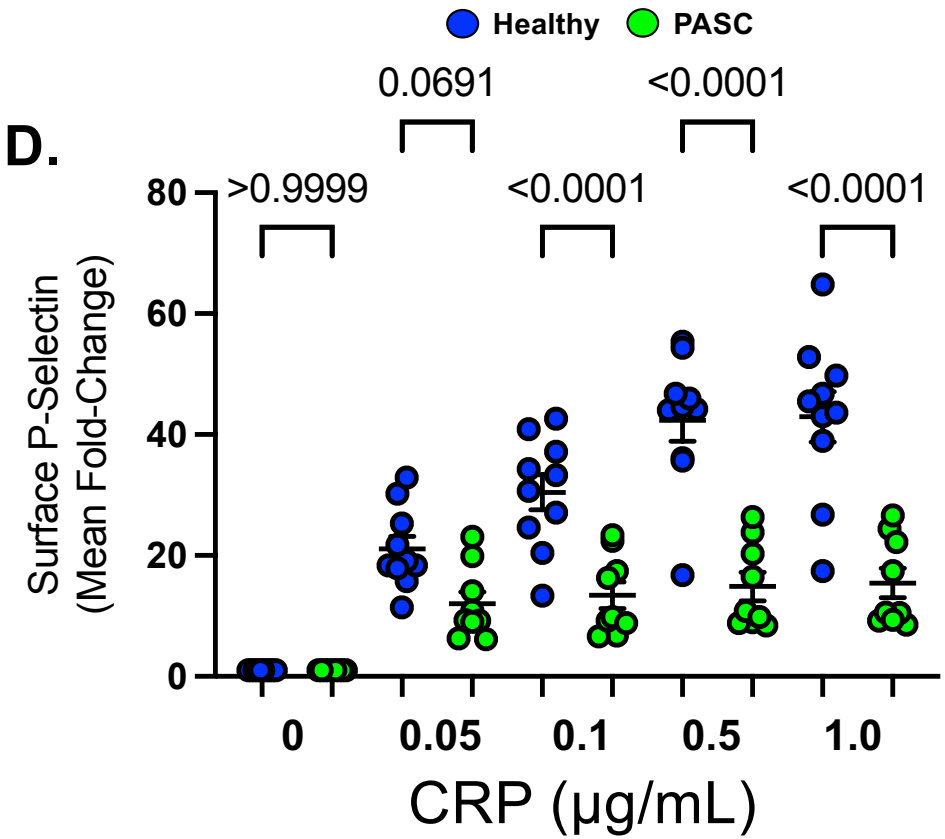


**Fig. 3D. Platelet Activation in PASC by Flow Cytometry.** Isolated, washed platelets were stimulated with collagen-related peptide (CRP) for 15 minutes, then reactivity was assessed by FACS by fold-change in surface CD62P (P-selectin) using a tagged antibody. Data are represented as mean fold-change ± SEM. Group differences were assessed by ANOVA, followed by the Bonferroni correction, n=10 for healthy individuals and n=9 for PASC.

