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Platelet activation and immune response against platelet factor 4 in COVID-19 patients and after SARS-CoV-2 vaccination

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List of Abbreviations

ACE2	Angiotensin-converting enzyme 2
ADAMTS13	A disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13
ADP	Adenosine diphosphate
AKT	Protein kinase B
CCP	COVID-19 convalescent plasma
COVID-2019	Coronavirus Disease 2019
CVST	Cerebral venous sinus thrombosis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medical Agency
FcγRIIa	Fc γ receptor IIa
FDA	Federal Drug Agency
GPIIb/IIIa	Glycoprotein IIb/IIIa
GPVI	Glycoprotein VI
HIPA	Heparin-induced platelet aggregation assay
HIT	Heparin-induced thrombocytopenia
ICU	Intensive care unit
Ig	Immunoglobulin
IgG	Immunoglobulin G
IVIG	Intravenous immunoglobulin
mAb IV.3	Monoclonal antibody IV.3
NET	Neutrophil extracellular trap
PAI-1	Plasminogen activator inhibitor type 1
PAR1	Protease-activated receptor 1
PAR4	Protease-activated receptor 4
PF4	Platelet factor 4
PI3K	Phosphatidylinositol 3-kinase
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
RBD	Receptor binding domain

SARS-CoV-2	Severe acute respiratory syndrome coronavirus type 2
TMPRSS2	Transmembrane protease serine 2
TRAP-6	Thrombin receptor activator peptide 6
VITT	Vaccine-induced immune thrombotic thrombocytopenia
vWF	von Willebrand factor
vWF:Ag	von Willebrand factor antigen

1. Introduction

SARS-CoV-2 (Severe acute respiratory syndrome coronavirus type 2) is a single-stranded RNA virus, which was first isolated from a cluster of patients with pneumonia of unknown cause in Wuhan City, China at the end of 2019 [1]. The disease caused by SARS-CoV-2 has been named later as Coronavirus Diseases 2019 (COVID-19). The World Health Organization declared on 11 March 2020 the COVID-19 outbreak as a global pandemic [2]. In the last 2.5 years, COVID-19 pandemic challenged humans, in particular medical staff, on several fronts and claimed more than 6 million lives [3].

The transmission of the infection occurs through droplets or aerosol particles containing the virus. The incubation period ranges between 0 to 14 days. Approximately 15% of the patients with COVID-19 develop severe disease and 5% require intensive care [4]. Initial observational studies have recognized the increased thrombotic events in hospitalized COVID-19 patients [5]. Thrombotic complications were associated with increased mortality [6,7]. Despite a significant derangement of hemostasis, only a small portion of the patients with COVID-19 fulfilled the criteria for disseminated intravascular coagulation or sepsis induced coagulopathy [8]. Later, it was suggested that COVID-19 causes a unique form of coagulopathy, which is mediated by the release of proinflammatory cytokines, endotheliopathy and microvascular thrombosis [9].

Several studies found platelet abnormalities in COVID-19 patients [10]. Platelet counts are slightly reduced in COVID-19 patients [11]. Furthermore, abnormalities in platelet morphology and function have been found in COVID-19 patients [12,13]. Our

group have previously demonstrated that platelets show a procoagulant phenotype in critically ill COVID-19 patients [14,15]. Platelet activation in COVID-19 is Fc γ receptor IIa (FcγRIIa) dependent, suggesting an immune mediated mechanism for platelet activation [14–17]. Understanding the underlying mechanism of platelet activation in COVID-19 might help us to minimize the thrombotic complications and reduce mortality.

Several patients report continuing or new onset symptoms after a COVID-19 infection [18,19]. Long COVID is a collective term for long-term health sequelae that may be present after an acute COVID-19 infection. The pathophysiologic mechanisms leading to Long COVID have not been adequately elucidated. One of the proposed mechanisms involves endothelial dysfunction and persistent hypercoagulability [20]. To our best knowledge platelet activation has not been investigated in patients who have recovered from COVID-19.

Vaccination is the most effective means to contain an infectious disease. Several vaccine candidates against SARS-CoV-2 have been developed and others are under development [21]. After an accelerated assessment process, a number of vaccines received approval from the Federal Drug Agency (FDA) in the USA and the European Medical Agency (EMA) in Europe. However, a rare but severe side effect of vector-based vaccines against SARS-CoV-2, vaccine-induced immune thrombotic thrombocytopenia (VITT), has been identified [22–26]. Patients with VITT suffer from thrombocytopenia and thrombosis in unusual locations [25,27,28]. The pathophysiology of VITT is not completely understood. VITT resembles serologically and clinically heparin-induced thrombocytopenia (HIT) and immune-mediated

platelet activation appears to be the cause of thrombotic complications and platelet clearance in VITT [25,29].

This dissertation focuses on understanding platelet activation in COVID-19 patients in the convalescent phase and immune response against platelet factor 4 (PF4) in VITT patients.

1.1. COVID-19-associated coagulopathy

Abnormalities in cellular and plasmatic coagulation is common in COVID-19. The pathogenesis of COVID-19-induced coagulopathy is not fully understood yet. Depending on the investigated patient population and the stage of the infection, different results have been reported in previous studies [10].

SARS-CoV-2 invades the cells through binding to angiotensin-converting enzyme 2 (ACE2) receptor, which is highly expressed on pulmonary alveolar epithelial cells [30]. Damaged endothelial cells release proinflammatory cytokines und chemokines [31]. Cytokine storm is coined to describe this excessive and uncontrolled release of proinflammatory cytokines [32]. Later on, inflammatory cells including neutrophils, CD4 helper T cells and CD8 cytotoxic T cells are sequestrated in the lung tissue [33]. Continuing viral replication and lung injury caused by inflammatory cells lead to diffuse alveolar damage in patients with COVID-19 [34,35].

Inflammatory and hemostatic pathways interact with each other in the pulmonary vasculature and lead to endotheliopathy and microvascular thrombosis [36,37]. The recruited immune cells lead to endothelial activation, inflammatory cell infiltration,

and vascular inflammation [36]. Autopsy studies showed the presence of neutrophils and macrophages in platelet-fibrin-rich microvascular thrombi in COVID-19. Interestingly, an increased platelet-neutrophil and platelet-monocyte aggregates have been observed in blood samples from COVID-19 patients, which are also associated with disease severity [38]. Platelets induces tissue factor expression on monocytes through CD62P and integrin α_{IIb}/β_3 signaling [39]. Platelet-monocyte aggregates induce tissue factor expression, which is a strong initiator of clotting cascade [37,40].

1.1.1. The role of platelets in COVID-19-associated coagulopathy

A number of studies demonstrated a direct interaction between SARS-CoV-2 and platelets [41,42]. Cell entry mechanism includes the binding of spike protein to ACE2 [43]. Transmembrane protease serine 2 (TMPRSS2), which cleaves S2' site, is also crucial for ACE2-mediated endocytosis [43]. Platelets can express both ACE2 and TMPRSS2 [41]. SARS-CoV-2 mRNA was detected in platelets of severe COVID-19 patients [44]. Others, however, failed to detect SARS-CoV-2 mRNA in platelets [45]. SARS-CoV-2 can directly induce programmed cell death and extracellular vesicle release in platelets [42]. Direct stimulation of platelets by SARS-CoV-2 and spike protein can stimulate the release of coagulation and inflammatory factors as well as the formation of platelet-leukocyte aggregates [41]. In fact, SARS-CoV-2 RNA in the blood stream correlates with platelet hyperactivity in patients with severe COVID-19 [41]. An ACE2-independent mechanism for the interaction of SARS-CoV-2 with platelets and megakaryocytes has been also described [46].

