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Full Length Article

Early but reversible haemostatic changes in a-symptomatic females expressing COVID-19 antibodies

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ABSTRACT

The coronavirus, COVID-19 pandemic spread across the globe in 2020, with an initial high case mortality in those requiring intensive care treatment due to serious complication. A vaccine programme was quickly developed and currently the UK is one of highest double vaccinated and boosted countries in the world. Despite tremendous efforts by the UK, new cases of COVID-19 are still occurring, due to viral mutation. A major problem associated with COVID-19 is the large a-symptomatic spread within the population.

Little investigation into the a-symptomatic population has been carried out and therefore we pose that the residual effects of a-symptomatic infection is still largely unknown. Prior to mass vaccination, a multi-phased single cohort study of IgM and IgG COVID-19 antibody prevalence and the associated haemostatic changes were assessed in a Welsh cohort of 739 participants, at three time points. Positive antibody participants with age and gender matched negative antibody controls were assessed at 0, 3 and 6 months. Antibody positive females appeared to have lower antibody responses in comparison to their a-symptomatic male counterparts. Despite this initial testing showed a unique significant increase in TRAP-6-induced platelet aggregation, prothrombin time (PT) and clot initiation time. Despite coagulation parameters beginning to return to normal at 3 months, significant decreases are observed in both haemoglobin and haematocrit levels. The production of extracellular vesicles (EV) was also determined in this study. Although the overall number of EV does not change throughout the study, at the initial 0 months' time point a significant increase in the percentage of circulating pro-coagulant platelet derived EV is seen, which does not appear to be related to the extent of platelet activation in the subject.

We conclude that early, but reversible changes in haemostatic pathways within the a-symptomatic, female, antibody positive COVID-19 individuals are present. These changes may be key in identifying a period of pro-coagulative risk for a-symptomatic female patients.

1. Introduction

COVID-19 is a novel coronavirus with a high infection rate, first identified in Wuhan, China in December 2019. The World Health Organization declared a pandemic and an international public health emergency on the 11th March 2020 [1]. COVID-19 is caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) and in severe cases can lead to pneumonia-like symptoms requiring hospitalisation [2]. Despite this, most cases are asymptomatic or linked with mild flu-like symptoms [4]. Management of COVID-19 presents a major healthcare challenge due to the potential for a large proportion of a-symptomatic cases spreading the highly infectious virus [3]. During the early phases, this was potentiated by focussing viral antigen testing by high sensitivity PCR to symptomatic subjects. Whilst this approach focussed the efforts of an overstretched health service and limited testing capacity, it resulted in the probable spread through the asymptomatic population and a serious incline in hospitalisation of patients with underlying co-morbidities and in the elderly. In response to the fast, wide spreading of COVID-19, the health research professionals in the UK moved quickly to develop an effective and efficient globally accepted vaccination programme. The research presented herein was carried out prior to vaccine mass roll out and is still imperative to consider in those who are not vaccinated and are still becoming infected with COVID-19, and perhaps in those becoming re-infected with COVID variants.

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Early observations pointed to widespread occurrence of coagulopathy in critically ill patients infected with COVID-19 with data suggesting that up to 49 % admitted to intensive care units (ICU) have an incidence of thrombotic complications [5]. This led to the international society of thrombosis and haemostasis releasing guidelines on COVID-19-associated coagulopathy (CAC) [6]. Common CAC laboratory findings, reported in 71.4 % of non-survivors [7] include a mild prolongation of the PT [7,8], increased D-Dimer (DD) [2,7,8] thrombocytopenia [9] and disseminated intravascular coagulation (DIC). CAC clinical and laboratory findings overlap with other coagulopathies including sepsis-induced coagulopathy and thrombotic microangiopathy, although, no exact equivalence to any [10]. Meta-analysis of multiple CAC studies revealed PT and DD directly correlates with disease severity, showing significantly higher levels in ICU patients but with no difference in platelet number and activated partial thromboplastin time (aPTT) [11]. A recent study investigating CAC in severe COVID-19 patients compared to patients with non-COVID-19 acute respiratory distress, reported significantly increased levels of pro-coagulation factors V, VIII and plasminogen activator inhibitor, with COVID-19 patients exhibiting higher clot strength values and raised C-reactive protein (CRP), supporting the view that systemic inflammation is the major contributor to CAC [12]. CAC is therefore a serious consequence of COVID-19, with unique characteristics when compared to similar diseases and coagulopathies.

EVs are nanosized, phospholipid membrane-bound vesicles (exosomes, microvesicles and apoptotic bodies) [13]. EV subsets have been well characterised and are broadly split dependent on mechanisms of production and release from the parent cell. EVs are crucial in transporting cell cargo, including RNA, DNA, lipids and proteins, whilst also playing an important role in intracellular communication [14]. EVs are largely representative of their parent cell, expressing traceable protein markers [15], allowing specific EV population isolation from a mixed plasma EV sample. EVs have been investigated within the coagulation cascade, primarily due to their largely negatively charged phospholipid membranes. Interaction of EVs with factors of the coagulation cascade, ultimately, can lead to increased activation of both the intrinsic [16,17] and extrinsic [18,19] arms. Recent studies have evaluated the role EVs could play in treatment of COVID-19 [20,21] as well as the transfer of viral particles to non-infected cells [22]. One study has looked at the role of EV and their surface antigen profile in severe COVID-19, suggesting a link between severe COVID-19 and CD142 surface expression [23].

To date, studies have primarily focussed on CAC in hospitalised and ICU patients. Whether similar effects are prevalent in the a-symptomatic population exposed to COVID-19 or those who have suffered with mild symptoms not requiring hospitalisation, is unknown. Given the presence of a growing population who are largely asymptomatic and the potential emergence of longer-term health effects, impacting haemostasis caused by exposure to COVID-19 [24], an underlying risk in the asymptomatic population exposed to COVID-19 is of great concern.

This study aimed to determine impact of COVID-19 exposure on markers of coagulation in an asymptomatic population, in which antibody prevalence (used as an index of exposure to COVID-19) was 3.65 % during the early phase of the pandemic [25]. A range of haematological tests were applied to evaluate CAC and the residual risk of thrombosis up to 6 months post COVID-19 exposure.

2. Methods

2.1. Study design and participants

All full-time Cardiff Metropolitan University staff were invited in July 2020 to participate in a multi-phased antibody-screening programme (ethics ID; Sta-2860). The cohort and screening methodology for SARS-CoV-2 IgM and IgG antibodies has been described in detail in our group's previous publication [25]. Briefly, capillary blood samples obtained from a finger prick were collected from 739 participants and applied to the FDA approved lateral flow immunoassay according to the manufacturer standardised operating procedure (Confirm Bio-Sciences®). Participants testing positive for IgM and/or IgG antibodies and a matched (age, gender) number of negative individuals were asked to provide a venous blood sample. Prevalence of IgG was independently confirmed by an immunoassay ran on serum samples (Abbott®) and specificity of the lateral flow screening was found to be 96 % with sensitivity of 95 %. These participants were invited for a re-test, 3 and 6 months later. The cohort was asymptomatic with no antibody-positive participants displaying severe symptoms with no hospitalisations recorded.

All participants completed a thorough health questionnaire to evaluate underlying health and cardiovascular risks, COVID-19 exposure, and other general health information.

2.2. Blood collection

Venous blood was collected from ante-cubital vein aseptically via a butterfly 20-gauge needle into vacutainers, depending on the assay; EDTA; Na-citrate; Serum SST™ (all Becton Dickinson) and Hirudin (Roche Diagnostics). Serum SST™, Na-citrate and EDTA were utilised for haematological analysis. Na-citrate was also used for EV quantification and soluble P-selectin (CD62P) analysis. One EDTA was used for flow cytometric analysis. Hirudin was used for platelet aggregation analysis. The first 5 mL of blood before sampling was discarded to avoid excessive platelet activation.