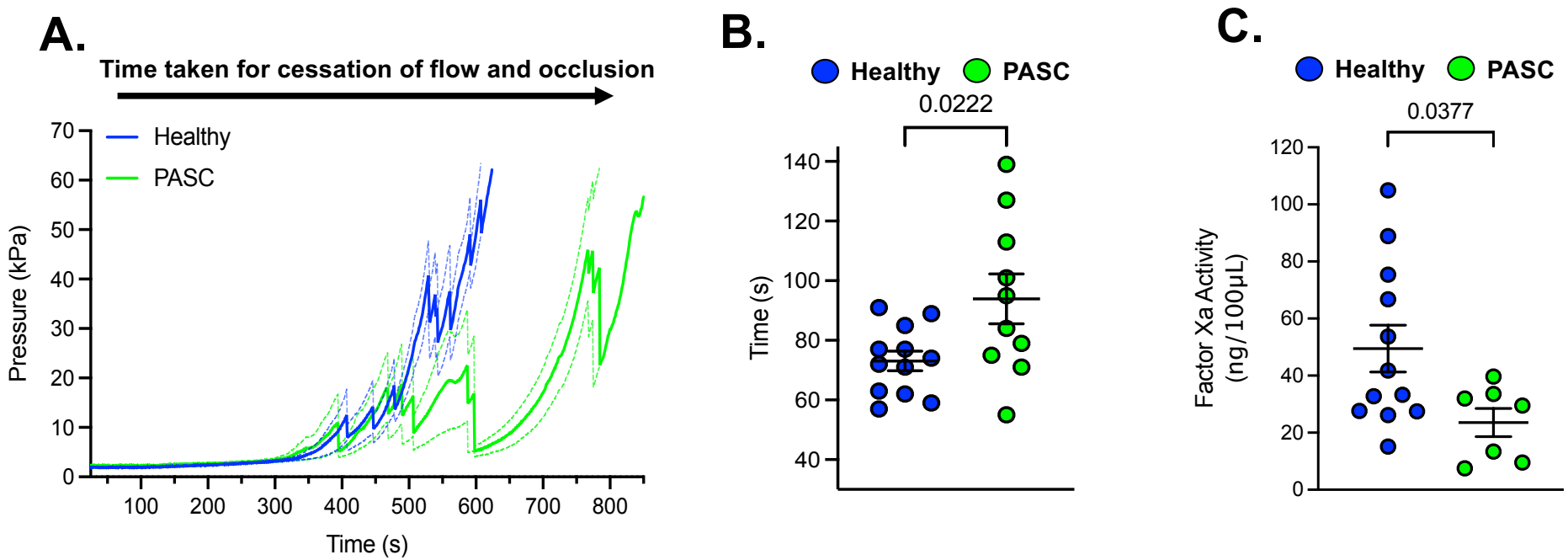


**Fig. 4. Whole Blood and Plasma Coagulation in PASC by Microfluidics and Factor Xa Activity. A.** Blood was isolated and passed through a microfluidics chip using the Total Thrombus formation Analysis System (T-TAS01) at roughly venous shear stress (600 $s^{-1}$). With clot generation, pressure in the microfluidics chamber (kPa) gradually increases until cessation of blood 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 for PASC patients (n=10) or or healthy volunteers (n=12). **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.

# Supplemental Data

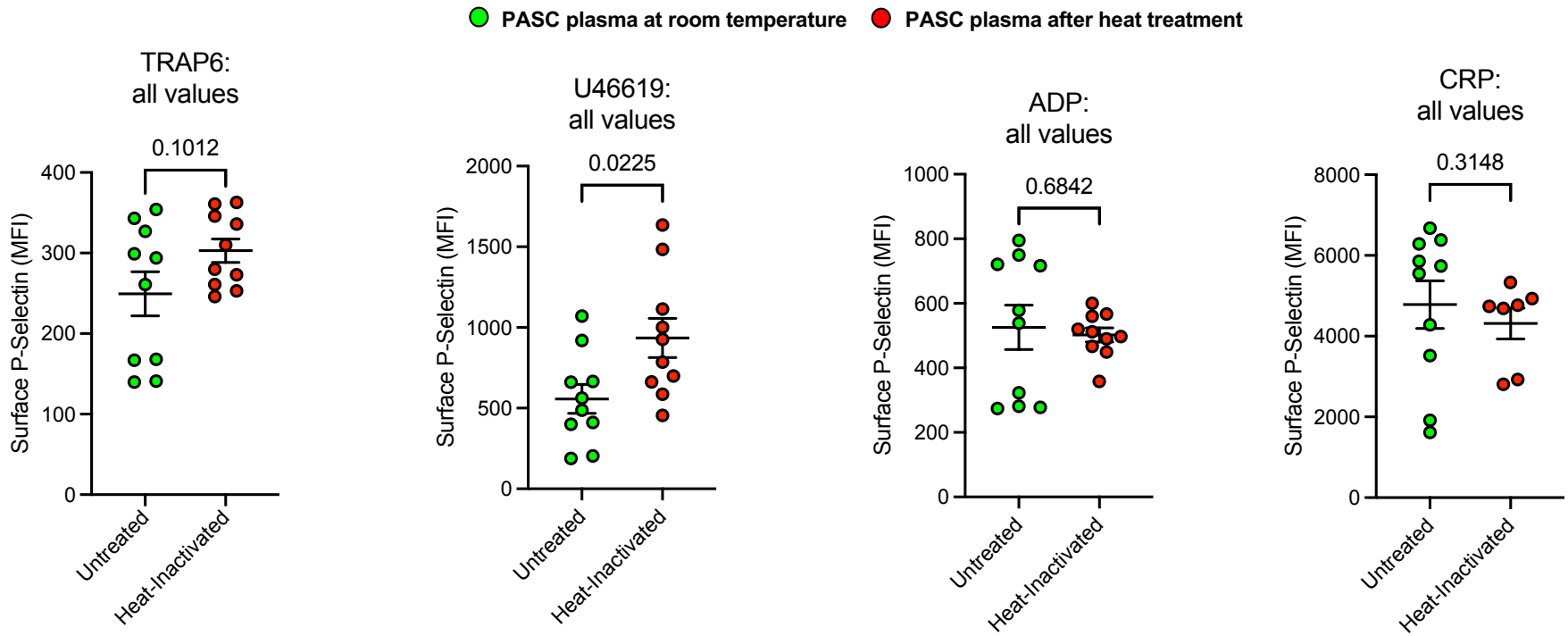


**Fig. S1. Activation of Platelets by PASC plasma persists after heat treatment.** Platelet-deplete citrate plasma from PASC patients already demonstrated to activate healthy male platelets divided in half and one half was heat-treated (55 ˚C, 15 minutes). Heat-treated and non-heat-treated plasma was incubated with washed platelets 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 (room temperature citrate plasma) or n=10 (heat-inactivated citrate plasma.