Neutrophil extracellular traps (NETs) might be another bridge between inflammation and thrombosis in COVID-19 patients [47]. Neutrophils release NETs, which consist of nucleosomes and histone proteins, to capture and inactivate pathogens. CD62P (P-selectin) promotes NET formation through binding to P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils [48]. Excessive formation of NETs has been shown in patients with severe COVID-19 [49,50]. NETs can trigger thrombus formation through a number of mechanisms, including intrinsic and extrinsic coagulation activation, platelet adhesion, and recruitment of platelet adhesion proteins [51]. Postmortem investigations revealed NETs in occluded microvessels in lung tissue of COVID-19 patients [49].

Platelet morphology is altered in patients with COVID-19. A reduced platelet count is common in hospitalized COVID-19 patients [11,52,53]. Impaired megakaryocyte maturation or increased platelet clearance are among the suggested mechanism of thrombocytopenia in COVID-19 patients [54,55]. Although, there is a relationship between thrombocytopenia and increased mortality, [6,56] platelet count is not related to thromboembolic events [57]. An increase in mean platelet volume and immature platelet fraction has been also shown in COVID-19 patients [58–60], the contribution of these morphological changes to increased thrombotic events is not clear.

Platelet hyperreactivity might contribute to COVID-19 coagulopathy [61,62]. The correlation between platelet activation markers (CD62P and PS externalization) and D-dimer in critically ill COVID-19 patients points to an association between COVID-19 induced coagulopathy and platelet activation [14,40]. A prothrombotic platelet

phenotype has been found in COVID-19 patients [14]. Platelets from COVID-19 patients are prone to delta granule release after low dose agonist stimulation [61]. Additionally, CD62P, LAMP-3 and glycoprotein IIb/IIIa (GPIIb/IIIa; CD41/CD61 complex) expression is increased in platelets from COVID-19 patients [63]. Compared to healthy controls, platelets from COVID-19 patients present a significantly higher CD62P expression after stimulation with adenosine diphosphate (ADP) and thrombin receptor activator peptide 6 (TRAP-6) [44]. On the other hand, PAC-1 binding, which is a maker of activation-induced conformational change in GPIIb/IIIa, decreases after platelet activation with TRAP-6 [44]. Furthermore, platelets from COVID-19 patients adhere more efficiently on collagen under flow conditions and aggregate even at suboptimal thrombin concentrations, suggesting that platelets in COVID-19 patients have a lower threshold for stimulation [62]. Although serum levels of PF4 and serotonin levels are increased, their levels in platelets are reduced, suggesting an increased platelet degranulation due to activation in COVID-19 patients [62]. Furthermore, platelets also release fibrinogen, von Willebrand factor (vWF), and factor XII in COVID-19 patients [64]. Together, these data suggest that platelets contribute to the hypercoagulable state in COVID-19 patients.

Hottz et al. showed that the expression of CD62P above the control group median was associated with in hospital mortality in COVID-19 patients [40]. On the other hand, platelets from COVID-19 patients with a mild or asymptomatic infection were not activated [40]. Similarly, soluble CD62P is higher in critically ill COVID-19 patients compared to non-critically ill COVID-19 patients [65]. CD62P and CD63 expression correlates with D-dimer levels in severe COVID-19 patients [40].

Another interaction between coagulation system and inflammation is the formation of platelet-monocyte aggregates [39]. Platelet-monocyte and platelet-granulocyte aggregates are significantly increased in COVID-19 patients [64]. COVID-19 patients in intensive care unit (ICU) has higher platelet-monocyte aggregate levels compared to controls and to patients with mild/asymptomatic infection [40]. Tissue factor expression is significantly higher on platelet-monocyte aggregates than monocytes without platelets [40]. Moreover, tissue factor expression on monocytes is associated with disease severity and in-hospital mortality [40].

1.1.2. Procoagulant platelets in COVID-19

Accumulating evidence suggests that platelets consist of subpopulations with distinct phenotype and functional properties [66]. Procoagulant platelets are one of these subpopulations, which are characterized by exposure of phosphatidylserine (PS) and CD62P on platelet surface [67]. On activated platelets, PS facilitates the assembly of coagulation factors (intrinsic tenase complex and prothrombinase complex), contributing to the thrombin burst in the propagation phase of blood coagulation [67].

The studies investigating the role of procoagulant platelets in COVID-19 are scarce. Althaus et al. showed that platelets from severe COVID-19 patients show an increased PS externalization [14]. PS externalization correlated with sequential organ failure assessment score and D-dimer level in critically ill COVID-19 patients [14]. Furthermore, patients with a thrombosis had a higher PS externalization compared to those without a thrombosis. Interestingly, sera and immunoglobulin G (IgG) fractions from critically ill COVID-19 patients induce a procoagulant phenotype

in platelets from healthy donors [14], suggesting an immune mediated mechanism for the formation of procoagulant phenotype [27].

FcγRIIa is a receptor for IgG that is expressed on platelets. FcγRIIa cross-linking leads to platelet activation and aggregation. Blocking FcγRIIa with monoclonal antibody IV.3 (mAb IV.3) inhibited strongly the COVID-19 sera induced PS externalization and mitochondrial inner membrane depolarization [14]. Similarly, platelet activation induced by sera from COVID-19 patients in serotonin release assay can be completely inhibited with mAb IV.3 [16].

In contrast to these findings, Denorme et al. demonstrated in a small study that platelets of hospitalized COVID-19 patients (n=11) showed lower PS externalization after dual stimulation (thrombin and convulxin) compared to those from healthy donors [68]. In another study, Khattab et al. showed that at baseline procoagulant platelet levels are lower than controls in moderate und severe COVID-19 patients [69]. However, baseline procoagulant platelet level were associated with increased mortality (adjusted hazard ratio of 40 for a procoagulant platelet level > 33.9%) [69]. The contrasting findings might suggest that generation of procoagulant platelet phenotype is limited to very severe COVID-19 patients and might be undetectable in small cohort of donors who had only mild SARS-CoV-2 infection. Further studies in patient groups with different disease severity are needed to better define pathophysiological and clinical significance, if any, of this phenomenon.

In two additional studies from our group, antibody-induced platelet activation in COVID-19 was investigated in detail [15,17]. Pelzl et al. showed that downstream

regulation of FcγRIIa coupling with IgG from severe COVID-19 patients is controlled by PI3K/AKT signaling pathway [17]. The inhibition of PI3K/AKT signaling pathway using specific inhibitors of either protein kinase B (AKT) or phosphatidylinositol 3-kinase (PI3K) blocks the formation of procoagulant platelets by IgG from COVID-19 patients [17]. A subsequent study found that upregulation of cyclic-adenosine monophosphate prevents in vivo procoagulant platelet generation and clot formation induced by IgGs from severe COVID-19 patients [15]. Both studies hint at potential therapies for COVID-19 induced coagulopathy. These hypotheses should be tested, however, in clinical trials.

Other mechanisms for antibody-mediated procoagulant platelet generation have also been reported previously. Stimulation of protease-activated receptors, protease-activated receptor 1 (PAR1) and PAR4, alongside with glycoprotein VI (GPVI), collagen receptor, on platelets increases intracellular Ca^{+2} content and leads to PS exposure [70]. Increased GPVI cleavage after stimulation with TRAP-6 have been shown in COVID-19 patients [63]. However, the contribution of mechanisms other than antibody-mediated activation to the generation of procoagulant platelets in COVID-19 remains the subject of further research.

