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Sustained VWF-ADAMTS13 axis imbalance and endotheliopathy in Long COVID syndrome is related to immune dysfunction

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ESSENTIALS

1. Reduced plasma ADAMTS13 levels and an increased VWF/ADAMTS13 ratio are common in convalescent COVID-19 patients
2. Levels of Platelet Factor 4, a putative VWF protector, are elevated in convalescent COVID-19 patients compared with healthy controls
3. Elevated levels of pro-angiogenic proteins, including Weibel Palade Body constituents Angiopoietin-2 and Osteoprotegerin are observed in a subset of patients
4. Intermediate monocytes and activated CD4+ and CD8+ T cell subsets are increased in convalescent COVID-19 and correlate with thrombin generation and endotheliopathy markers, respectively.

ABSTRACT

Background: Prolonged recovery is common after SARs-CoV-2 infection, however the pathophysiological mechanisms underpinning Long COVID syndrome remain unknown. VWF-ADAMTS13 imbalance, dysregulated angiogenesis and immuno-thrombosis are hallmarks of acute COVID-19. We hypothesized that VWF-ADAMTS13 imbalance persists in convalescence together with EC activation and angiogenic disturbance. Additionally, we postulate that ongoing immune cell dysfunction may be linked to sustained EC and coagulation activation.

Patients and Methods: Fifty patients were reviewed at a minimum of 6 weeks following acute COVID-19. ADAMTS13, WPB proteins and angiogenesis-related proteins were assessed and clinical evaluation and immunophenotyping performed. Comparisons were made with healthy controls (n=20) and acute COVID-19 patients (n=36).

Results: ADAMTS13 levels were reduced (p=0.009) and the VWF/ADAMTS13 ratio was increased in convalescence (p=0.0004). Levels of Platelet Factor 4 (PF4), a putative protector of VWF, were also elevated (p=0.0001). A non-significant increase in WPB proteins Angiopoietin-2 (Ang-2) and Osteoprotegerin (OPG) was observed in convalescent patients and WPB markers correlated with EC parameters. Enhanced expression of 21 angiogenesis-related proteins was observed in convalescent COVID-19. Finally, immunophenotyping revealed significantly elevated intermediate monocytes and activated CD4+ and CD8+ T cells in convalescence, which correlated with thrombin generation and endotheliopathy markers, respectively.

Conclusion: Our data provide insights into sustained EC activation, dysregulated angiogenesis and VWF/ADAMTS13 axis imbalance in convalescent COVID-19. In keeping with the pivotal role of immuno-thrombosis in acute COVID-19, our findings support the hypothesis that abnormal T cell and monocyte populations may be important in the context of persistent EC activation and hemostatic dysfunction during convalescence.

KEYWORDS - Endothelial cell (EC) activation, Weibel Palade Body (WPB) exocytosis, Immune dysfunction, Long COVID, Convalescent COVID-19

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INTRODUCTION

Prolonged functional impairment following COVID-19 disease, referred to as Long COVID or post-acute COVID-19 syndrome (PACS), is estimated to occur in 30-40% of infected individuals.[1, 2] Typical features include fatigue, breathlessness and reduced exercise tolerance. However, the clinical phenotype is heterogeneous and proposed definitions vary.[3, 4] Despite the global morbidity associated with Long COVID, the underlying pathogenic mechanisms are poorly understood. However, recent studies have demonstrated that, similar to acute illness, persistent coagulation activation is common in convalescent COVID-19 patients.[5-9] In particular, several groups have reported sustained increases in endogenous thrombin potential and D-dimer levels in patients between 4 and 12 months following acute illness.[6, 7, 9] Ongoing hemostatic dysfunction was more common in patients who required intensive care support during their acute COVID-19 illness and in patients aged >50 years.[6, 7] Interestingly, however, prolonged increased D-dimer levels and coagulation activation were also seen in some patients who had mild COVID-19 and were managed exclusively as out-patients.[6, 7]