2.3. Platelet aggregation

Platelet aggregation was assessed via impedance aggregometry using a Multiplate analyser (Roche Diagnostics), explained in detail in previous studies [26]. Briefly, whole blood (300 μL) collected in a hirudin vacutainer was diluted 1:1 with saline and incubated at 37 °C for 3 min. Following incubation, platelets were stimulated by the addition of ADP (adenosine-5′-diphosphate; final concentration 6.5 μM), or TRAP-6 (thrombin receptor activating peptide-6; final concentration 32 μM). The increase in impedance is transformed to arbitrary units whereby aggregation is measured as the area under the curve (AUC) and expressed in units (U). Analysis was performed within 30 min of blood collection.

2.4. Routine coagulation evaluation

Blood samples were analysed at the University Hospital Wales haematology and biochemistry laboratories. Standard clinical coagulation testing included PT time (data was collected but is not shown for aPTT; fibrinogen levels; DD; and CRP). All samples were tested within 4 h of bleeding. 3 month and 6 month re-testing included all the above testing plus a full blood count (FBC) on an EDTA sample.

2.5. Turbidimetric clotting and lysis assay

Fresh frozen plasma (25 μL) was added to assay buffer (0.05 mol/L Tris-HCl, 0.1 mol/L NaCl, pH 7.4) (65 μL) in a 96 well-plate. 12.5 ng of tissue plasminogen activator (Technoclone, Austria) was added to the assay buffer (83 ng/mL final concentration) before adding the activation mix (final concentrations: 0.03 U/mL thrombin (Merck, Germany) and 7.5 mmol/L calcium (30 μL)). Fluorescence was calculated every 12 s at 340 nm for 1 h. The reaction caused fibrin clot formation and complete lysis within an hour.

2.6. Extracellular vesicle isolation

Na-citrated blood was subjected to an 800 ×*g* centrifugation to isolate platelet poor plasma for 20 min before a 2000 ×*g* spin for 20 min twice to render the plasma acellular. Acellular plasma (500 mL) was

applied to a size exclusion chromatography column (Beckman Coulter ®), where fractions 5–10 were collected based on extensive work carried out previously by the research group [15]. Use of size exclusion chromatography fulfils the requirements and guidelines for extracellular vesicle isolation set by the international society of extracellular vesicles [27].

2.7. Nanoparticle tracking analysis

Concentration and size distribution of EVs were determined using nanoparticle tracking analysis (NTA), based on tracking movement of EVs illuminated by a 405 nm laser source as previously described [15]. Briefly, EV samples were diluted in PBS to range from 10^6 to 10^9 particles per mL (p/mL), prior to being measured and NTA software used to analyse the results.

2.8. Flow cytometry

EV characterisation was performed on EV isolates, used a CD9 positive section method paired with a violet side scatter size gate to confirm EVs (gating strategy is described in Supplementary information). EVs (80 μL) were labelled with Phycoerythrin (PE)-conjugated anti-CD9 (20 μL). EV origin markers (5 μL) were either allophycocyanin (APC)-conjugated anti-CD41 (platelet), APC-conjugated anti-CD144 (endothelial), APC-conjugated anti-CD235a (red blood cell) or APC-conjugated anti-CD11b (WBC). These were incubated for 20 min in the dark and diluted using PBS up to 250 μL. 20,000 events or total sample volume was collected and analysed on a Cytoflex Cytometer (Beckman Coulter) for dual % expression.

For CD62P expression on resting platelets (3 & 6 months only), EDTA anti-coagulated whole blood was centrifuged at 120 ×*g* for 20 min. After a resting period of 30 min, platelet-rich plasma was diluted 1:10 in Dulbecco's phosphate buffered saline and labelled with APC-conjugated anti-CD41 and fluorescein isothiocyanate (FITC)-conjugated anti-CD62P (BD Biosciences) for 30 min at room temperature. Isotype-matched controls were used to monitor non-specific binding with each sample. Forward and side scattering characteristics and binding of CD41 distinguished platelets, allowing a retrospective gate to be set (gating strategy described in Supplementary information). 20,000 platelet-gated events were collected and analysed on a Cytoflex flow cytometer (Beckman Coulter) for CD41/CD62 expression (%).

2.9. Soluble P-selectin analysis

Plasma concentration of soluble CD62P (sCD62P) was assessed using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Quantikine; R&D Systems) according to the manufacturer's instructions.

2.10. Statistical analysis

Statistical analysis was performed with Graphpad Prism (version 5.0, GraphPad Software). Prior to analysis, a Shapiro Wilks test was used to determine if data was normally distributed. A *t*-test was used to compare antibody positive and negative participants. An ANOVA with a Bonferroni post hoc was used to compare antibody negative participants versus antibody positive participants over the three time points (0, 3 & 6 months respectively). $p \leq 0.05$ was considered statistically significant. All graphs represent mean ± SEM. N numbers differ for each analysis and can be found in the appropriate figure legends, antibody negative N are reported first followed by, antibody positive N, respectively throughout.

3. Results

The results collected over the three testing time points (0, 3, 6 months) are detailed below. 0 months refers to the original time of testing; the first antibody test.

3.1. Cohort characteristics

The average age of positive participants was 44.5 years vs 42.91 years for negative participants. Of those participants who tested positive for COVID-19 antibodies, 34.6 % were aged 40 and under. The highest prevalence of COVID-19 antibodies was observed in males aged between 61 and 70 (6.67 % prevalence). Individuals who tested positive for COVID-19 antibodies and a matched set of antibody negative gender controls (total N = 64) participated in detailed laboratory haematological assessment at the point of initial lateral flow test. Lateral flow antibody tests and blood samples analysis was repeated at both 3 (67.19 % of 0 months) and 6 months (83.72 % of 3 months). General characteristics, underlying conditions and medications are summarised in Table 1. Thorough medical history was collected for all participants, and conditions declared by participants are outlined in Table 1.

Of those who originally tested negative 96.43 % remained negative and 3.57 % (1 participant) showed new antibody positivity at the 3-month retest therefore was excluded from further analysis. Of those who originally tested positive 78.26 % remained positive, however 21.74 % had a negative antibody result (data not shown). Of those whom sero-converted from antibody positive to antibody negative 80 % were female.

The results presented herein focus on the haemostatic changes observed in the female cohort. No significant changes were observed within the male population.

3.2. Functional platelet changes in antibody positive females

Despite demonstrating lower antibody titre against COVID-19, positive female participants had a significantly increased TRAP-6 aggregation compared to antibody negative females (106.5 ± 5.15 U vs 124.4 ± 6.29 U, p = 0.045, Fig. 1A). There was no difference in ADP response of platelets, between antibody negative and positives or over time (Supplementary Fig. 4). This is coupled with a significant (11.31 ± 0.12 s vs 11.84 ± 0.19 s, p = 0.02, Fig. 1B) increase in PT time in antibody positive females in comparison with antibody negative females. Follow up studies after 3 months show that PT time is reducing towards that of the antibody negative population (11.27 ± 0.12 s vs 11.70 ± 0.24 s, p = 0.09, Fig. 1D) and is comparable by 6 months post original sampling point (Fig. 1F). TRAP-6 aggregation returns to normal at 3 months (Fig. 1C) post sampling and this is maintained at the 6-month point (Fig. 1E).

3.3. Haematological changes in antibody positive females

Although both TRAP-6 aggregation and PT time are returning to normal at the 3 months follow up point antibody positive females exhibited a significantly decreased haematocrit (0.3957 L/L ± 0.01172 vs 0.423 L/L ± 0.006068, p = 0.0309, Fig. 2A) and haemoglobin (132.6 $(\times 10^9)$/L ± 4.347 vs 142.2 $(\times 10^9)$/L ± 2.132, p = 0.0359, Fig. 2B) compared to antibody negative females. At 3 months, there was no measured difference in mean corpuscular haemoglobin (MCH) (Fig. 2C). Both haematocrit and haemoglobin measurements become comparable with the negative antibody population at the 6 months retest (Fig. 2D, E). At 6 months a significant increase in MCH (29.98 pg ± 0.32 v 30.86 pg ± 0.27 p = 0.05, Fig. 2F) is observed in antibody positive females. All other measures from full blood count, including mean corpuscular volume, red cell distribution width and platelet number did not change between antibody negative and positives, throughout this study (data not shown). Levels of transferrin were unaltered, ferritin levels increased in phase 3 females when compared to antibody negative participants (Supplementary data Table 1).