1.1.3. Post-acute COVID-19 coagulopathy

Several patients report continuing or new onset symptoms after a COVID-19 infection [18,19]. An increased readmission rate (3.5 fold, 95%CI: 3.4-3.6) and post-discharge mortality rate (7.7 fold, 95%CI: 7.2-8.3) in COVID-19 patients have been reported [71]. The term "Long COVID" includes symptoms that persist or are new more than 4 weeks after the onset of COVID-19. The most commonly reported

persistent symptoms after discharge are fatigue, headache, joint pain, chest pain, anxiety, and depression [18,72]. The pathophysiologic mechanisms leading to long COVID have not been adequately elucidated. One of the proposed mechanisms involves endothelial dysfunction and persistent hypercoagulability [20].

Follow-up studies investigated the rate of thromboembolic events after discharge in patients with COVID-19. The rate of thrombosis in these studies ranged between 0-2.6% [73–78]. Due to limitations of these studies such as retrospective design and lack of a comprehensive screening, it is possible that the true incidence of thromboembolic events is even higher.

A number of studies investigated cellular and plasmatic components of the coagulation system after acute COVID-19 infection. von Meijenfeldt et al. measured markers of coagulation system and performed functional testing in 52 COVID-19 patients at hospital admission and 4 months after hospital discharge [79]. Although, factor VIII was reduced after discharge it was still higher than controls 4 months after discharge [79]. Plasminogen activator inhibitor type1 (PAI-1) levels remained high and a prolonged clot lysis time was observed at 4 months. Although, D-dimer was normal at 4 months; endogenous thrombin generation potential was higher in COVID-19 patients. They suggested that COVID-19 patients sustain a hypercoagulable and hypofibrinolytic state several months after acute infection [79]. Townsed et al. investigated 69 hospitalized and 81 non-hospitalized COVID-19 patients after a median of 80.5 (range 44-155) days after initial diagnosis [80]. Markers of coagulation and inflammation had returned to normal range in most of the

patients during follow-up. However, elevated D-dimer levels were observed in 25% of patients [80].

Rotational thromboelastometry provides a global evaluation of coagulation status in COVID-19 patients [81]. Magomedov et al. investigated clot dynamics using intrinsically (INTEM) and extrinsically (EXTEM) activated viscoelastic test assays in 13 COVID-19 patients during ICU stay and 3 months after ICU discharge [82]. Maximum clot firmness was reduced significantly within 12 weeks after discharge in COVID-19 patients [82]. Furthermore, compared to ICU values, maximum lysis increased in both tests at 3 months follow-up, suggesting a substantial normalization of fibrinolytic activity in COVID-19 patients after discharge [82]. Another study investigating 22 COVID-19 patients using tissue-type plasminogen activator rotational thromboelastometry reported a normalized maximum clot firmness in all patients 6 months after discharge from ICU [83]. However, the lysis time remained over the normal range in 4 of 22 (18%) patients [83].

The relationship between abnormal hemostatic parameters and post-COVID-19 syndrome is not clear. An increased FVIII level has been found in one quarter of patients with post-acute COVID-19 syndrome [84]. Furthermore, an increased von Willebrand factor antigen (VWF:Ag)/ADAMTS13 ratio (≥1.5) is associated with an impaired exercise capacity in patients with post-acute COVID-19 syndrome [84]. Similarly, vWF:Ag and vWF propeptide levels correlate with 6 minute walking distance in COVID-19 convalescent patients, suggesting a relationship between persistent endotheliopathy and the development of long COVID-19 [85]. In contrast, a recent study showed that the markers of coagulopathy (FVIII, vWF, PAI-1 and D-

Dimer) are not different in patients with and without persistent symptoms after COVID-19 infection [86].

Although the studies discussed here suggest an association between long COVID and abnormal coagulation markers, further studies are needed to confirm these findings. Most importantly, to our knowledge, platelet functions in patients recovered from COVID-19 have not been studied previously.

1.1.4. COVID-19 convalescent plasma

Convalescent plasma refers to plasma collected from individuals after the infection has resolved and antibodies to the pathogen have developed [87]. Passive immunotherapy with COVID-19 convalescent plasma (CCP) was proposed based on historical experiences for the treatment of patients with COVID-19 [87]. Convalescent plasma has been used previously to treat other viral infections such as H1N1 influenza, severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle Eastern respiratory syndrome coronavirus (MERS-CoV) [88,89].

The neutralizing antibodies against SARS-CoV-2 are the main reason of the beneficial effects in CCP [90,91]. CCP has been used so far in several thousands of patientswith COVID-19 [91–93]. The effectiveness of CCP on mortality and morbidity in COVID-19 patients is yet to be proven [94]. Recent evidence suggest that CCP units with a high neutralizing antibody content might be beneficial [91].

Safety of CCP is another of aspect that needs to be clearly addressed. Concerns have been raised about whether the plasma constituents in CCP can put the already

imbalanced coagulation system into a hypercoagulable state [90,92]. A recent study found that coagulation factor levels in CCP units are not different from fresh frozen plasma in CCP [95]. On the other hand, soluble CD62P level is increased in CCP donors (35810548). To our knowledge, the platelet phenotype and activation in COVID-19 convalescents have not been studied yet.

1.2. Vaccine induced thrombosis and thrombocytopenia (VITT)

1.2.1. Definition

VITT is a rare but serious complication of adenoviral vector based vaccines against SARS-CoV-2 [22,25]. In most cases, the disease manifests with severe thrombocytopenia and arterial and/or venous thrombosis within 4 to 28 days after vaccination [96].

As of 18 May 2022, a total of 443 cases (51 cases after 2. dose) with thrombosis and thrombocytopenia have been reported to Medicines and Healthcare Products Regulatory Agency in the UK after vaccination with ChAdOx1 nCov19 (Vaxzevria, Oxford/AstraZeneca), which counts for an estimated incidence of 15.5 per million doses after the first vaccine and 2.1 per million doses after the second vaccination [97]. Ad26.Cov2.S (Janssen/Johnson & Johnson) is another adenoviral vector-based vaccine used mainly in the US. See et al. calculated an incidence of 3.5 per million doses after vaccination with Ad26.Cov2.S in US [28]. However, since some mild cases were not diagnosed properly the incidence of VITT might be even higher.

Although early reports defined women under 60 years of age as the risk group [22,23,25,26], further monitoring of the cases revealed that the sex and age imbalance were smaller than previously observed [96,97].

1.2.2. Pathophysiology

The simultaneous occurrence of thrombocytopenia and thrombosis and the manifestation of disease symptoms approximately 5 days after inoculation suggest an immune-mediated underlying mechanism similar to HIT type II [98]. In HIT type II, immune complexes consisting of anti-PF4 antibody, PF4, and heparin activate platelets via FcγRIIa [98]. Similarly, in VITT anti-PF4 antibodies bind to PF4 and activate platelets via FcγRIIa [22,25]. In contrast to HIT Type II, patients develop anti-PF4 antibodies in VITT without any previous exposure to heparin. Anti-PF4 antibodies in VITT and HIT show different binding properties [99]. Huyn et al. isolated anti-PF4 antibodies from VITT patients and showed that they strongly bind to PF4 at heparin binding site [100]. Thus, they mimic the effect of heparin by causing PF4 tetramers to group and form big immune complexes, which is necessary for FcγRIIa coupling and platelet activation [100]. This difference might explain the heparin induced dissociation of bound anti-PF4-antibodies isolated from VITT patients [101].