Post-mortem studies have highlighted that acute SARS-CoV-2 infection is associated with specific deleterious effects on endothelial cells (EC).[10, 11] Features include loss of normal tight EC junctions leading to increased EC permeability, enhanced EC apoptosis and abnormal angiogenesis.[11-14] In keeping with the concept that SARS-CoV-2 infection is associated with marked EC activation, plasma levels of von Willebrand factor antigen (VWF:Ag), VWF propeptide (VWFpp) and angiopoietin-2 (Ang-2) are all significantly increased and correlate with clinical severity.[15-17] In addition, reduced plasma ADAMTS13 levels and abnormal ultra-large VWF multimers have been observed in acute COVID-19.[18, 19] Collectively, these data suggest that acute EC activation and dysregulation of the normal VWF-ADAMTS13 axis play important roles in the pathobiology underlying pulmonary immunothrombosis and microvascular occlusion in acute COVID-19. Interestingly, recent studies have further reported that plasma VWF:Ag, VWFpp and FVIII levels all remain significantly elevated after 3 months in convalescent COVID-19 patients compared to healthy controls.[6] Based on these data, roles for sustained EC activation in the pathogenesis of persistent hemostatic dysfunction and Long COVID symptomatology have been proposed. In this study, we sought to further investigate the nature of ongoing endotheliopathy and VWF-ADAMTS13 axis dysfunction in convalescent COVID-19.

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METHODS

Sequential convalescent COVID-19 patients (n=50) who had blood samples taken at time of outpatient review in the Long COVID clinic at St James's Hospital (SJH) between May-September 2020, were included. Acute COVID-19 patients from Beaumont Hospital and SJH (n=36) and a control group of age- and gender-matched asymptomatic controls (n=20) were also recruited. Informed written consent was obtained from participants and ethical approval granted by the Hospital Research Ethics Committee. All acute (n=36) and convalescent COVID-19 patients (n=50) had coagulation samples (3.2% sodium citrate tubes) taken. Samples for immunophenotyping were also taken from a subset of patients (n=37 convalescent and n=32 acute COVID-19 patients). Levels of VWF:Ag, VWF propeptide (VWFpp), Interleukin-6 (IL-6), and soluble thrombomodulin (sTM) were quantified as before.[6, 20] Factor VIII activity was measured by chromogenic assay (FVIII:C) in the National Coagulation Centre reference laboratory at SJH. Thrombin generation was performed in a Fluouroskan Ascent Fluorometer with Thrombinoscope software (Stago) using PPP Low reagent (1 pM tissue factor, 4 mM phospholipids) as before[6, 21]. Characterisation of peripheral blood monocyte and T lymphocyte populations by flow cytometry were performed as previously described.[22] Plasma ADAMTS13 (R&D Systems #DY4245-05), Platelet Factor 4 (PF4) (R&D Systems #DY795), Angiopoietin-2 (Ang-2) (R&D Systems #DY623) and Osteoprotegerin (OPG) (R&D Systems #DY805) levels were measured using commercial ELISAs. Angiogenesis profiles were assessed by membrane-based antibody array according to the manufacturers' instructions (R&D Systems #ARY007). Clinical assessment included: chest x-ray and 6-minute walk test (6MWT) measuring distance covered and lowest arterial oxygen saturation. Statistical analyses were performed using the Mann-Whitney U tests, Kruskal-Wallis tests and the Spearman rank correlation test in GraphPad Prism 9.0 (GraphPad Software, USA) with a p-value of <0.05 considered statistically significant.

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RESULTS AND DISCUSSION

Convalescent COVID-19 patients (n=50, 60% male, median age 50 [IQR 36 – 63] years) were assessed at a minimum of 6 weeks (median 68 [IQR 61 – 72] days), following either symptom resolution or hospital discharge, in keeping with previous definitions of Long COVID **(Table 1)**.[1] The majority of patients (37/50, 74%) required hospitalization, while just over one quarter (13/50, 26%) managed their acute illness at home. Comorbidities were identified in 31/50 (62%) patients **(Table 1)**. Acute hospitalized COVID-19 patients received weight- and renally-adjusted LMWH prophylaxis whereas non-hospitalized and convalescent patients did not.