Table 1
Cohort characteristics. Descriptive characteristics of all participants who underwent a venous blood sample at 0-months, following the initial LFT.

Cohort characteristics					
	Whole population	Positive males	Negative males	Positive females	Negative females
Number of participants	64	15	17	13	19
0 months		23.44 %	26.56 %	20.31 %	29.69 %
Number of participants	43	11	11	7	14
3 months	67.19 %	25.58 %	25.58 %	16.28 %	35.56 %
Number of participants	36	9	11	7	9
6 months	83.72 %	25 %	30.5 %	19.4 %	25 %
Mean age (years)	43.45	45.79	43.52	43.21	42.3
Number of asthmatics	13	4	2	4	3
	20.31 %	30.77 %	15.38 %	30.77 %	23.08 %
Number of Type 1 diabetics	2	0	1	1	0
	3.125 %		50 %	50 %	
Number of hypertensive	2	1	0	1	0
	3.125 %	50 %		50 %	
Number of Type 2 diabetics	1	0	1	0	0
Number of atrial fibrillation and cardiomyopathy	1	1	0	0	0
Number of Chron's disease	1	0	1	0	0
Number of hypothyroidism	1	0	0	0	1
Number of chronic migraine	1	0	0	1	0
No reported co-morbidity	42	9	13	6	14
	65.63 %	21.43 %	30.95 %	14.29 %	33.33 %

Medication review					
	Whole population	Positive males	Negative males	Positive females	Negative females
Ventolin	9	4	1	2	2
	14.06 %	44.4 %	11.1 %	22.2 %	22.2 %
Contraceptive pill	3	0	0	1	2
	4.69 %			33.3 %	66.6 %
Fostair	2	1	0	1	0
	3.125 %	50 %		50 %	
Omeprazole	2	0	1	1	0
	3.125 %		50 %	50 %	
Gabapentin	2	0	1	1	0
	3.125 %		50 %	50 %	
Metformin	1	0	0	1	0
Losartan potassium	1	0	0	1	0
Bendroflumethiazide	1	0	0	1	0
Flecainide	1	1	0	0	0
Insulin	1	0	1	0	0
Cimetidine	1	0	1	0	0
Mebeverine	1	0	1	0	0
Levothyroxine	1	0	0	0	1
Clenil	1	1	0	0	0
Oromorph	1	0	1	0	0
Dihydrocodeine	1	0	1	0	0
Sertraline	1	1	0	0	0
Amitriptyline	1	0	0	1	0
Propanolol	1	0	0	1	0
Citalopram	1	0	0	0	1
Fluoxetine	1	0	0	1	0
Flixotide	1	0	1	0	0
No reported regular medication	41	9	11	5	16
	64.06 %	21.95 %	26.83 %	12.20 %	39.02 %

3.4. Turbidimetric clotting and lysis assay

The time taken for a blood clot to be initiated in response to thrombin in an ex vivo assay was significantly increased following the initial positive antibody test when compared with antibody negative females (212.3 s ± 5.65 v 238.3 s ± 10.72, p = 0.025, Fig. 3A). Despite the clot formation being delayed, the maximum clot size (Fig. 3B) and the overall time to reach this point (Fig. 3C) was not significantly affected by a positive COVID-19 antibody test. Similarly, the 50 % lysis time was also unaffected (Fig. 3D). As shown within the PT time the change observed in clot initiation time returned to a normal level following both 3 and 6 months (Fig. 3E, I). A significantly increased time to form the maximum clot size was seen at 3 months in antibody positive females (9.88 min ± 0.13 v 11.27 min ± 0.83, P = 0.029, Fig. 3G). No further significant changes were seen at 6 months post initial testing (Fig. 3J–L).

3.5. Extracellular vesicle quantification

Extracellular vesicles were separated from plasma samples using size exclusion chromatography. Both the number of EV and the mean size of EV were calculated in antibody negative and antibody positive female participants and were compared at the 3 time points across this study. There were no reported significant changes in EV number or size in comparison with the negative population (Fig. 4A, negative vs 0 months, p = 0.2487; negative vs 3 months, p = 0.9581; negative vs 6 months, p = 0.9891; 0 vs 3 months, p = 0.8133; 0 vs 6 months, p = 0.3742 and 3 vs 6 months, p = 0.9222). There was also no reported significance in EV size across the study (Fig. 4B, negative vs 0 months, p = 0.2379; negative