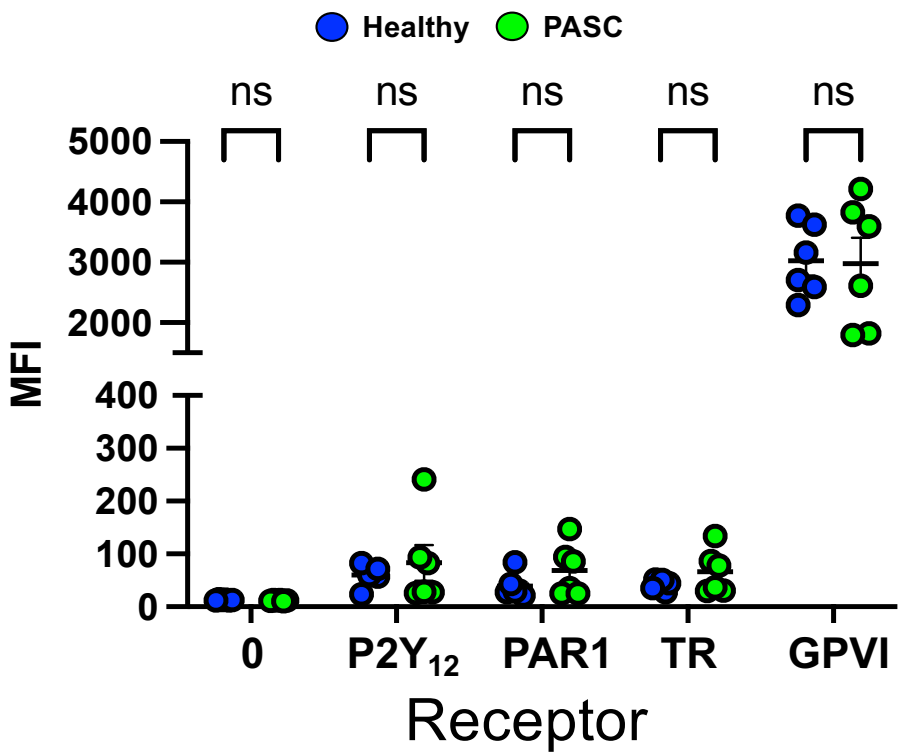


**Fig. S2. Platelet Surface Receptor Expression in PASC Patients.** Blood was drawn from healthy individuals (n=6) or PASC patients (n=6) and isolated, washed platelets were assessed by FACS for expression the following receptors tagged with fluorescent antibodies: $P2Y_{12}$, PAR1, Thromboxane Receptor (TR), GPVI, or 0 (control Immunoglobulin). Data are represented as MFI (Mean Fluorescence Intensity) of surface receptor expression ± SEM. Group differences were assessed by t-test with no differences noted between groups, ns=not significant)