In keeping with previous studies, we confirmed significant increases in plasma VWF:Ag levels (**Supplementary Fig. 1A**) and concurrent reductions in plasma ADAMTS13 levels (**Fig. 1A**) in our acute COVID-19 subgroup. As a result, the VWF/ADAMTS13 ratio was markedly elevated >10 fold in acute COVID-19 patients compared to controls (**Fig. 1B**). In patients with convalescent COVID-19, we observed that plasma ADAMTS13 levels were significantly reduced compared to controls (median 598ng/ml versus 630ng/ml, p=0.009; **Fig.1A**). Marked inter-individual variation was apparent, with ADAMTS13 levels ranging from 220ng/ml to 900ng/ml in the convalescent cohort. Pertinently, plasma ADAMTS13 levels below the lower limit of our local normal reference range (399ng/ml) were seen in 15/50 (30%) convalescent COVID-19 patients, with a median ADAMTS13 level of 384ng/ml in this subgroup. ADAMTS13 levels were significantly lower in convalescent COVID-19 patients who had required hospitalisation compared to those managed entirely as outpatients (p=0.04) (data not shown). The absolute reduction in ADAMTS13 levels in convalescent COVID-19 patients was significantly less marked than that seen in patients with acute COVID-19 (**Fig. 1A**). Importantly, however, the reduction in ADAMTS13 levels coupled with elevated plasma VWF:Ag levels, meant that the VWF/ADAMTS13 ratio remained significantly elevated in convalescent COVID-19 patients compared to controls (median 2.3 versus 1.0, p=0.0004; **Fig.1B)**. In fact, 35/50 (70%) convalescent COVID-19 patients studied still had VWF/ADAMTS13 ratios above the upper limit of the normal reference range (1.8). On univariate analysis, the VWF/ADAMTS13 ratio was significantly higher in patients who had required hospitalization, those with ≥2 comorbidities and those with reduced exercise tolerance (6MWT below the median distance, 430m) (data not shown). Interestingly, Prasannan *et al*, recently reported similar VWF/ADAMTS13 ratios in a convalescent COVID-19 cohort using an ADAMTS13 FRETS assay, in which VWF/ADAMTS13 ratios > 1.5 were noted in 28% and were associated with impaired exercise tolerance.[23]

In addition to the abnormal VWF/ADAMTS13 ratio in acute COVID-19, previous studies have reported significant increases in a number of putative VWF protectors, including platelet factor 4

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(PF4) and interleukin-6 (IL-6), which can regulate VWF cleavage by ADAMTS13.[18, 24, 25] We observed that PF4 levels remained significantly increased in convalescent COVID-19 compared to controls (median 481pg/ml versus 250pg/ml, p=0.0001; **Fig. 1C)**. Notably, 23/50 (46%) of the convalescent COVID-19 cohort had persistent PF4 levels >500ng/ml, levels similar to those seen in acute COVID-19. While it has been shown that PF4 binds to VWF and PF4-VWF complexes exist in thrombotic thrombocytopenic purpura[25], further studies are needed to determine the biological mechanisms underlying this sustained increase in plasma PF4 levels and whether similar PF4-VWF complexes exist in convalescent COVID-19. Finally, in contrast to the PF4 findings, we observed that plasma IL-6 levels had returned to within the normal range in the majority (46/50, 92%) of convalescent COVID-19 patients (**Fig. 1D**).

The increases in plasma VWF:Ag, VWFpp and FVIII:C levels seen in convalescent COVID-19 suggest ongoing EC activation and Weibel Palade body (WPB) exocytosis. Importantly WPB also store other important pro-inflammatory and pro-angiogenic molecules, including Angiopoietin-2 (Ang-2) and Osteoprotegerin (OPG).[26, 27] To further investigate the hypothesis that sustained EC activation may be associated with ongoing exocytosis of WPB constituents in convalescent COVID-19, we next investigated Ang-2 and OPG levels. Overall, plasma Ang-2 levels were non-significantly increased in the convalescent cohort compared to healthy controls (median 1009pg/ml versus 859pg/ml, p=0.23) **(Fig. 2A).** However, significant inter-patient variation was observed, with plasma Ang-2 levels above the local normal range observed in 22% (11/50) of convalescent COVID-19 patients (**Fig. 2A)**. Similarly, marked inter-individual variability in plasma OPG levels was also seen in the convalescent COVID-19 cohort (**Fig. 2B**). Overall, there was a non-significant increase in convalescent OPG levels compared to controls (median 835pg/ml versus 818pg/ml, p=0.9; **Fig. 2B**). However, 14/50 (28%) of convalescent COVID-19 patients had plasma OPG levels above the upper limit of the normal healthy controls. Consistent with the concept of ongoing endotheliopathy and WPB exocytosis in patients with convalescent COVID-19, significant correlations were observed between OPG, VWFpp, FVIII and Ang-2 were observed (**Figs. 2C-2E**).