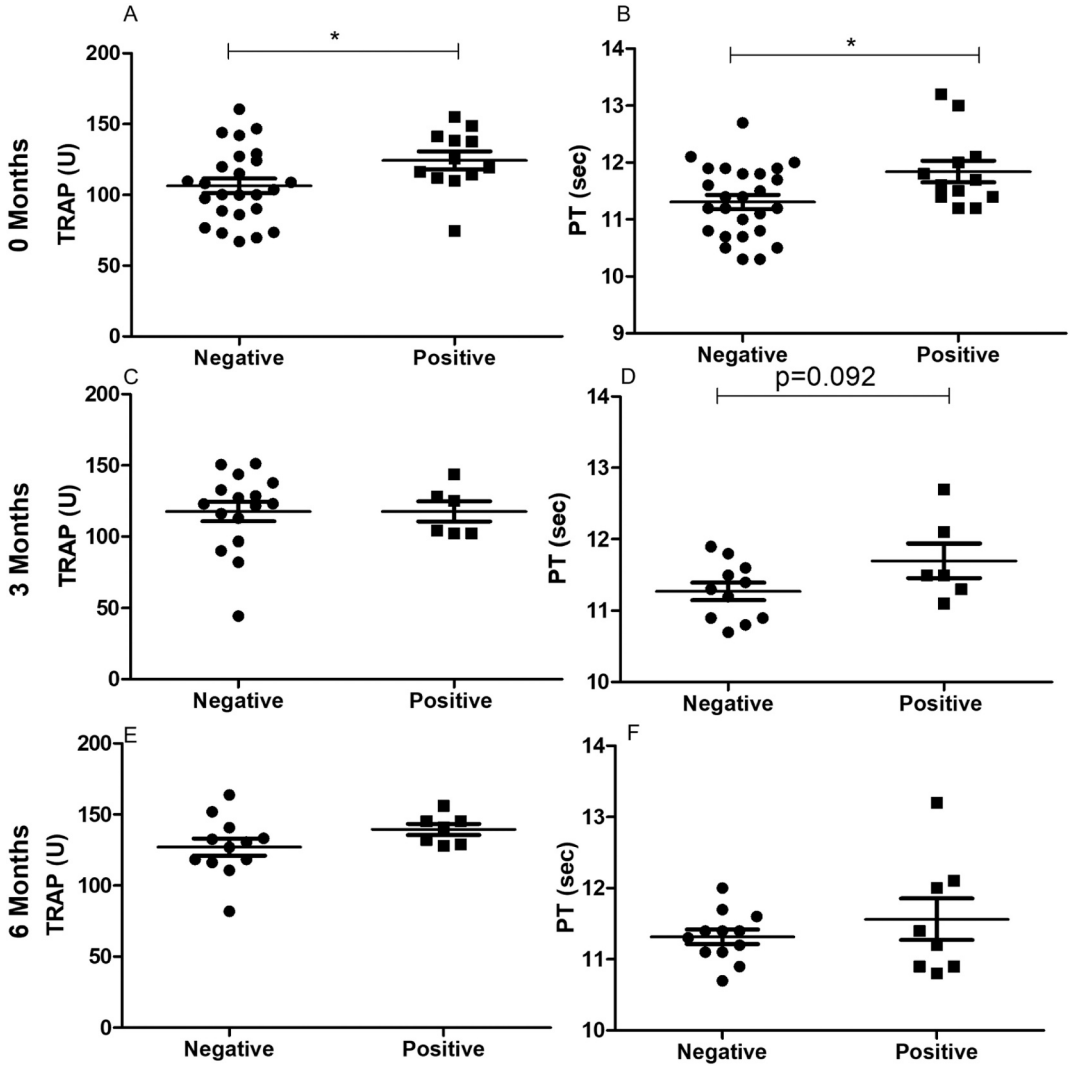

Fig. 1. Impedance aggregometry assessment of platelets. Functional platelet responsiveness to TRAP-6 as an agonist was measured at the initial sampling point (0 months N = 25, N = 12, A), 3 months (N = 16, N = 6, C) and 6 months (N = 12, N = 7, E) and was compared between female participants who had a negative COVID-19 antibody test and those who tested positive. Clinical PT time was measured in seconds at 0 months (N = 26, N = 12, B), 3 months (N = 11, N = 6, D) and 6 months (N = 12, N = 8, F). * = $p \leq 0.05$. Bar charts represent mean ± SEM.

vs 3 months, p = 0.5832; negative vs 6 months, p = 0.6691; 0 vs 3 months, p = 0.0870; 0 vs 6 months, p = 0.9824 and 3 vs 6 months, p = 0.2660).

3.6. Extracellular vesicle characterisation

Flow cytometry was used to identify the cellular origin of EV within blood, utilising a CD9+ selection method. Platelet EV (CD9+CD41+), endothelial EV (CD9+CD144+), red blood cell EV (CD9+CD235a+) and white blood cell EV (CD9+CD11b+) were identified. Percentage was assessed by using a fixed number of events to compare EV sub-type. A significant increase was seen between negative and positive female participants at the initial point of testing (0 months, Fig. 5A) in the production of platelet derived CD9+CD41+ EV (34.25 % ± 3.73 v 60.46 % ± 6.36, $p < 0.01$). The production of platelet EV returned to a normal level at both 3 and 6 months follow ups and were both significantly different from the initial reading (21.39 % ± 3.73 and 17.13 % ± 2.43 v 60.46 % ± 6.36, $p < 0.01$, $p < 0.001$ respectively). No difference was observed at any time point for endothelial (Fig. 5B), red blood cell (Fig. 5C) or white blood cell (Fig. 5D) derived EV.

3.7. Platelet activation

The activation state of platelets was determined by measuring the production of both soluble and membrane bound P-selectin on resting platelets. No difference was seen in the production of either soluble P-selectin in plasma samples (Fig. 6A, B, D), or membrane bound P-selectin in whole blood samples (Fig. 6C, E) at any time point between antibody positive and antibody negative female participants.

4. Discussion

This study adds to a growing body of evidence highlighting the impact of COVID-19 on the residual risk of thrombosis in recovered individuals. The temporal nature of CAC is seen as a substantive feature of infection by the virus [28] and to the best of our knowledge for the first time; we have assessed this risk within an asymptomatic population.

At the time of completion 3.65 % of participants were positive for COVID-19 antibodies. This level of antibody prevalence was lower than that reported by UK REACT study which showed 6 % antibody prevalence in July and 4.4 % in September 2020 [29]. Prevalence is also lower than suggested by UK Biobank (4.7 %) based on a similar sized Welsh cohort [30]. A potential reason for a lower incidence in this population

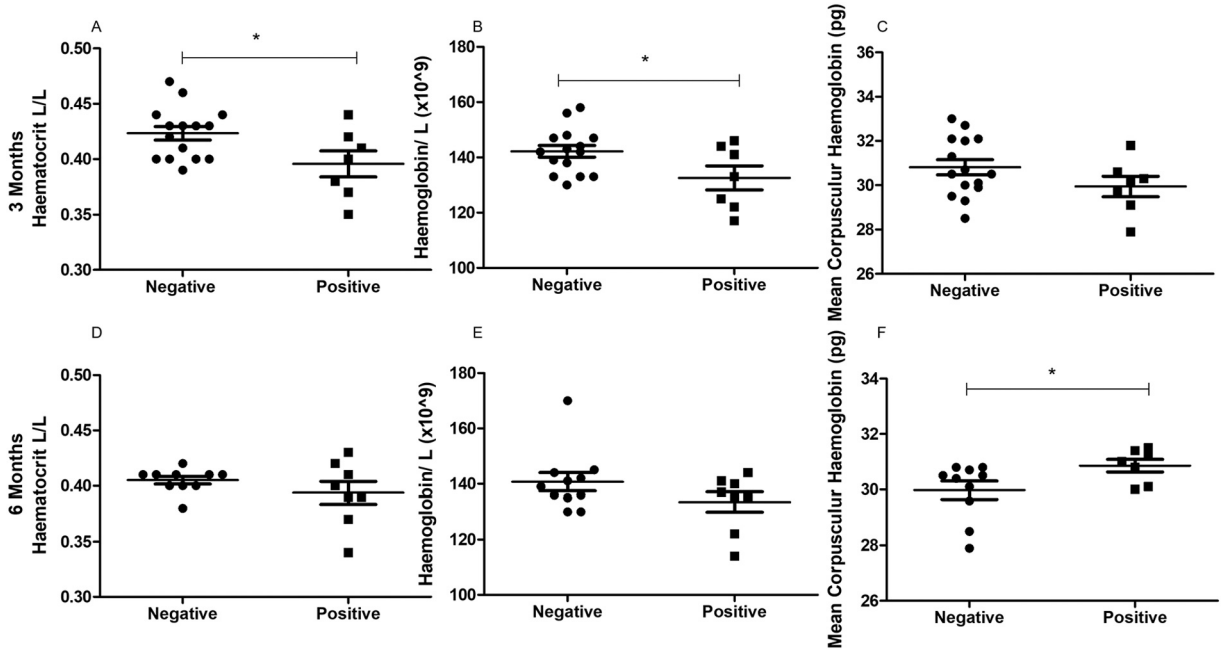

Fig. 2. Haematological indices. Haematological changes were measured in all female participants at both 3 and 6 months post initial COVID-19 antibody test. Specifically, haematocrit (N = 15, N = 7, A), (N = 10, N = 8, D), haemoglobin (N = 15, N = 7, B), (N = 11, N = 8, E) and mean corpuscular haemoglobin (N = 15, N = 7, C), (N = 10, N = 7, F) were assessed. * = $p \leq 0.05$. Bar charts represent mean ± SEM.

may be linked to a rapid enforcing by the university requiring and supporting home working from early March 2020, resulting in reduced risk of viral exposure. Despite mass vaccination and the expectation of widespread natural antibodies as we approach the middle of 2022, new COVID-19 cases are still emerging, in both the double vaccinated and boosted individuals and, importantly, in those who have previously suffered with COVID-19 infection.

Significant increases in TRAP-6-induced platelet aggregation is unique to antibody positive females and was not observed in response to other platelet agonists (Supplementary Fig. 4), suggesting hyper-reactivity of the protease-activated receptor-1. Platelets from hospitalised COVID-19 patients aggregate faster and have a lower threshold of activation to low dose agonists compared to healthy controls [31], suggesting a virus-induced sensitisation of platelets. In comparison, our results confirm female asymptomatic patients exhibit hyper-reactivity to thrombin only. It is hypothesised that following COVID-19 infection there is an alteration to the surface expression of the mobile transmembrane receptors that are found on the platelet membrane. Changes in these receptors, including thrombin receptors; PAR-1 and PAR-4 may explain the increased platelet responsiveness to TRAP-6. PAR-1/4 receptors have been abundantly recognised for their roles in the development of chronic inflammatory diseases. Further work is required to determine if PAR expression is the cause of TRAP-6 responsiveness [45].