The role of other immune cells in the pathophysiology of VITT is also discussed. Holm et al. found increased levels of NETosis markers (circulating H3Cit, dsDNA, and myeloperoxidase-DNA complex) in blood samples of VITT patients [102]. Furthermore, in thrombus material they found IgG deposits together with NETs [102].

The initial trigger for the development of anti-PF4 antibodies is not known. It is proposed that vaccine components such as human and non-structural viral proteins or free DNA might bind to PF4, which will be recognized by innate immune system as a neoantigen [103–105]. Another theory includes cross-reactivity between spike protein of the virus and PF4 [106]. Konstantinou found an 85% similarity between signaling peptide of the SARS-CoV-2 spike protein and PF4 [107]. This topic requires further research.

1.2.3. Clinical picture

The disease become manifest within the 2 weeks after vaccination in most cases. However, delayed presentations to a medical institution due to non-specific symptoms have also been reported [108]. Petechiae or bruising might be the earliest symptom in patients with severe thrombocytopenia; however, hematoma is rarely reported in patients with VITT. Thrombosis can be seen in different locations, however, cerebral venous sinuses is seen almost 50% of patients with VITT [96,109,110]. The mortality rate is markedly high in patients with a cerebral venous sinuses thrombosis (CVST) [111]. Patients with a cerebral venous sinuses thrombosis develop due to congestion and thrombocytopenia intracranial hemorrhage [112,113], which is associated with mortality [109]. Severe headache is common in these patients [109,113]. Since headache is also a frequent side effect of vaccination, it is often neglected, which leads to delayed diagnosis [108,114,115]. Patients with CVST can also present with altered mental status and/or focal neurologic deficits [111]. Interestingly, splanchnic vein thrombosis is also common in patients with VITT [110]. Patients with splanchnic vein thrombosis have often non-specific symptoms such as abdominal pain, nausea, distention, vomiting or diarrhea.

Less commonly, patients may also have pulmonary embolism or lower extremity venous thrombosis. A systematic screening for thrombosis is necessary in patients with VITT.

Mortality is high in patients with VITT. Initial case series reported a mortality rate as high as 60% [22,23,111]. The mortality rate has decreased with the recognition of the phenomenon and publication of diagnostic and treatment guidelines. Current case series report a mortality rate around over 20% [96,111,112,116].

1.2.4. Diagnosis

Diagnosis of VITT depends on clinical and laboratory findings [96,117,118]. Diagnostic algorithm based mainly on the experience in HIT Type II. Laboratory diagnosis includes the detection of anti-PF4 antibodies. Rapid immunoassays are not suitable for the detection of anti-PF4 antibodies in VITT [119–121]. Furthermore, commercially available anti-PF4 IgG or IgG, IgM, IgA ELISA assays have different levels of sensitivities in VITT [121]. An appropriate anti-PF4 ELISA assay should be preferred in the diagnosis of VITT [118,121]. Since not all anti-PF4 antibodies induce platelet activation, additionally laboratory confirmation of the diagnosis with a functional platelet activation assay is recommended [118]. However, these assays are only available in a number of specialized centers.

Pavord et al. recommended a set of clinical and laboratory criteria to evaluate the likelihood of VITT in patients presenting with symptoms after COVID-19 vaccination. [96] These criteria include the following: 1) symptom onset within 5-30 days after vaccination with an adenoviral vector-based COVID-19 vaccine (AstraZeneca and

Johnson&Johnson/Jannsen), 2) documented venous or arterial thrombosis, 3) thrombocytopenia (150 000/microliter), 4) D-dimer > 4000 fibrin equivalent units (FEU), and a 5) a positive Anti-PF4 IgG ELISA. If all five criteria are met, the diagnosis of VITT is considered definite; if one criterion is missing, the diagnosis is considered probable. In these cases, anticoagulation and intravenous immunoglobulin (IVIG) may be considered based on clinical and laboratory findings.

Patients might not present classical symptoms at first presentation [115]. Salih et al. defined cases who initially presented with anti-PF4 antibodies and later developed thrombosis [122]. Delayed onset thrombocytopenia is also described [123]. The disease can progress rapidly and patients might develop new thrombosis or bleeding. Close follow up is crucial to reduce additional morbidity and mortality.

1.2.5. Treatment

National and international societies published recommendations to help clinicians to manage patients with VITT [124]. Until VITT can be ruled out, patients should be hospitalized and monitored closely. Special attention should be given to patients with CVST. Since they may require endovascular or neurosurgical intervention, they should be transferred to a center with these capabilities.

Patients should be anticoagulated to avoid further thrombotic complications [103]. Before this phenomenon was recognized, heparin was used for anticoagulation [111,125–127]. However, published societal guidelines recommend non-heparin anticoagulants [117,124]. In vitro studies demonstrated the inhibition of platelet activation as well as dissociation of PF4-antibody complexes with therapeutic dose

heparin [101]. In addition, a meta-analysis of published cases did not demonstrate different outcomes for patients treated with heparin and those treated with a non-heparin anticoagulant [113]. Nevertheless, clinical data on the efficacy and safety of heparin in VITT is very limited and a non-heparin anticoagulant is recommended [29,103]. Anticoagulation should continue at least 3 months after the normalization of the platelet count.

IVIG competes with anti-PF4 antibodies for FcγIIa [128,129]. The recommended dose for IVIG is 1 g/kg/day for two consecutive days. The dose can be repeated if platelet count did not respond in 48 hours. Given that confirmatory assays are only available in a limited number of centers, the diagnosis of VITT may be delayed by several hours or even days. When VITT is suspected, prompt initiation of treatment is critical to contain disease activity and reduce disease-related morbidity. IVIG therapy should be initiated in suspected cases without waiting for the results of functional tests. Perry et al. reported lower mortality rate in VITT patients with CVST receiving IVIG than in those who did not (40% vs 73%; p=0.022) [126]. Other immunomodulatory therapies such as steroids have also been used in patients with VITT [29]. However, retrospective case series did not show their benefit [126]. Favorable outcomes have been reported with therapeutic plasma exchange in refractory cases [130,131]. Therapeutic plasma exchange removes IgG antibodies causing VITT from the circulation [98,132].

It is important to note that platelet transfusions should be avoided unless a life-threatening bleeding occurs or immediate major surgery is required [29].

2. Aims

The aims of the dissertation are as follows:

1. To investigate the procoagulant platelet phenotype after mild COVID-19 infection
2. To evaluate the coagulation and fibrinolytic system in the long term after mild COVID-19 infection
3. To measure the effect of sera from COVID-19 convalescent subjects on healthy platelets
4. To investigate the contribution of anti-SARS-CoV-2 antibodies in the development of VITT
5. To analyze the platelet response to IVIG therapy in patients with VITT
6. To investigate the clinical use of IVIG therapy in patients with VITT

3. Results

3.1. Platelets and sera from donors of convalescent plasma after mild COVID-19 show no procoagulant phenotype.

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Summary of the study:

Thrombotic complications are common in patients with COVID-19 and associated with a negative outcome. Furthermore, high rehospitalization and mortality rate have been shown after discharge in COVID-19 patients suggesting a continuing disease progression even in the convalescent phase. COVID-19 associated derangements in the plasmatic coagulation system can remain several months after the acute disease. This study addressed the following questions:

1) **Do platelets express a procoagulant platelet phenotype for long time after a mild infection in COVID-19 patients?**

To answer this question, we investigated PS externalization, CD62P expression, and GPVI shedding both in platelet rich plasma as well as after incubation of washed healthy platelets with CCP donors' sera using flow cytometry in CCP donors. Furthermore, coagulation and fibrinolysis systems were assessed with thromboelastometry.