In an autopsy study of fatal acute COVID-19, Ackermann *et al* reported significantly increased new vessel growth, predominantly through a mechanism of intussusceptive angiogenesis in the pulmonary vasculature.[11] While data specifically on intussusceptive angiogenesis in acute COVID-19 is limited, a larger study of 144 autopsy samples from fatal COVID-19 similarly identified abnormal angiogenesis in multiple organ sites, with 139 angiogenesis-related proteins being significantly dysregulated compared with 74 control tissues from non-COVID patients.[28] Subsequent studies have found circulating pro-angiogenic factors (including VEGF-A, PDGF-AA

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and PDGF-AB/BB) were significantly elevated in acute COVID-19 and correlated with disease severity.[29] Since VWF, Ang-2 and OPG can influence EC angiogenesis, we next performed a pilot study to assess a panel of 24 angiogenesis-related proteins in a subset (n=9) of our convalescent COVID-19 patients and healthy controls (n=3). Significant inter-patient heterogeneity was again seen (**Fig. 2F**). Overall, significantly ($p<0.05$) enhanced expression of 21 angiogenesis-related proteins was observed in the convalescent COVID-19 subgroup compared to controls (**Fig. 2F**). In keeping with previous studies in acute COVID-19, elevated pro-angiogenic markers included Ang-2, as well as members of the vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), placental growth factor (PGF) and fibroblast growth factor (FGF) families.[17, 29] Conversely, plasma levels of Endostatin and Serpin F1 (both inhibitors of angiogenesis) were significantly reduced in convalescent COVID-19 (**Fig. 2F**)**.** These preliminary data can serve as a platform for future larger studies exploring the concept that dysregulated angiogenesis is not only a feature of acute COVID-19, but may also be sustained during convalescence in a subset of patients.

The molecular mechanisms responsible for persistent hemostatic abnormalities in convalescent COVID-19 have not been defined. However, recent studies have described persistent immune profile abnormalities in convalescent COVID-19 patients, including abnormal monocyte profiles and T cell activation.[22] We hypothesized that sustained inflammatory cell abnormalities may be important in triggering ongoing thrombin generation and/or endotheliopathy. Consistent with previous reports, immunophenotyping revealed increased HLA-DR+CD14+CD16+ intermediate monocytes in convalescent (n=37) and acute COVID-19 patients (n=32) compared with controls (n=20) **(Fig. 3A)**, whereas classical and non-classical monocyte profiles were unchanged in convalescence **(Supp. Fig. 1B-C)**. We and others have previously reported sustained increases in endogenous thrombin potential (ETP) and peak thrombin generation in convalescent COVID-19 patients compared to controls.[6] Interestingly, intermediate monocyte percentage correlated significantly ($p=0.007$) with ETP and peak thrombin ($p=0.02$) in our patient cohort **(Figs. 3B-C; Supplementary Table 1)**. This is noteworthy as TF induction on intermediate monocytes has been shown to contribute to platelet activation in acute COVID-19.[30] Finally, similar to previous studies, we observed significant reductions in naïve CD4+ and CD8+ T cell subsets in both convalescent (n=37) and acute COVID-19 patients (n=32) compared to controls (n=20) **(Figs. 3D-3E)** with concomitant elevations in activated CD4+ and CD8+ T cell subsets **(Figs. 3F-3G)**. Furthermore, activated CD4+ and CD8+ T cells correlated positively with EC and WPB parameters, including plasma soluble thrombomodulin (sTM), OPG and VWF:Ag levels **(Figs. 3H-3J)** whereas naïve CD4+ and CD8+ T cells correlated inversely with these markers

(Supplementary Table 2). Importantly, no significant associations were noted between (i) intermediate monocytes and EC activation markers or (ii) T cell activation subsets and thrombin generation parameters **(Supplementary Tables 1-2)**.

In conclusion, our data provide novel insights into the nature of sustained EC activation, WPB exocytosis and VWF/ADAMTS13 axis imbalance in convalescent COVID-19. In keeping with the pivotal role of immuno-thrombosis in acute COVID-19, our findings support the hypothesis that abnormal T cell and monocyte profiles may be important in the context of persistent EC activation and ongoing hemostatic dysfunction during convalescence. Further studies will be required to determine how this prolonged EC and immune activation, together with dysregulated angiogenesis in convalescent patients may contribute to functional impairment post-COVID-19.

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LEGENDS

Table 1: Demographic and laboratory parameters of Acute and Convalescent COVID-19 cohorts

Figure 1

Comparisons are shown between acute COVID-19 patients (n=36), convalescent COVID-19 patients (n=50) and healthy controls (n=20) including: **(A)** plasma ADAMTS13:Ag levels, (**B)** plasma VWF/ADAMTS13 ratios, **(C)** plasma Platelet Factor 4 (PF4) levels and **(D)** plasma interleukin-6 (IL-6) levels. Dotted red lines denote the lower limit of the local reference range for plasma ADAMTS13:Ag and IL-6 levels and the upper limit of the local reference range for the VWF/ADAMTS13 ratio and PF4 levels. Data are presented as median and the interquartile range. Comparisons between groups were assessed using the Kruskal-Wallis test. (ns = not significant, **p<0.01,***p<0.001, ****p<0.0001).