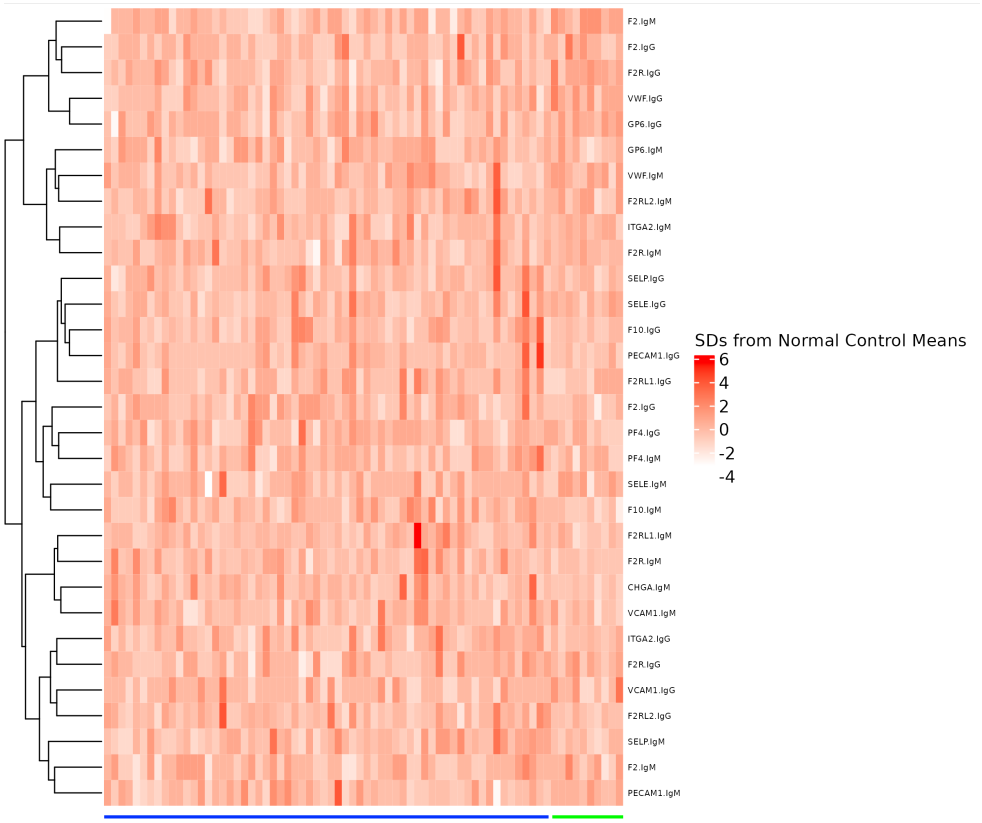


**Fig. S3. Plasma Antibody Analysis.** Blood was isolated from patients with PASC (n=66, blue) or matched controls (n=10, green) and antibody profiling was performed using the HuProt 4.0 array of unique human proteins known to be exclusively expressed in platelets: Platelet Factor 4 (PF4), Glycoprotein VI (GP6), integrin alpha2 gene (ITGA2). Other proteins highly expressed in platelets were also evaluated: von Willebrand Factor (vWF), Platelet And Endothelial Cell Adhesion Molecule 1 (PECAM1), E-selectin (SELE), P-selectin (SELP), Vascular Cell Adhesion Molecule 1 (VCAM1). Coagulation cascade protein or receptors were also evaluated: Factor II (F2), factor II receptor (FIIR), Factor X (F10). A heat map was generated to show each antibody concentration expressed as standard deviations (SD) above or below the mean concentration found in healthy individuals.

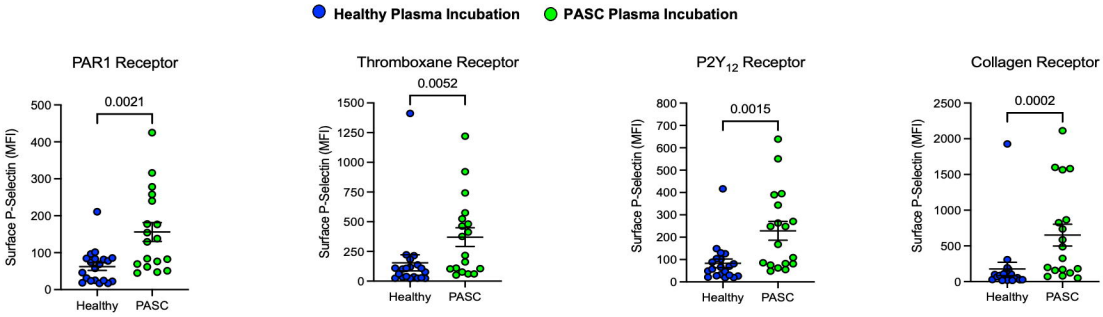


**Fig. 1. Activation of Platelets by PASC but not Healthy Plasma.** Isolated, washed platelets were pre-incubated for 30 minutes with 5 µL of age- and sex-matched healthy plasma or PASC plasma, then stimulated with TRAP-6, 5 µM (PAR1), U46619, 1 µM (Thromboxane receptor), ADP, 0.1 µM ($P2Y_{12}$ Receptor), 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.

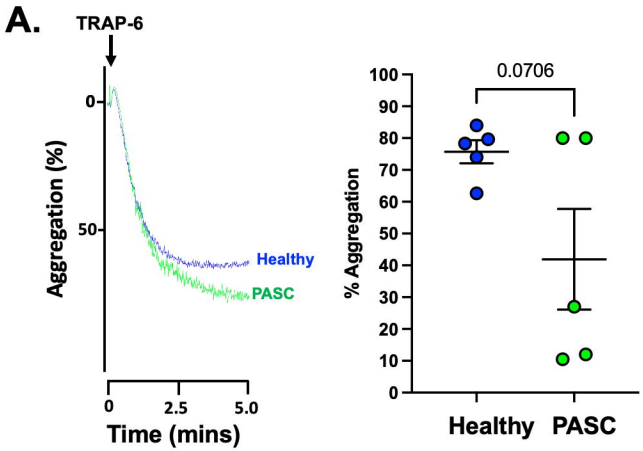


**Fig. 2A. Platelet Activation in PASC by Aggregometry.** Blood was drawn from healthy individual or PASC patients and PRP was isolated prior to stimulation with TRAP-6, 5μM until platelet aggregation was noted. Platelet aggregation was evaluated by LTA and data reported as mean % maximum aggregation ± 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.