The study included forty-seven CCP donors [22 Male, 25 Female; and mean age (±SD) 41.4±13.7 years] with a history of mild COVID-19 infection. Platelets from CCP

donors did not show increased PS externalization, CD62P expression, or GPVI shedding.

2) Can CCP activate healthy platelets?

Our study showed that sera from CCP donors do not induce PS externalization or GPVI shedding in healthy platelets. None of the patients had a thrombotic complication during or after COVID-19 infection.

3) Are there any alterations in the fibrinolytic system after a mild COVID-19 infection?

In the thromboelastometry, all but one patient showed a normal clot formation and clot lysis.

In conclusion, we did not observe a prolonged coagulopathy after a mild COVID-19 infection.

Platelets and Sera from Donors of Convalescent Plasma after Mild COVID-19 Show No Procoagulant Phenotype

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Abstract

Coronavirus disease-2019 (COVID-19) is associated with increased thromboembolic complications. Long-term alteration in *the* coagulation system after acute COVID-19 infection is still a subject of research. Furthermore, the effect of sera from convalescent subjects on platelets is not known. In this study, we investigated platelet phenotype, coagulation, and fibrinolysis in COVID-19 convalescent plasma (CCP) donors and analyzed convalescent sera-induced effects on platelets. We investigated CCP donors who had a history of mild COVID-19 infection and donors who did not have COVID-19 were used as controls. We analyzed phosphatidylserine (PS) externalization, CD62p expression, and glycoprotein VI (GPVI) shedding both in platelet-rich plasma (PRP) and after incubation of washed healthy platelets with donors' sera using flow cytometry. Coagulation and fibrinolysis systems were assessed with thromboelastometry. Forty-seven CCP donors (22 males, 25 females; mean age (±SD): 41.4 ± 13.7 years) with a history of mild COVID-19 infection were included. Median duration after acute COVID-19 infection was 97 days (range, 34–401). We did not find an increased PS externalization, CD62p expression, or GPVI shedding in platelets from CCP donors. Sera from CCP donors did not induce PS externalization or GPVI shedding in healthy platelets. Sera-induced CD62p expression was slightly, albeit statistically significantly, lower in CCP donors *than* in plasma donors without a history of COVID-19. One patient showed increased maximum clot firmness and prolonged lysis time in thromboelastometry. Our findings suggest that procoagulant platelet phenotype is not present after mild COVID-19. Furthermore, CCP sera do not affect the activation status of platelets.

Keywords
- procoagulant platelets
- platelet activation
- convalescent plasma
- COVID-19

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Introduction

Thromboembolic complications are common in patients suffering from severe coronavirus disease-2019 (COVID-19).[1,2] Various organs are affected by micro and macro thrombosis as a result of COVID-19-induced coagulopathy.[3,4] Thrombotic complications can lead to multiorgan failure and mortality in severe cases.[5] Both cellular and plasma elements of the coagulation system show abnormalities in COVID-19.[6] Platelets contribute not only to the hypercoagulable state in COVID-19 patients but also to the systemic inflammatory response (cytokine storm) by releasing inflammatory mediators.[7] The expression of P-selectin and CD63 is correlated with D-dimer in severe COVID-19 patients, suggesting an association between platelet activation and COVID-19-associated coagulopathy.[8] We have previously shown that platelets of COVID-19 patients express *a* procoagulant phenotype.[9,10] Furthermore, a correlation between procoagulant platelets and thrombosis as well as mortality has been shown in COVID-19 patients.[10,11] As more people recover from COVID-19 infection and continue to experience symptoms,[12] discussion has begun over the possibility of persistent coagulopathy even after the acute infection period.[13–15]

COVID-19 convalescent plasma (CCP) is used in the treatment of COVID-19.[16–18] However, concerns have been expressed whether plasma components in CCP can shift the already imbalanced coagulation system to a more hypercoagulable state.[16] Furthermore, we have previously shown that immunoglobulin G fractions from severe COVID-19 patients induce a procoagulant phenotype in healthy platelets.[10] To our best knowledge, the effect of CCP on platelet phenotype and activation has not been investigated earlier.

The aims of this study were (1) to investigate the procoagulant platelet phenotype and platelet activation after acute infection in COVID-19 patients, (2) to investigate the effect of CCP on healthy platelets, (3) to measure the viscoelastic properties of blood using a rotational thromboelastometry in CCP donors.

Methods

Study Cohort

This study was conducted between January 2021 and July 2021. Plasma donors who had *a* mild COVID-19 infection at least 4 weeks before plasma donation were invited to participate in the study (CCP donors). Donors who were hospitalized for COVID-19 infection or who did not have a positive SARS-CoV-2 polymerase chain reaction test or SARS-CoV-2 antibody enzyme immunoassay were excluded from the study. Information on medical history and COVID-19 infection was obtained with a questionnaire. Blood samples were collected before plasma donation and either tested immediately or frozen for later analysis. Additionally, citrated blood samples and native blood samples were also collected from plasma donors who did not have a COVID-19 infection to establish control values for flow cytometry (FC) measurements. Written informed consent was obtained from all study participants.

Assessment of Platelet Phenotype

Platelets were isolated from citrated blood of the healthy plasma donors and/or CCP donors and tested within 3 hours. In brief, whole blood was centrifuged (120 *g*, 20 minutes [min*] at room temperature [RT], without brakes), and PRP was gently separated and used for further analysis. Where indicated, PRP was incubated with buffer or thrombin receptor activating peptide (TRAP-6, 2.5 and 10 µM; Hart Biologicals, Hartlepool, UK) for 15 minutes at RT. Platelets were then stained with annexin V-FITC and CD62p-APC (ImmunoTools, Friesoythe, Germany) and directly analyzed by FC. Test results were determined as fold increase (FI) of the percentage of double phosphatidylserine (PS)/CD62p-positive events in platelets.

For the assessment of GPVI shedding, platelets were stained with 1 µL of phycoerythrin (PE)-labeled anti-GPVI monoclonal antibodies (BD, San Jose, CA) for 15 minutes at RT in the dark and analyzed by FC. Where indicated, platelets treated with collagen-related peptide (CRP, 2.5 µg/mL) (CambCol laboratories, Ely, UK) served as positive control. Changes in GPVI expression on the platelet surface were quantified as percentage of reduction in the GPVI-positive platelet population and normalized to baseline.

Investigation of Antibody-Mediated Effects on Platelets

The ability of sera to induce procoagulant platelets was determined by incubating the sera from healthy controls, plasma donors, and CCP donors with washed platelets from blood donors. Platelets were obtained from blood donors, whose platelets are known to have a good response in the heparin-induced platelet activation assay. Each sample was tested with platelets from one donor.