Figure 2

Comparisons are shown between acute COVID-19 patients (n=36), convalescent COVID-19 patients (n=50) and healthy controls (n=20) including: **(A)** plasma Angiopoietin-2 levels and **(B)** plasma Osteoprotegerin (OPG) levels. Dotted red lines denote the upper limit of the local reference range for plasma Ang-2 and OPG. Data are presented as median and the interquartile range. Comparisons between groups were assessed using the Kruskal-Wallis test. Correlations are shown between plasma levels of OPG and EC activation parameters including: **(C)** VWF propeptide (VWFpp), **(D)** Factor VIII:C and **(E)** Angiopoietin-2. Correlations were evaluated using the Spearman rank correlation test. **(F)** Heatmap visualization indicating angiogenesis-related protein expression detected in each subject (columns) for each protein (rows), comparing a subset of convalescent COVID-19 patients (n=9, 6/9 males) and healthy controls (n=3, 2/3 males). This subset of convalescent patients and controls were selected to match the age and gender profile of the overall convalescent cohort. Protein levels were measured via membrane-based antibody array and data are represented by mean pixel intensity with red indicating higher and green indicating lower levels of the protein of interest. Comparisons between groups were assessed using the Mann-Whitney U test. (ns = not significant, ***p<0.001, ****p<0.0001).

Figure 3

(A) Intermediate monocytes in peripheral blood were assessed using flow cytometry in convalescent COVID-19 patients (n=37), acute COVID-19 patients (n=32) and healthy controls

(n=20). Correlations between intermediate monocytes and **(B)** Endogenous Thrombin Potential (ETP) and **(C)** Peak thrombin generation respectively. T lymphocyte subsets in peripheral blood were assessed using flow cytometry in convalescent COVID-19 patients (n=37), acute COVID-19 patients (n=32) and healthy controls (n=20) including percentage of **(D)** Naïve CD4, **(E)** Naïve CD8, **(F)** Activated CD4 and **(G)** Activated CD8 positive T cells. Correlations are shown between activated CD8+ T cells and plasma levels of **(H)** soluble thrombomodulin (sTM), **(I)** OPG and **(J)** VWF:Ag. Data are presented as median and the interquartile range. Comparisons between groups were assessed by the Mann-Whitney U test. Correlations were evaluated using the Spearman rank correlation test. (ns=not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

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Author Contributions

Contribution: HF, SEW, LT, EK, SE, NC, CB, CNC, JOS & JOD - conception, patient enrollment, data collection and interpretation. All authors contributed to literature review, final draft writing and critical revision. All the authors have participated sufficiently in this work, take public responsibility for the content and have made substantial contributions to this research

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Conflict of Interest

J.S.O'D has served on the speaker's bureau for Baxter, Bayer, Novo Nordisk, Sobi, Boehringer Ingelheim, Leo Pharma, Takeda and Octapharma. He has also served on the advisory boards of Baxter, Sobi, Bayer, Octapharma CSL Behring, Daiichi Sankyo, Boehringer Ingelheim, Takeda and Pfizer. J.S.O.D has also received research grant funding awards from 3M, Baxter, Bayer, Pfizer, Shire, Takeda, 3M and Novo Nordisk. The remaining authors have no conflict-of-interests to declare.

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Appendix

Additional investigators of the Irish COVID-19 Vasculopathy Study (ICVS):
Niamh O'Connell (National Coagulation Centre, St James's Hospital, Dublin), Kevin Ryan (National Coagulation Centre, St James's Hospital, Dublin), Dermot Kenny (Irish Centre for Vascular Biology, Royal College of Surgeons in Ireland), Judicael Fazavana (Irish Centre for Vascular Biology, Royal College of Surgeons in Ireland).