Haematological differences in antibody positive females are further implied by an increased PT in antibody positives. Prolonged PT, but normal aPTT (as observed in our study) has also been seen in COVID-19 hospitalised patients [32]. These findings might indicate a defect in the extrinsic pathway or possible factor VII deficiency [33]. COVID-19 has been reported to cause endothelial dysfunction and alveoli damage [34] which in turn causes an increase in tissue factor (TF) release due to high expression of TF in alveolar cells [35]. In severe COVID-19, TF release and binding to FVII causes extrinsic pathway activation and in conjunction with an overwhelming inflammatory response and loss of anti-coagulation control leads to DIC. Our participants were asymptomatic, yet SARS-CoV-2's attachment to ACE receptors on endothelial cells may have modulated TF levels or circulating concentrations [36], reducing FVII, thus prolonging PT. To explore the prolonged PT time in greater depth we applied an in house turbidimetric clotting and lysis assay to assess the whole coagulation process. The assay allows analysis of four major components of clot formation and lysis: lag time; time to maximum clot formation; maximum clot size and resistant to lysis (50 % lysis time). The assay confirmed that the prolonged PT was caused by an increased lag time, suggesting a slower initiation of clot formation.

Our FBC results are consistent with numerous studies in COVID-19 patients that report no significant difference in platelet number, WBC, RBC and other haematological parameters [32], although a meta-analysis suggested thrombocytopenia is seen in ICU patients [37]. However, our data showed antibody positive females had a lower haematocrit and haemoglobin in phase 2 compared to antibody negative females. The participants in this study are not clinically anaemic, as defined by the relevant reference ranges for adult females [46] and the changes observed in haemoglobin remain within the normal clinical range, the findings reflect data to show lower haematological indices in both severe and non-severe COVID patients [38] with a meta-analysis confirming anaemia enhances the risk of COVID-19 due to the viral-haemoglobin interaction leading to changes to the β chain of haemoglobin and formation of methaemoglobin [39]. Low levels of folate cannot be excluded as the cause of increased MCH and low initial haematocrit. However, other indicators of such deficiency are not observed. The decrease in both haematocrit and haemoglobin appears to correct at the 6 month time point, however, MCH is significantly increased, although within the normal range, suggesting potential prolonged impact. The results found in female participants are not surprising as there is vast data suggesting red blood cells in females are often affected due to infection [40]. Further data on MCH levels is required in asymptomatic females to determine the full impact of symptomless COVID-19 disease and the presentation of anaemia.

The production of EV from platelets was increased significantly at the initial point of testing, this returned to normal at both follow up time points. A publication [41] by Capellano et al. has shown that platelet EV are increased in severe COVID, despite platelet number remaining the same in severe COVID-19. Our data would support this finding and for the first time to our knowledge shows this phenomenon within the asymptomatic cohort. The increase in platelet EV appears to be

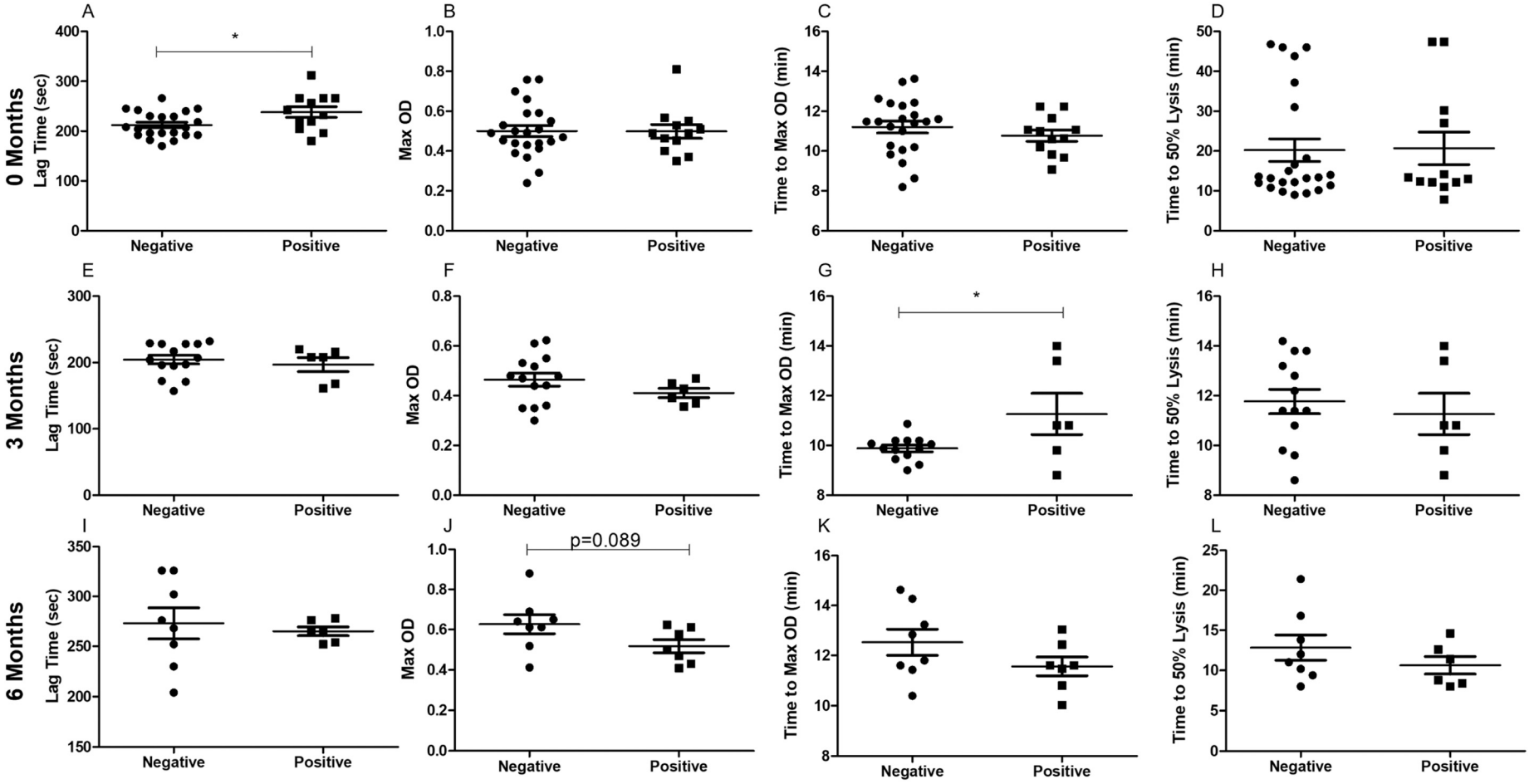

Fig. 3. In-house coagulation assessment. Turbidimetric clotting and lysis assays were carried out at 0 months (N = 23, N = 12, A–D), 3 months (N = 14, N = 6, E–H) and 6 months (N = 8, N = 7, I–L). Each assay produces data for time for clot formation to begin – lag time (A, E, I), the maximum size of clot – max optical density (OD) (B, F, J), the time to form the maximal clot size – time to max OD (C, G, K) and the time to 50 % lysis of the formed clot (D, H, L). * = $p \leq 0.05$. Where p is between 0.06 and 0.1 the p value is given in the graph. Bar charts represent mean ± SEM.