Prior to use, all sera samples were heat-inactivated at 56 °C for 30 minutes, followed by a sharp centrifugation step at 5,000 *g*. The supernatant was collected in a fresh tube. For the determination of procoagulant platelets, 5 µL serum was incubated with 25 µL washed platelets (7.5×10^6) for 1 hour under rotating conditions at RT. Platelets co-incubated with TRAP-6 (10 µM; Hart Biologicals, Hartlepool, UK) and ionomycin (5 µM, 15 minutes* at RT [Sigma-Aldrich, St. Louis, MO]) were used as a positive control for procoagulant platelets. Afterwards, samples were washed once (7 minutes, 650 *g*, RT, without brake) and gently resuspended in 75 µL of phosphate-buffered saline (PBS; Biochrom, Berlin, Germany). Platelets were then stained with annexin V-FITC and CD62-APC (ImmunoTools, Friesoythe, Germany) and directly analyzed by FC. Test results were determined as FI of the percentage of double PS/CD62p-positive events in platelets upon incubation with donors' sera compared with healthy controls.

For the determination of GPVI shedding, aforementioned sera-treated washed platelets were stained with 1 µL of PE-labeled anti-GPVI (BD for 15 minutes at RT in the dark. After incubation, platelets were filled up with PBS to a final volume of 500 µL and immediately assessed by FC. Platelets incubated with TRAP-6 (10 µM; Hart Biologicals, Hartlepool, UK) and ionomycin (5 µM, 15minutes at RT [Sigma-Aldrich, St. Louis, MO]) were used as a positive control. Changes in GPVI

expression on the platelet surface were quantified as percentage of reduction in the GPVI-positive platelet population, and normalized to washed platelets that were treated with sera from healthy controls.

Thromboelastographic Assays

Citrated blood samples from CCP donors were analyzed within 2 hours using a viscoelastic test system (ClotPro; Enicor GmbH, Munich, Germany). Blood coagulation is determined by elastic motion (clockwise and anticlockwise) of a cylindrical cup including blood mixed with activator reagents around a fixed pin. The motion of the cup is recorded and the data are converted into thromboelastographic amplitude values that are plotted over time. The rotation of the cup is progressively reduced depending on the elastic properties of the formed clot. We used the extrinsic assay (EX test), fibrinogen assay (FIB test), and the tissue plasminogen activator assay (tPA test) according to the manufacturer's instructions. In brief, in the EX test, clotting is triggered by tissue factor. This test appears to be sensitive to anticoagulation, fibrinogen, factor XIII, and hyperfibrinolysis. In the FIB test, platelets are inhibited by cytochalasin D and a synthetic GP2b3a antagonist. The FIB test indicates fibrinogen levels and fibrin polymerization in citrated blood. The tPA test is similar to the EX test but contains an additional 650 to 700 ng/mL of recombinant tPA (r-tPA), an activator of plasmin, to determine fibrinolysis resistance. The following parameters were estimated during the study: clotting time, maximum clot firmness, lysis time (time from the beginning of the clot formation until 50% of clot lysis), and maximum clot lysis. The normal range specified by the manufacturer was used in all measured parameters.

Ethics Statement

The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all volunteers prior to any study-related procedure. The study protocol was approved by the Institutional Review Board of the University of Tübingen.

Data Sharing Statement

Data may be requested for academic collaboration from the corresponding author.

Statistics

Statistical analyses were performed using GraphPad Prism 7 (La Jolla, CA). *t*-Test was used to analyze normally distributed results. Nonparametric test (Mann–Whitney test) was used when data failed to follow a normal distribution as assessed by the D'Agostino–Pearson omnibus normality test. A *p*-value of <0.05 was assumed to represent statistical significance.

Results

Study Cohort

Forty-nine CCP donors were included in the study. Two donors were excluded later because of the negative SARS-CoV-2 antibody test results (data not shown). The results of the remaining 47 CCP donors (25 females, 22 males) were analyzed. Mean age (±SD) of CCP donors was 41.4 ± 13.7 years. Median duration after acute COVID-19 infection was 97 days (range, 34–401). Patient characteristics are presented in ►Table 1. None of the CCP donors developed a thrombotic event during or after COVID-19 infection until study inclusion. None of them was vaccinated against SARS-CoV-2 at the time of blood collection. In the control cohort, 51 (22 females, 29 males) plasma donors with a mean age of 38.9 ± 18.4 years, who did not have SARS-CoV-2 infection, were enrolled.

Table 1 Demographic and clinical characteristics of the CCP donors

Demographic data	
Age (years)	41.4 ± 13.7
Sex	
Female	25 (53%)
Male	22 (47%)
SARS-CoV-2 infection	
Duration of infection (days)	8.6 ± 6.5
Symptoms	
Fever	24 (51%)
Anosmia	25 (53%)
Taste disorder	20 (43%)
Eyes (redness, inflammation)	3 (6%)
Headache	33 (70%)
Sore throat	18 (38%)
Congestion or runny nose	26 (55%)
Cough	27 (57%)
Shortness of breath or difficulty breathing	18 (38%)
Pneumonia	0 (0)
Nausea/vomiting/diarrhea	5 (11%)
Fatigue	37 (79%)
Limb/joint/back pain	27 (57%)
Skin rash	1 (2%)
Time to blood collection after the acute infection (days)	97 (34–401)
Comorbidities	
Arterial hypertension	5 (11%)
Diabetes mellitus type II	2 (4%)
Asthma	1 (2%)
Benign prostate hypertrophy	1 (2%)

Abbreviation: CCP, COVID-19 convalescent plasma.
Note: Data are represented as mean ± standard deviation or median (range) for continuous data and *n* (%) for categorical data.

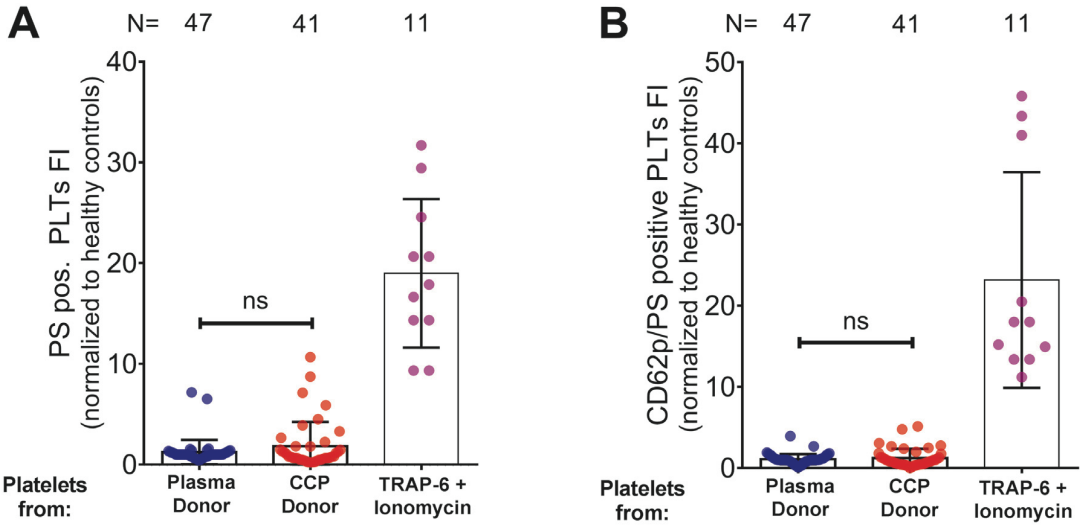

Fig. 1 Assessment of procoagulant platelet phenotype in COVID-19 convalescent plasma (CCP) donors. Procoagulant platelet phenotype was analyzed by assessing phosphatidylserine (PS) externalization (**A**) in platelets from plasma donors and CCP donors. Furthermore, procoagulant platelets were assessed (**B**) by using flow cytometry analysis of CD62p/PS double positive cells in platelets from healthy plasma and CCP donors (blue: plasma donors; red: CCP donors). Data are presented as mean ± standard deviation of the fold increase (FI) of the mean fluorescence intensity (MFI) or percentage (%). ns, not significant.