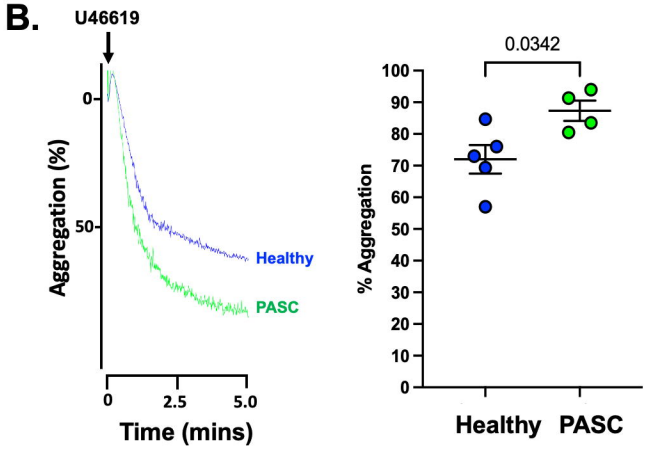


**Fig. 2B. Platelet Activation in PASC by Aggregometry.** Blood was isolated from healthy individual or PASC patients and PRP was isolated prior to stimulation with U466119, 5μM until platelet aggregation was noted. Platelet aggregation was evaluated by LTA and data reported as mean % maximum aggregation ± 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.

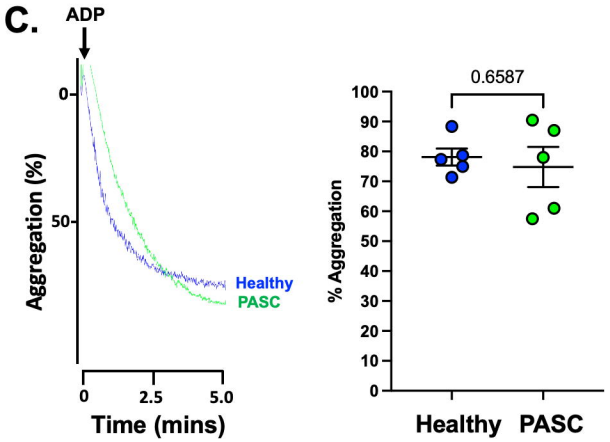


**Fig. 2C. Platelet Activation in PASC by Aggregometry.** Blood was isolated from healthy individual or PASC patients and PRP was isolated prior to stimulation with ADP, 1 μM until platelet aggregation was noted. Platelet aggregation was evaluated by LTA and data reported as mean % maximum aggregation ± 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.

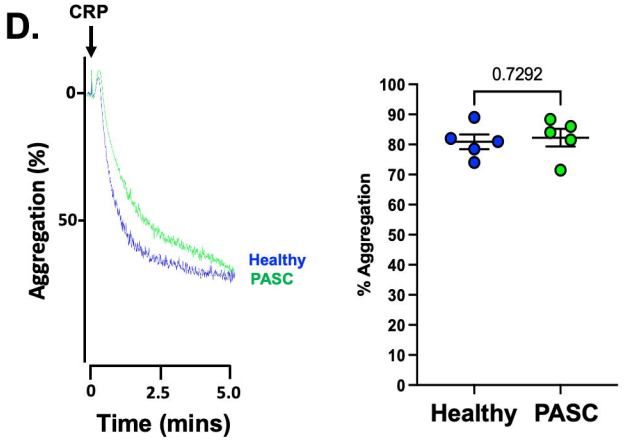


**Fig. 2D. Platelet Activation in PASC by Aggregometry.** Blood was isolated from healthy individual or PASC patients and PRP was isolated prior to stimulation with CRP, 1 µg/mL until platelet aggregation was noted. Platelet aggregation was evaluated by LTA and data reported as mean % maximum aggregation ± 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.

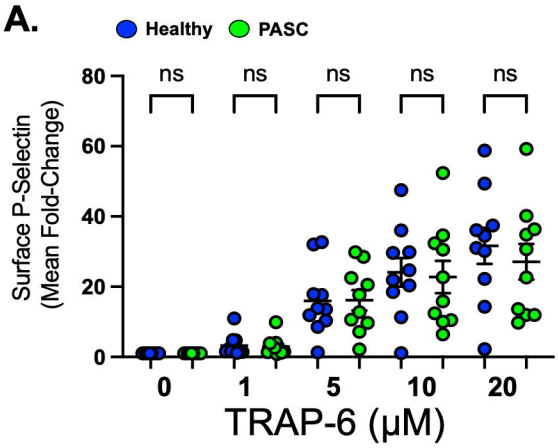


**Fig. 3A. Platelet Activation in PASC by Flow Cytometry.** Isolated, washed platelets were stimulated with TRAP-6 for 15 minutes, then reactivity was assessed by FACS by fold-change in surface CD62P (P-Selectin) using a tagged antibody. Data are represented as mean fold-change ± SEM. Group differences were assessed by the Kruskal-Wallis test, n=10 for healthy individuals and n=9 for PASC. NS=not significant.