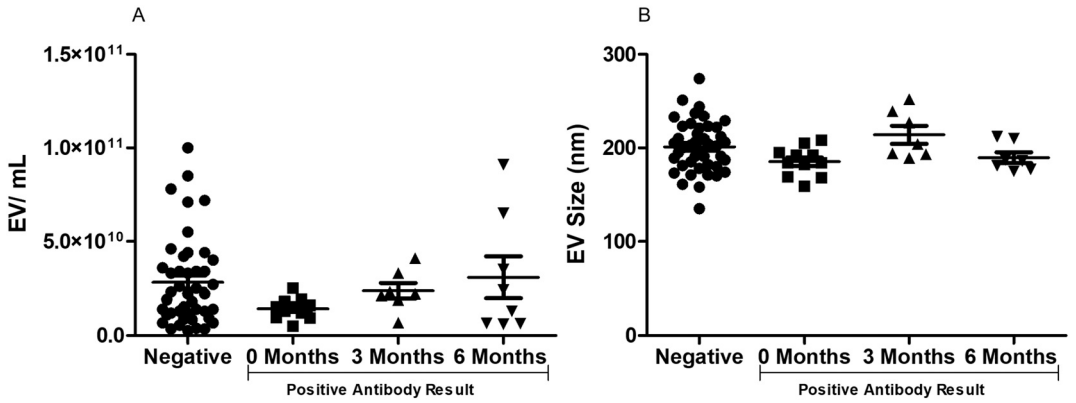

Fig. 4. Extracellular vesicle concentration and size. Extracellular vesicles (EV) were isolated and counted using nanosight tracking analysis, from whole blood samples from female participants who tested negative for COVID-19 antibody (N = 44) and those who tested positive at 0 (N = 11), 3 (N = 7) and 6 months (N = 8) (A). The size of the EV was also determined in the same samples (B). Bar charts represent mean ± SEM.

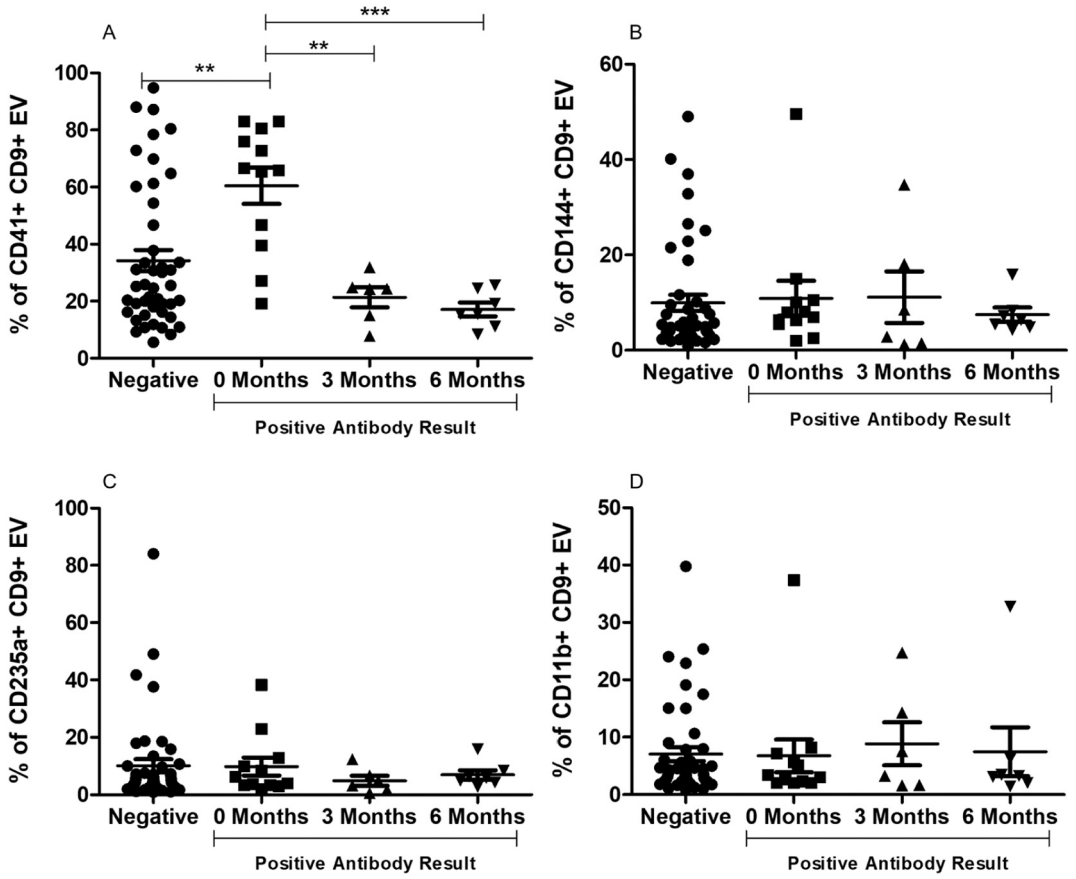

Fig. 5. Extracellular vesicle sub-type. Extracellular vesicles were classified into 4 major families based on a positive CD9 selection from whole blood samples from female participants who tested negative for COVID-19 antibodies (N = 45) and those who tested positive at 0 (N = 12), 3 (N = 6) and 6 months (N = 7). Platelet derived EV were determined using CD9+CD41+ selection (A), endothelial derived EV were determined using CD9+CD144+ selection (B). Whilst red blood cell EV were distinguished using CD9+CD235a+ selection (C) and white blood cell EV using CD9+CD11b+ selection (D). ** = $p \leq 0.01$, *** = $p \leq 0.001$. Bar charts represent mean ± SEM.

independent of platelet activation state, but is associated with alterations to clot formation, as measured by PT time. It is suggested that following stimulation with an agonist, such as, TRAP-6, platelet EV may be responsible for the increased responsiveness. As previously discussed, thrombocytopenia is a clinical hallmark in severe COVID-19 and it has been shown that an increase in platelet derived EV correlates with the occurrence of venous thromboembolism [42]. As platelet EV number decreases over time in our study, it could be suggested that platelets either are no longer being activated, or that the platelets are experiencing an exhausted phenotype. Platelet hypo-responsiveness has been previously shown in symptomatic COVID-19 [47].

In order to test whether the increase in platelet EV production was associated with platelet activation, flow cytometric analysis of membrane bound CD62P expression, along with sCD62P in plasma was assessed as an index of basal platelet activation without prior stimulation. Previous studies have shown that increased CD62P correlates with