Table 1

Parameters	Normal range	Convalescent COVID-19 (n=50)	Acute COVID-19 (n=36)
Demographic data			
Age, median (IQR)		50 (27)	62 (16)
Male – n (%)		30 (60)	27 (75)
BMI, median (IQR)		28 (7)	30 (9)
Comorbidity count, median (IQR)		1 (3)	3 (2)
Hospitalization – n (%)		37 (74)	36 (100)
ICU – n (%)		8 (16)	18 (50)
Laboratory parameters at time of blood sampling – median (IQR)			
Leukocytes (x10^9/L)	4 – 11	6.3 (1.7)	7.8 (5.3)
Lymphocytes (x10^9/L)	1.5 – 3.5	1.9 (0.7)	1.1 (0.7)
Neutrophils (x10^9/L)	2 – 7.5	3.3 (1.2)	5.9 (5.4)
Platelets (x10^9/L)	140 – 450	272 (60)	276 (145)
Creatinine (µmol/L)	45 – 84	73 (26)	72 (34)
Fibrinogen (g/L)	1.9 – 3.5	2.9 (0.8)	4.8 (2.8)
D-dimer (ng/mL)	0 – 500	377 (251)	980 (1619)
CRP (mg/mL)	0 – 5	1.1 (1)	110 (173)

IL-6 (pg/mL)	0 – 7.26	1.5 (3.6)	76.6 (144.4)
Clinical parameters Convalescent COVID-19 only – median (IQR) unless otherwise stated			
Time to follow-up (days)		68 (11)	NA
6 MWT distance (m)	400 – 700	430 (160)	NA
Lowest desaturation (%)		95 (2)	NA
Abnormal Chest X-ray – n (%)		3 (6)	NA