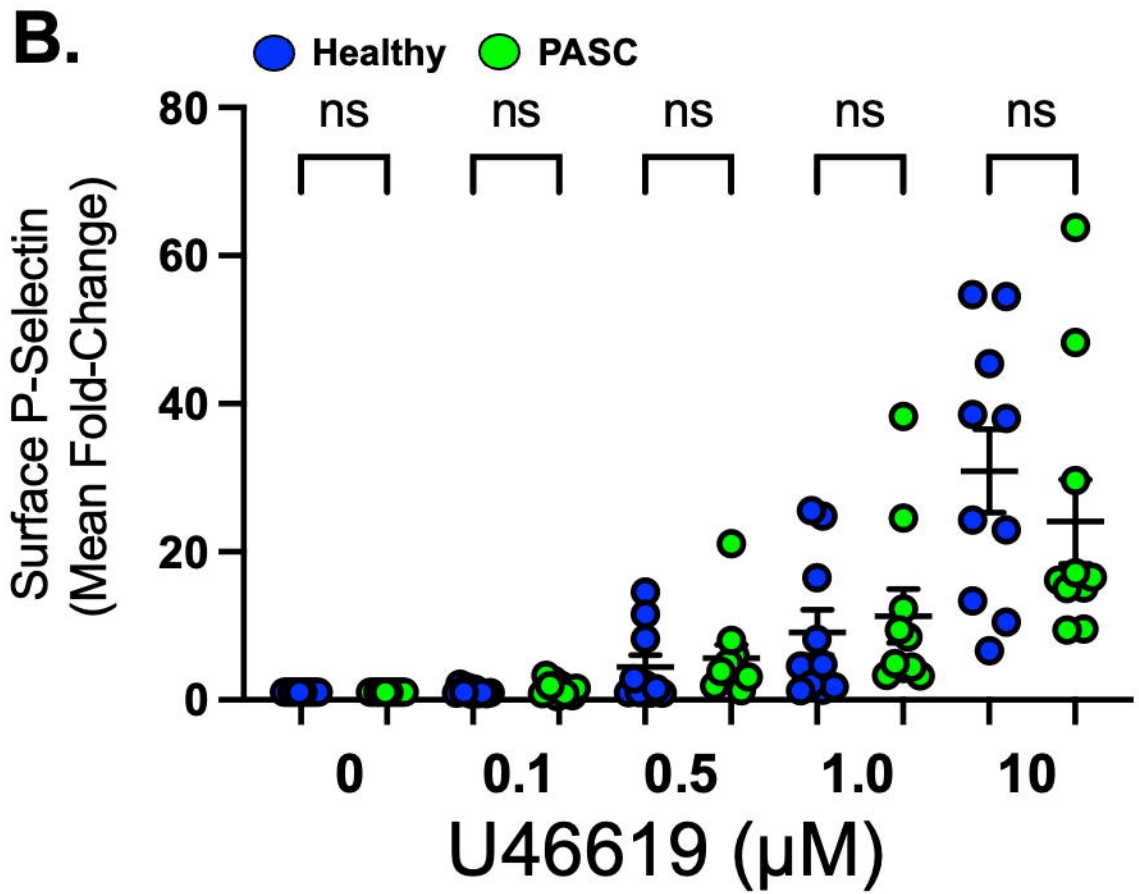


**Fig. 3B. Platelet Activation in PASC by Flow Cytometry.** Isolated, washed platelets were stimulated with U46619 for 15 minutes, then reactivity was assessed by FACS by fold-change in surface CD62P (P-selectin) using a tagged antibody. Data are represented as mean fold-change ± SEM. Group differences were assessed by ANOVA, followed by the Bonferroni correction, n=10 for healthy individuals and n=10 for PASC.

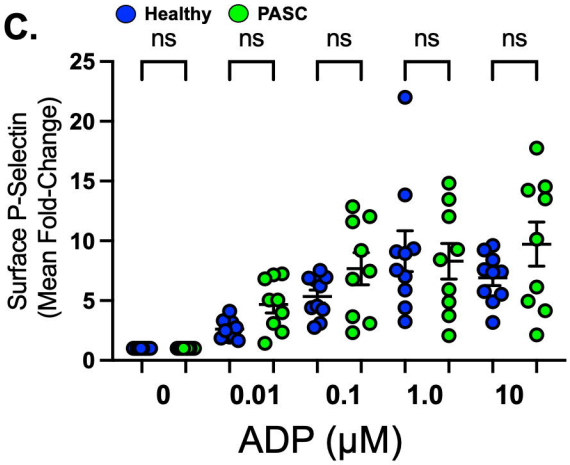


**Fig. 3C. Platelet Activation in PASC by Flow Cytometry.** Isolated, washed platelets were stimulated with ADP for 15 minutes, then reactivity was assessed by FACS by fold-change in surface CD62P (P-selectin) using a tagged antibody. Data are represented as mean fold-change ± SEM. Group differences were assessed by ANOVA, followed by the Bonferroni correction, n=10 for healthy individuals and n=9 for PASC.