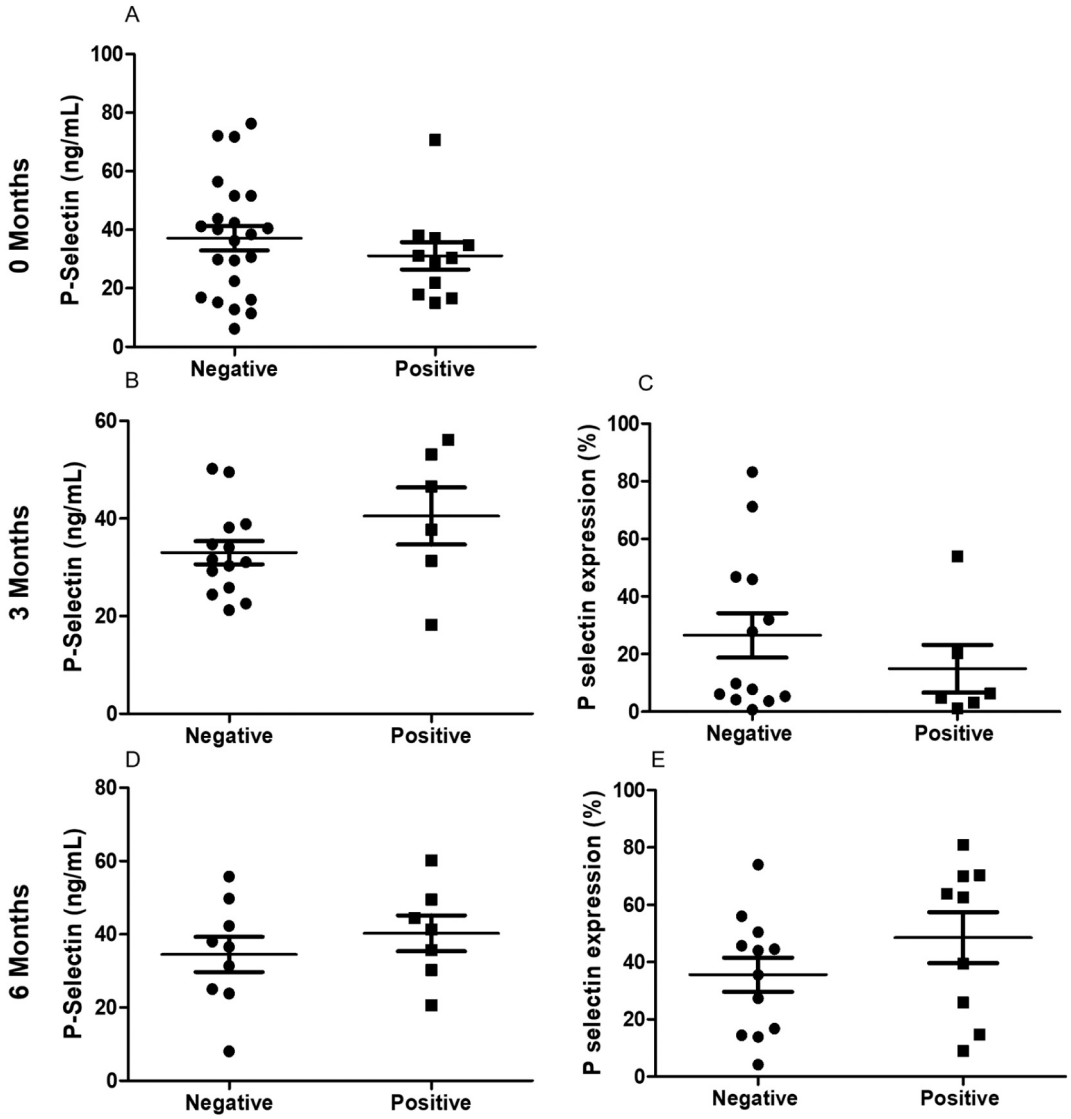

Fig. 6. P-selectin expression on resting platelets. Platelet activation was determined by measuring soluble P-selectin in plasma samples between COVID-19 antibody negative and antibody positive female participants at 0 (N = 23, N = 11, A), 3 (N = 14, N = 6, B) and 6 months (N = 9, N = 7, D). Further studies analysed changes in bound P-selectin at 3 (N = 13, N = 6, C) and 6 Months (N = 12, N = 9, E) post initial COVID-19 antibody tests in both negative and positive female participants. Bar charts represent mean ± SEM.

disease severity in hospitalised COVID-19 patients [43]. Our results show no significant difference in CD62P, suggesting COVID-19 is not causing systemic platelet activation, therefore does not explain the increase in platelet EV production. A limitation to this finding is that we based this on CD62p expression alone to reflect resting platelets. To further investigate the role of possible platelet activation in EV production other relevant platelet activation receptors, such as, GPIIb/IIIa, GPVI and PAR-1, should be characterised.

The authors wish to acknowledge certain limitation to the study including that not all haematological measures were included in phase 1 of testing (specifically FBC, HCT); basal platelet activation was not assessed at the initial screening and PCR measurement was not applied to confirm presence of circulating viral antigen. Whilst at first glance this may seem significant, we are keen to point out that our interest was focussed on the population and laboratory measures associated with CAC risk. Furthermore, at the time of study, viral antigen PCR was limited to symptomatic patients. Importantly, at initial screening only 3 individuals expressed IgM only (no IgG) and CRP levels were not significantly different between antibody positive and negative participants, consistent with COVID-19 exposure of >15 days previously [44]. Furthermore, we are keen to point out that this is a proof-of-principle study which is reflected by the small sample size. This therefore has limited the statistical power associated with the data presented.

The findings in this study are of importance to the ever-growing information on the impact of COVID-19 and show asymptomatic females exposed to the virus, exhibit an altered haemostatic response in the early phase post viral exposure. This risk appears reversible over time, however, suggests a window of high risk of co-morbidity. Whilst our study was limited to otherwise healthy individuals without pre-existing conditions, increased CAC risk in the aging population/ those with pre-existing cardiovascular risk suggests that the consequences of COVID-19 and appropriate application of anti-coagulation therapy in the short-to-medium term post-exposure will need careful evaluation in the general population.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2022.07.012.