No Increased Levels of Procoagulant Platelets in CCP Donors

We first measured PS externalization using annexin V. The rate of PS-positive cells was not different between plasma donors and CCP donors (FI of PS-positive platelets: 1.24 ± 1.21 (95% confidence interval [CI]: 0.89–1.6) vs. 1.83 ± 2.40 (95% CI: 1.07–2.6), respectively, $p = 0.43$; ►Fig. 1A). Similarly, the rate of double positive cells (CD62p and annexin V) was similar between plasma donors and CCP donors (FI of CD62p/PS-positive platelets: 1.09 ± 0.62 [95% CI: 0.91–1.27] vs. 1.22 ± 1.51 [95% CI: 0.85–1.58], respectively, $p = 0.29$; ►Fig. 1B). Baseline CD62p expressions (FI in mean fluorescence intensity [MFI]: 1.0 ± 0.13 [95% CI: 0.95–1.04] vs. 1.31 ± 1.20 [95% CI: 0.93–1.69], $p = 0.93$) were not statistically different between plasma donors and CCP donors. Moreover, CD62p release was comparable between both groups upon activation with TRAP-6 at 2.5 µM (FI of MFI: 1.59 ± 1.58 [95% CI: 1.1–2.08] vs. 1.69 ± 1.18 [95% CI: 1.28–2.01], $p = 0.43$) as well as with TRAP-6 at 10 µM (FI of MFI: 4.62 ± 4.42 [95% CI: 3.52–6.12] vs. 4.18 ± 3.28 [95% CI: 3.15–5.22], $p = 0.92$; ►Fig. 2). Furthermore, GPVI shedding at baseline (FI of GPVI-negative platelets: 1.0 ± 0.12 [95% CI: 0.96–1.04] vs. 1.31 ± 0.81 [95% CI: 1.06–1.55], $p = 0.08$) and after activation with 2.5 µg/mL CRP (FI of GPVI-negative platelets: 2.16 ± 1.16 [95% CI: 1.83–2.48] vs. 2.31 ± 1.21 [95% CI: 1.96–2.68], $p = 0.49$) were also similar between CCP donors and plasma donors (►Fig. 3).

Antibody-Mediated Procoagulant Platelets and GPVI Using Washed Platelets

Compared with sera from noninfected plasma donors, sera from CCP donors did not induce higher PS externalization (FI of PS-positive platelets: 1.16 ± 0.66 [95% CI: 0.61–1.72] vs. 1.51 ± 0.74 [95% CI: 1.3–1.74], respectively, $p = 0.11$; ►Fig. 4A) or increased the rate of CD62p/PS double positive procoagulant phenotype (FI in CD62p/PS-positive platelets: 1.86 ± 0.87 [95% CI: 1.13–2.59] vs. 1.37 ± 0.63 [95% CI: 1.19–1.56], respectively, $p = 0.10$; ►Fig. 4B) in platelets from healthy persons. Of note, CD62p expression in healthy platelets after incubation with sera from CCP plasma donors was significantly lower compared with sera from noninfected donors (FI in CD62p: 2.09 ± 1.36 [95% CI: 0.95–3.24] vs. 1.16 ± 0.45 [95% CI: 1.03–1.30], $p < 0.01$; ►Fig. 4C). Sera-mediated GPVI shedding was similar between the groups (1.07 ± 0.16 [95% CI: 0.94–1.21] vs. 1.27 ± 0.91 [95% CI: 0.99–1.54], $p = 0.52$, ►Fig. 4D).

Thromboelastographic Assays

We assessed coagulation and fibrinolysis in whole blood samples using a thromboelastometry (►Table 2, ►Fig. 5). The thromboelastometry was available in 39 CCP donors. We determined clotting time and maximum clot firmness in EX test as well as in FIB test to evaluate the coagulation. Only one CCP donor (case no. 7) had increased maximum clot firmness in EX test and FIB test. These parameters were *within the* normal range in other CCP donors. Second, we determined maximum clot lysis in EX test and in tPA test and lysis time in tPA test to evaluate the fibrinolysis. Again, one CCP donor (case no. 7) had increased lysis time in tPA test. This donor had arterial hypertension and diabetes mellitus type 2. Other CCP donors had normal fibrinolysis values.

Discussion

Recent studies have repeatedly shown that platelets are composed of different subpopulations that fulfill different

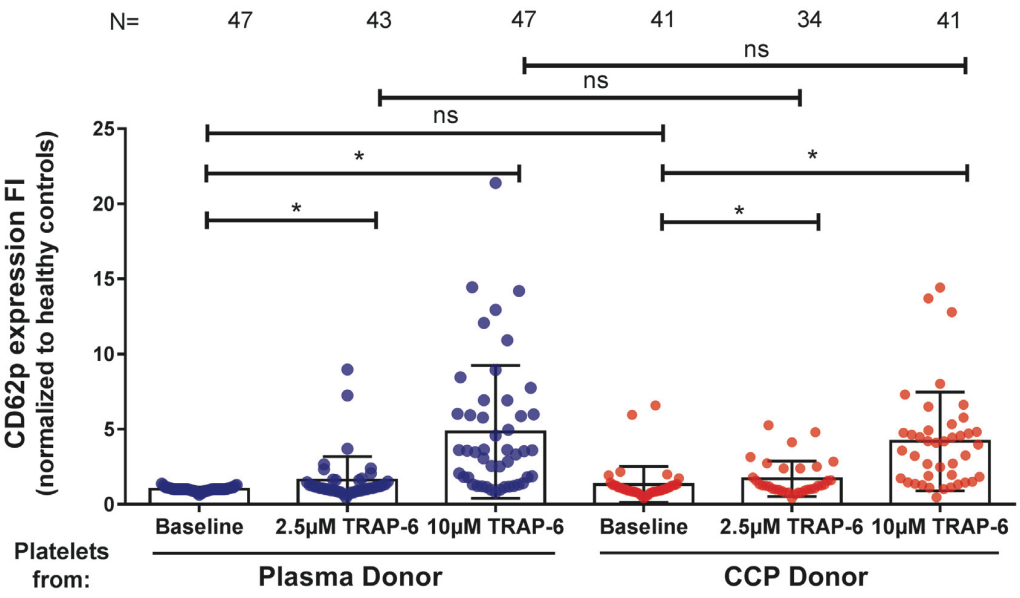

Fig. 2 CD62p expression on platelets from COVID-19 convalescent plasma (CCP) donors. The basal expression of P-selectin was determined on the surface of platelets from plasma and CCP donors. Where indicated, platelets were treated with thrombin receptor activating peptide (TRAP-6, 2.5 and 10 µM) before staining with anti-CD62p (blue: plasma donors; red: CCP donors). Data are presented as mean ± standard deviation of the fold increase (FI) of mean fluorescence intensity (MFI) or percentage (%). ns, not significant; $^{*}p < 0.05$.