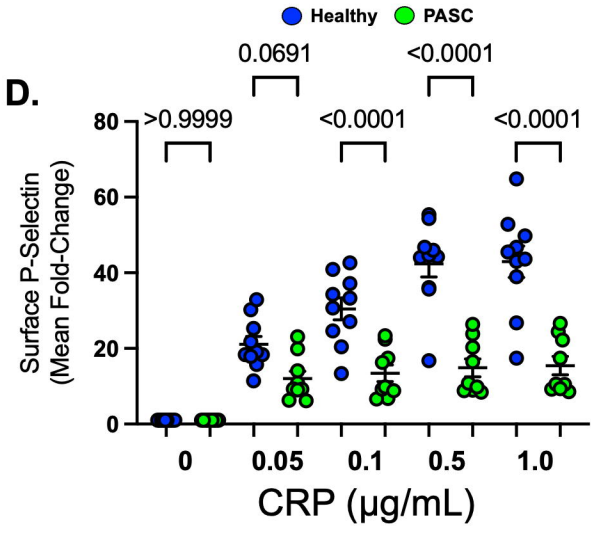


**Fig. 3D. Platelet Activation in PASC by Flow Cytometry.** Isolated, washed platelets were stimulated with collagen-related peptide (CRP) for 15 minutes, then reactivity was assessed by FACS by fold-change in surface CD62P (P-selectin) using a tagged antibody. Data are represented as mean fold-change ± SEM. Group differences were assessed by ANOVA, followed by the Bonferroni correction, n=10 for healthy individuals and n=9 for PASC.

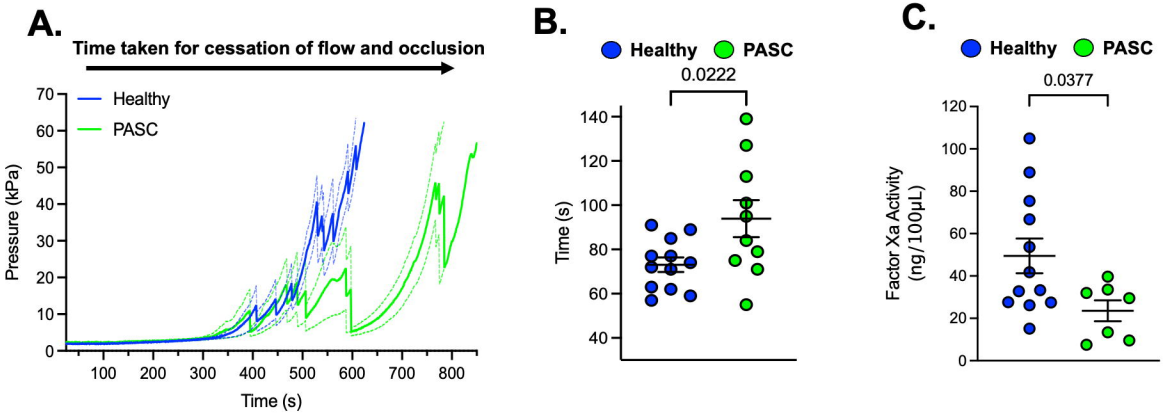


**Fig. 4. Whole Blood and Plasma Coagulation in PASC by Microfluidics and Factor Xa Activity. A.** Blood was isolated and passed through a microfluidics chip using the Total Thrombus formation Analysis System (T-TAS01) at roughly venous shear stress (600 $s^{-1}$). With clot generation, pressure in the microfluidics chamber (kPa) gradually increases until cessation of blood 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 for PASC patients (n=10) or or healthy volunteers (n=12). **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.