References

[1] World Health Organisation, Rolling updates on coronavirus disease (COVID-19), Available from: https://www.who.int/emergencies/diseases/novel-coronavirus-2019/events-as-they-happen, 2020.
[2] W.J. Guan, Z.Y. Ni, Y. Hu, W.H. Liang, C.Q. Ou, J.X. He, et al., Clinical characteristics of coronavirus disease 2019 in China, N. Engl. J. Med. 382 (18) (2020) 1708–1720.
[3] W. Cao, T. Li, COVID-19: towards understanding of pathogenesis, Cell Res. 30 (5) (2020) 367–369.
[4] M. Day, Covid-19: four fifths of cases are asymptomatic, China figures indicate, BMJ 369 (2020), m1375.
[5] The Lancet Haematology, COVID-19 coagulopathy: an evolving story, Lancet Haematol. 7 (6) (2020), e425.
[6] J. Thachil, N. Tang, S. Gando, A. Falanga, M. Cattaneo, M. Levi, et al., ISTH interim guidance on recognition and management of coagulopathy in COVID-19, J. Thromb. Haemost. 18 (5) (2020) 1023–1026.
[7] N. Tang, D. Li, X. Wang, Z. Sun, Abnormal coagulation parameters are associated with poor prognosis in patients with novel coronavirus pneumonia, J. Thromb. Haemost. 18 (4) (2020) 844–847.
[8] C. Huang, Y. Wang, X. Li, L. Ren, J. Zhao, Y. Hu, et al., Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China, Lancet 395 (10223) (2020) 497–506.
[9] M. Aggarwal, J. Dass, M. Mahapatra, Hemostatic abnormalities in COVID-19: an update, Indian J. Hematol. Blood Transfus. 1–11 (2020).
[10] T. Iba, J.H. Levy, J.M. Connors, T.E. Warkentin, J. Thachil, M. Levi, The unique characteristics of COVID-19 coagulopathy, Crit. Care 24 (1) (2020) 360.
[11] M. Xiong, X. Liang, Y.D. Wei, Changes in blood coagulation in patients with severe coronavirus disease 2019 (COVID-19): a meta-analysis, Br. J. Haematol. 189 (6) (2020) 1050–1052.
[12] P. Masi, G. Hékimian, M. Lejeune, J. Chommeloux, C. Desnos, M. Pineton De Chambrun, Systemic inflammatory response syndrome is a major contributor to COVID-19-associated coagulopathy: insights from a prospective, Single-Center Cohort Study, Circulation 142 (6) (2020) 611–614.
[13] G. Van Neil, G. D'Angelo, G. Raposo, Shedding light on the cell biology of extracellular vesicles, Nat. Rev. Mol. Cell Biol. 19 (2018) 213–228.
[14] A. Gaceb, M.C. Martinez, R. Andriantsitohaina, Extracellular vesicles: new players in cardiovascular diseases, Int. J. Biochem. Cell Biol. 50 (2014) 24–28.
[15] K. Connolly, I. Guschina, Characterisation of adipocyte-derrived extracellular vesicles released pre- and post-adipogenesis, J. Extracellular Vesicles (2015) 29159. Oct.
[16] M.N. Abid Hussein, A.N. Böing, E. Biró, et al., Phospholipid composition of in vitro endothelial microparticles and their in vivo thrombogenic properties, Thromb. Res. 121 (2008) 865–871.
[17] H. Kagawa, Y. Komiyama, S. Nakamura, et al., Expression of functional tissue factor on small vesicles of lipopolysaccharide-stimulated human vascular endothelial cells, Thromb. Res. 91 (1998) 297–304.
[18] E. Biró, K.N. Sturk-Maquelin, G.M.T. Vogel, et al., Human cell-derived microparticles promote thrombus formation in vivo in a tissue factor-dependent manner, J. Thromb. Haemost. 1 (2003) 2561–2568.
[19] V. Combes, A.C. Simon, G.E. Grau, et al., In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant, J. Clin. Invest. 104 (1999) 93–102.
[20] Yan-Yan Yan, Wen-Min Zhou, Yu-Qing Wang, Qiao-Ru Guo, Fu-Xi Zhao, Zhuang-Yan Zhu, Yan-Xia Xing, Hai-Yan Zhang, Aljofan Mohamad, Jarrahi Alireza Mosavi, Makabel Bolat, Jian-Ye Zhang, The potential role of extracellular vesicles in COVID-19 treatment: opportunity and challenge, Front. Mol. Biosci. 8 (2021), P695, https://doi.org/10.3389/fmolb.2021.699929.
[21] G. Pocsfalvi, R. Mammadova, A.P. Ramos Juarez, R. Bokka, F. Trepiccione, G. Capasso, COVID-19 and extracellular vesicles: an intriguing interplay, Kidney Blood Press Red 45 (2020) 661–670.
[22] M. Hassanpour, J. Rezaie, M. Nouri, Y. Panahi, The role of extracellular vesicles in COVID-19 virus infection, Infect. Genet. Evol. 85 (2020), 104422, https://doi.org/10.1016/j.meegid.2020.104422.
[23] C Balbi J Burrello S Bolis E Lazzarini V Biemmi E Pianezzi et al. Circulating Extracellular Vesicles are Endowed With Enhanced Procoagulant Activity in SARS-CoV-2 Infection. Doi:10.1016/j.ebiom.2021.103369.
[24] Calcaterra I. Di Minno, R. Lupoli, A. Storino, G.A. Spedicato, M. Maniscalco, A. Di Minno, P. Ambrosino, Hemostatic changes in patients with COVID-19: a meta-analysis with meta-regressions, J. Clin. Med. 9 (7) (2020 Jul 15) 2244.
[25] J.O. Williams, L. Watkeys, J. Nash, C. Whelan, A.J. Davies, J. Evans, K.M. Morris, P.E. James, A two phase single cohort study of COVID-19 antibody sera-surveillance, Ann. Epidemiol. Public Health (Feb 2021) 2639–4391.
[26] E.I. Peerschke, D.D. Castellone, A.K. Stroobants, J. Francis, Reference range determination for whole-blood platelet aggregation using the multiplate analyzer, Am. J. Clin. Pathol. 142 (5) (2014) 647–656.
[27] C Thery K Witwer E Aikawa MJ Alcaraz JD Anderson et al. Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): A Position Statement of the InternationalSociety of Extracellular Vesicles and Update of the MISEV2014 Guidelines. doi:10.1080/20013078.2018.1535750.
[28] C. Pawlowski, T. Wagner, A. Puranik, et al., Inference from longitudinal laboratory tests characterizes temporal evolution of COVID-19-associated coagulopathy (CAC), elife 9 (2020), e59209.
[29] H. Ward, G. Cooke, C. Atchison, Declining Prevalence of Antibody Positivity to SARS-CoV-2: A Community Study of 365 000 Adults, Preprint, 2020, https://doi.org/10.1101/2020.10.26.20219725.
[30] UK Biobank, SARS_CoV-2 Serology Study Weekly Report, 21 July 2020.
[31] Shane P. Comer, Sarah Cullivan, Paulina B. Szklanna, COVID-19 Induces a Hyperactive Phenotype in Circulating Platelets, Preprint, 2020, https://doi.org/10.1101/2020.07.24.20156240.
[32] X. Jin, Y. Duan, T. Bao, J. Gu, Y. Chen, Y. Li, et al., The values of coagulation function in COVID-19 patients, PLoS One 15 (10) (2020), e0241329.
[33] A.H. Kamal, A. Tefferi, R.K. Pruthi, How to interpret and pursue an abnormal prothrombin time, activated partial thromboplastin time, and bleeding time in adults, Mayo Clin. Proc. 82 (7) (2007) 864–873.
[34] R.J. José, A. Williams, A. Manuel, J.S. Brown, R.C. Chambers, Targeting coagulation activation in severe COVID-19 pneumonia: lessons from bacterial pneumonia and sepsis, Eur. Respir. Rev. 29 (157) (2020).
[35] B. Østerud, E. Bjørklid, Sources of tissue factor, Semin. Thromb. Hemost. 32 (1) (2006) 11–23.
[36] W. Ni, X. Yang, D. Yang, J. Bao, R. Li, Y. Xiao, et al., Role of angiotensin-converting enzyme 2 (ACE2) in COVID-19, Crit. Care 24 (1) (2020) 422.
[37] J. Lin, H. Yan, H. Chen, C. He, C. Lin, H. He, et al., COVID-19 and coagulation dysfunction in adults: a systematic review and meta-analysis, J. Med. Virol. 93 (2) (2020) 934–944.
[38] Yu-Qing Cai, Xiao-Bin Zhang, Hui-Qing Zeng, Prognostic Value of Neutrophil-To-Lymphocyte Ratio, Lactate Dehydrogenase, D-Dimer and CT Score in Patients with COVID-19, Preprint, 2020, https://doi.org/10.21203/rs.3.rs-30959/v1.
[39] J.M. Jin, P. Bai, W. He, F. Wu, X.F. Liu, D.M. Han, et al., Gender differences in patients with COVID-19: focus on severity and mortality, Front. Public Health 8 (2020) 152.
[40] D.K. Djakpo, Z. Wang, R. Zhang, X. Chen, P. Chen, M.M.L.K. Antoine, Blood routine test in mild and common 2019 coronavirus (COVID-19) patients, Biosci. Rep. 40 (8) (2020), BSR20200817, https://doi.org/10.1042/BSR20200817.
[41] G. Cappellano, D. Raineri, R. Rolla, M. Giordano, C. Puricelli, B. Vilardo, M. Manfredi, V. Cantaluppi, P.P. Sainaghi, L. Castello, et al., Circulating platelet-derived extracellular vesicles are a Hallmark of sars-Cov-2 infection, Cells 10 (2021) 85, https://doi.org/10.3390/cells10010085.
[42] A. Fricke, P.V. Ullrich, A.F.V. Cimniak, C. Becherer, M. Follo, J. Heinz, J. Scholber, G.W. Herget, O. Hauschild, U.A. Wittel, et al., Levels of activated platelet-derived microvesicles in patients with soft tissue sarcoma correlate with an increased risk of venous thromboembolism, BMC Cancer 17 (2017) 527.
[43] B.K. Manne, F. Denorme, E.A. Middleton, I. Portier, J.W. Rowley, C. Stubben, et al., Platelet gene expression and function in patients with COVID-19, Blood 136 (11) (2020) 1317–1329.
[44] Guoxin Huang, Gaojing Qu, Hui Yu, Meiling Zhang, Xiaoming Song, Features of C-reactive Protein in COVID-19 Patients Within Various Period: A Cohort Study, Preprint, 2020, https://doi.org/10.1101/2020.10.26.20219360.
[45] Dorothea Heuberger, Reto Schuepbach, Protease-activated receptors (PARs): mechanism of action and potential therapeutic modulators in PAR-driven inflammatory diseases, Thromb. J. (2019), https://doi.org/10.1186/s12959-019-0194-8.
[46] G. Moore, G. Knight, A. Blann, Haematology (Fundamentals of Biomedical Science), 3rd Edition, Oxford University Press, 2021.
[47] Ercan, Schrottmaier, Pirabe, Platelet phenotype analysis of COVID-19 patients reveals progressive changes in the activation of integrin αIIbβ3, F13A1, the SARS-CoV-2 target EIF4A1 and Annexin A5, Front. Cardiovas. Med. 8 (779073) (2021).