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Figure 1

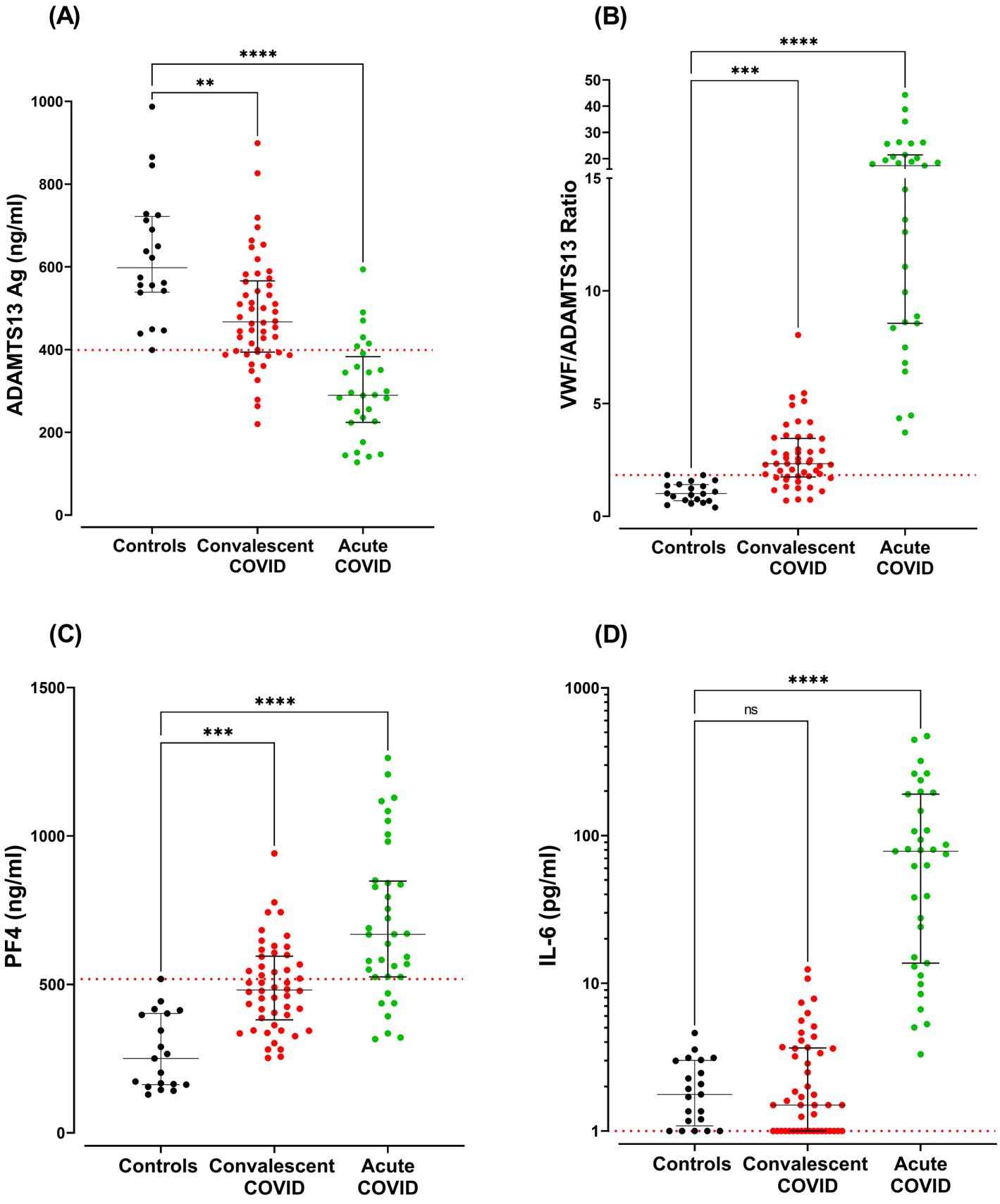

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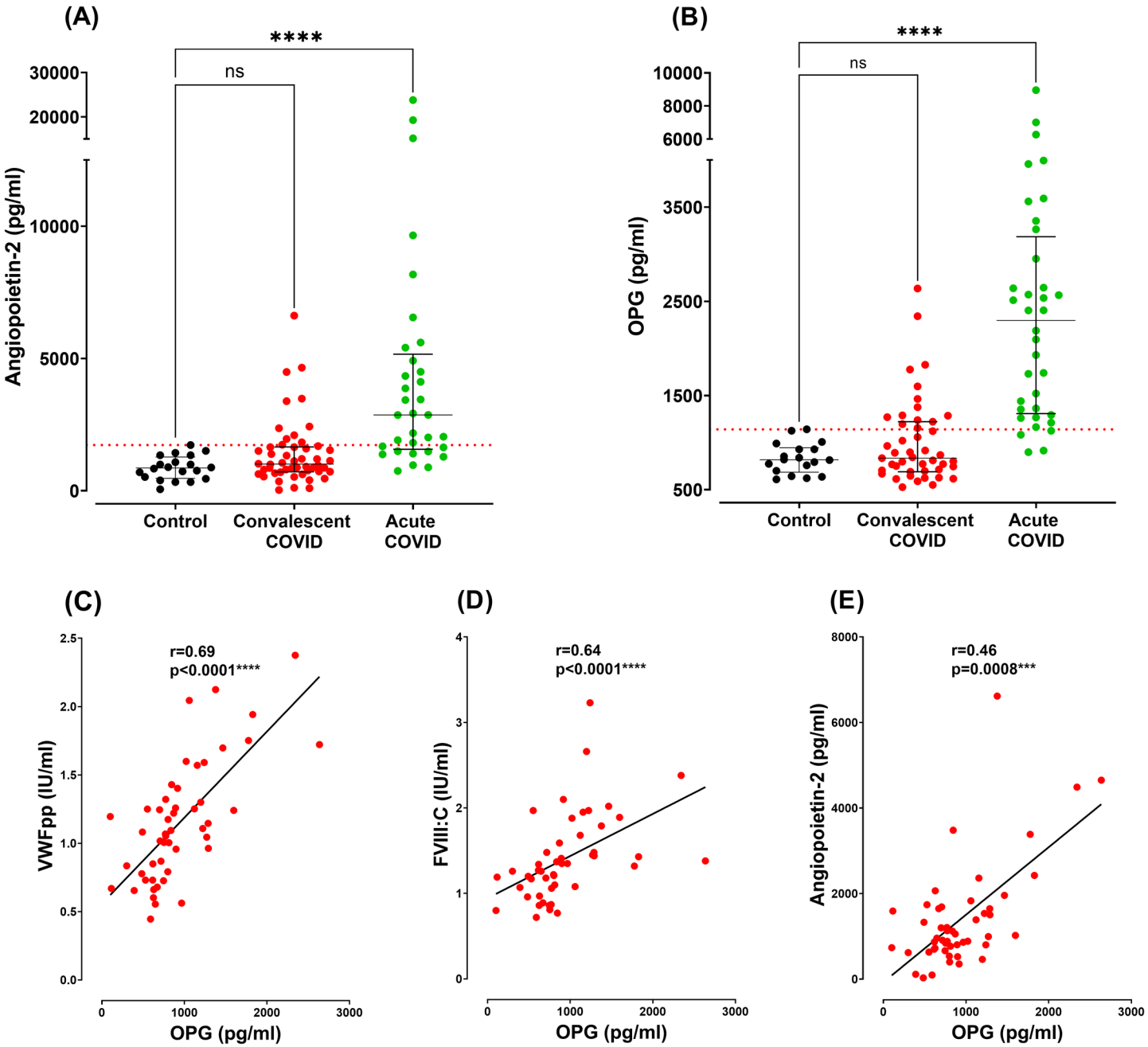
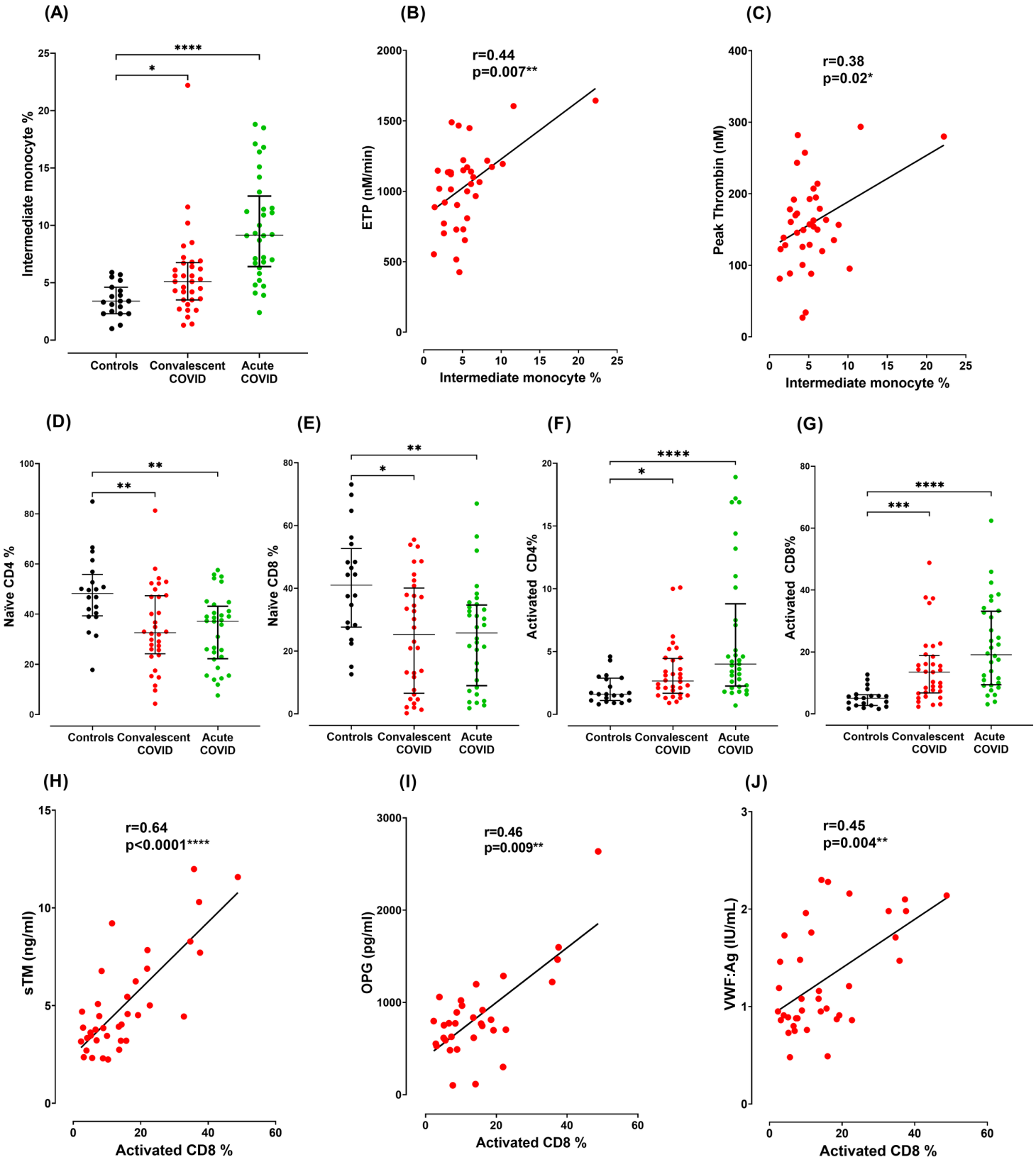
Figure 2



(F)

Expression Increased	Control	Convalescent COVID
Angiopoietin-2		
LAP (TGF-beta1)		
Leptin		
MMP-8		
Pentraxin 3 (PTX3)		
PD-ECGF		
PDGF-AA		
PDGF-AB/PDGF-BB		
PlGF		
TIMP-4		
VEGF		
VEGF-C		
Activin A		
Angiostatin/Plasminogen		
Amphiregulin		
Artemin		
EG-VEGF		
FGF acidic		
FGF basic		
FGF-4		
FGF-7		
Expression Decreased	**Control**	**Convalescent COVID**
Endostatin/Collagen XVIII		
IGFBP-3		
Serpin F1		

Figure 3



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