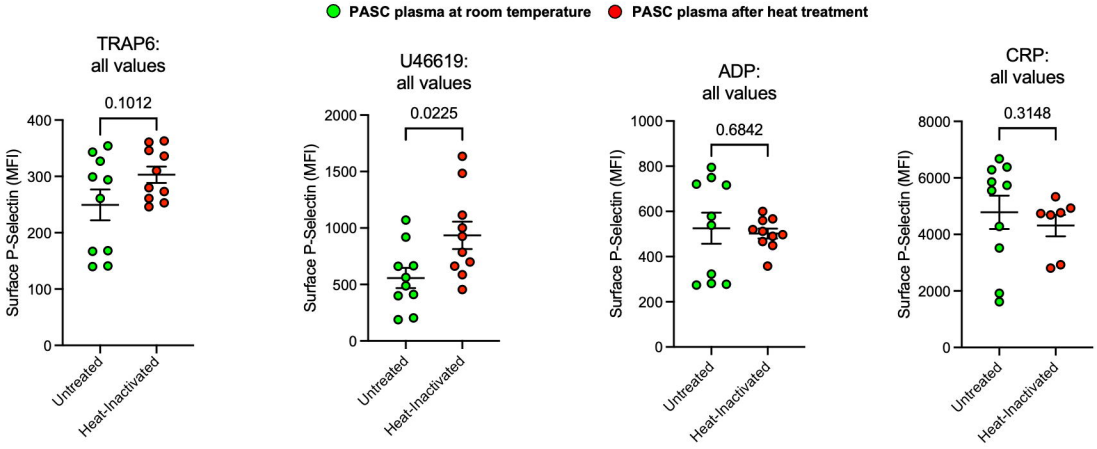


**Fig. S1. Activation of Platelets by PASC plasma persists after heat treatment.** Platelet-deplete citrate plasma from PASC patients already demonstrated to activate healthy male platelets divided in half and one half was heat-treated (55 ˚C, 15 minutes). Heat-treated and non-heat-treated plasma was incubated with washed platelets 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 (room temperature citrate plasma) or n=10 (heat-inactivated citrate plasma).

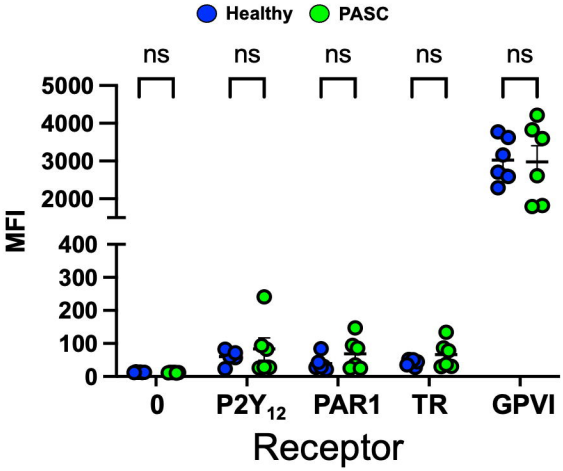


**Fig. S2. Platelet Surface Receptor Expression in PASC Patients.** Blood was drawn from healthy individuals (n=6) or PASC patients (n=6) and isolated, washed platelets were assessed by FACS for expression the following receptors tagged with fluorescent antibodies: $P2Y_{12}$, PAR1, Thromboxane Receptor (TR), GPVI, or 0 (control Immunoglobulin). Data are represented as MFI (Mean Fluorescence Intensity) of surface receptor expression ± SEM. Group differences were assessed by t-test with no differences noted between groups, ns=not significant)

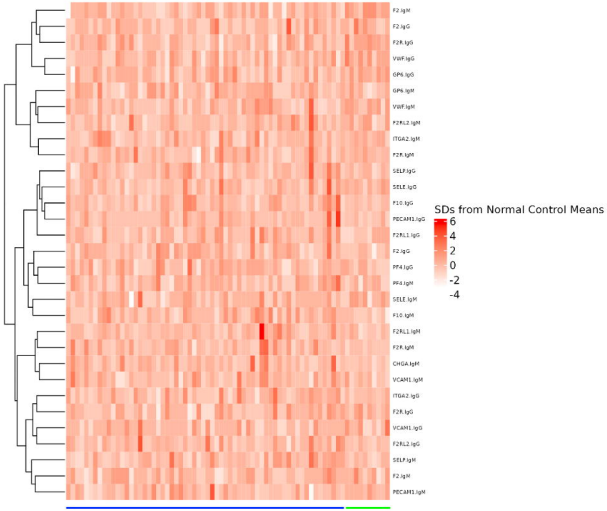


**Fig. S3. Plasma Antibody Analysis.** Blood was isolated from patients with PASC (n=66, blue) or matched controls (n=10, green) and antibody profiling was performed using the HuProt 4.0 array of unique human proteins known to be exclusively expressed in platelets: Platelet Factor 4 (PF4), Glycoprotein VI (GP6), integrin alpha2 gene (ITGA2). Other proteins highly expressed in platelets were also evaluated: von Willebrand Factor (vWF), Platelet And Endothelial Cell Adhesion Molecule 1 (PECAM1), E-selectin (SELE), P-selectin (SELP), Vascular Cell Adhesion Molecule 1 (VCAM1). Coagulation cascade protein or receptors were also evaluated: Factor II (F2), factor II receptor (FIIR), Factor X (F10). A heat map was generated to show each antibody concentration expressed as standard deviations (SD) above or below the mean concentration found in healthy individuals.