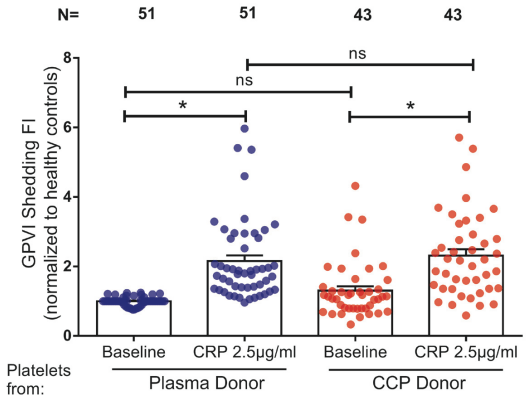

Fig. 3 GPVI shedding from platelet surface. Reduction in the surface expression of GPVI on the platelets of plasma and CCP donors was analyzed with or without incubation with collagen-related peptide (CRP, 2.5 µg/mL) by anti-GPVI-PE antibody staining (blue: plasma donors; red: CCP donors). Data are presented as mean ± standard deviation of the fold increase (FI) of percentage of GPVI-negative platelets (%). ns, not significant; $^{*}p < 0.05$.

roles in coagulation.[19] Procoagulant platelets are a distinct subgroup that externalize PS on their surfaces and support fibrin formation.[20] Despite the vast amount of studies on coagulation in COVID-19, very few studies investigated procoagulant platelets during acute COVID-19 infection. Althaus et al have shown an increase in PS externalization in critically ill COVID-19 patients compared with noncritically ill COVID-19 patients.[10] They also showed that PS externalization is associated with thrombosis and high SOFA scores in this patient group.[10] Interestingly, Denorme et al found a reduced PS externalization after dual agonist stimulation in COVID-19 patients compared with healthy donors.[21] Similarly, Khattab et al demonstrated that procoagulant platelet levels are lower than controls in moderate and severe COVID-19 patients, but an increase in procoagulant platelets is associated with mortality in COVID-19 patients.[11] Previous studies reported on antibody-mediated increase in PS exposure as a marker for procoagulant platelets during severe COVID-19, via active engagement of FcγRIIA.[9,10,22] However, this phenomenon seems to be limited to very severe COVID-19 patients and might be undetectable in small cohort of donors who had only mild SARS-CoV-2 infection. To our best knowledge, PS externalization of platelets in COVID-19 convalescent individuals has not been investigated before. In this study, we did not find a difference between CCP donors and controls in terms of PS externalization.

Surface expression of CD62p (P-selection) is a marker of platelet activation. Manne et al showed that CD62p expression is increased compared with controls in hospitalized COVID-19 patients.[23] Hottz et al found an increased CD62p expression in severe COVID-19 patients but not in patients with a mild or asymptomatic COVID-19 infection.[8] Furthermore, CD62p surface expression at admission was correlated with D-dimer and associated with the need for mechanical ventilation as well as with in-hospital mortality, suggesting an association between platelet activation and COVID-19-associated coagulopathy.[8] We found that CD62p expression in CCP donors at baseline and after stimulation with TRAP-6 was not higher compared with controls. These findings suggest that platelets of CCP donors are not activated.

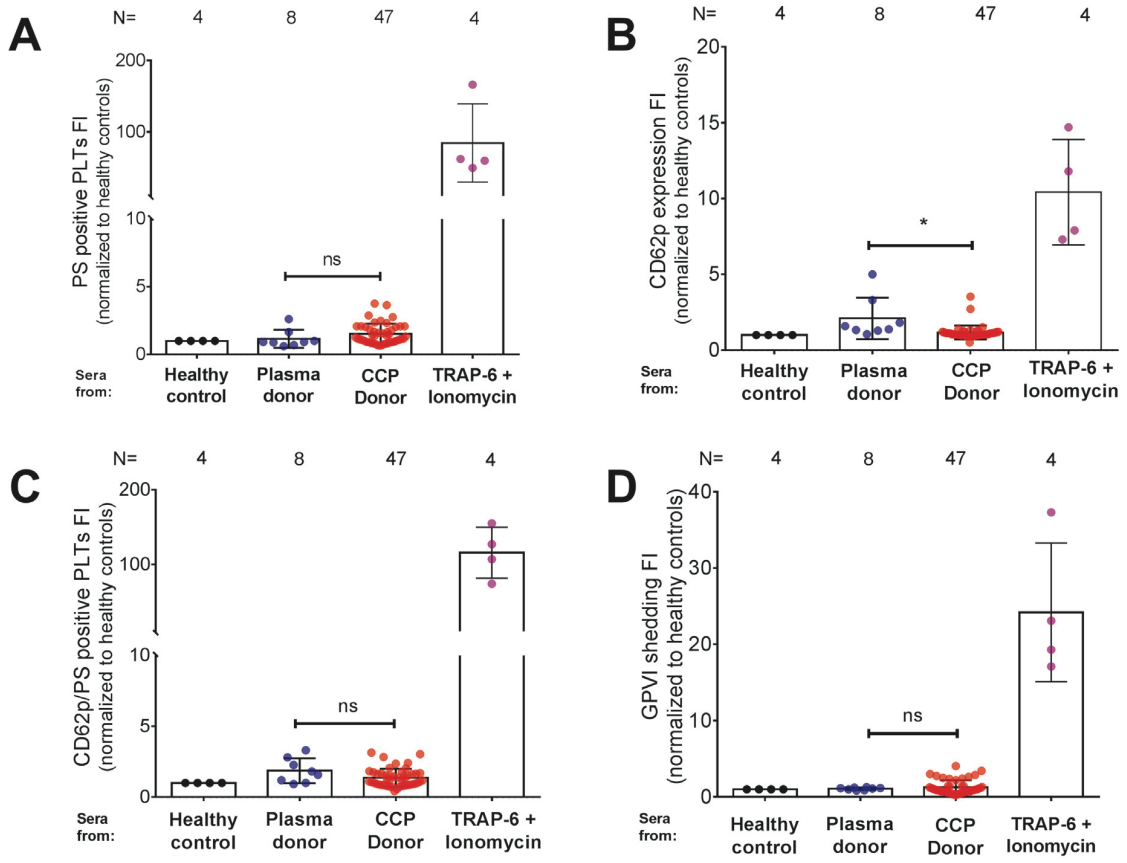

Fig. 4 Serum-induced effects on platelet's phenotype. Sera from healthy controls, plasma donors, and COVID-19 convalescent plasma (CCP) donors were incubated with washed platelets isolated from healthy donors, and PS externalization (**A**), procoagulant platelet formation (**B**), platelet activation (**C**), and GPVI shedding (**D**) were determined (blue: plasma donors; red: CCP donors). Each sample was tested with platelets from one donor. Data are presented as mean ± standard deviation of the fold increase (FI) of healthy donors. ns, not significant; PS, phosphatidylserine; $^{*}p < 0.05$.

Table 2 Viscoelastic properties of clots formed in blood samples from CCP donors

Parameter	Test	Reference rage	Mean	Standard deviation	Lower 95% CI of mean	Upper 95% CI of mean
Clotting time (s)	EX test	38–65	48	4.8	46	49
Maximum clot firmness (mm)	EX test	53–68	62	3.5	60	63
Maximum lysis (%)	EX test	0–12	5.6	2	4.9	6.2
Maximum clot firmness (mm)	FIB test	9–27	15	4.2	14	17
Maximum lysis (%)	tPA test	92–100	95	1.2	94	95
Lysis time (s)	tPA test	<300	185	39	173	198

Abbreviations: CCP, COVID-19 convalescent plasma; CI, confidence interval.

Recent randomized trials showed increased survival in COVID-19 patients receiving CCP with high-dose neutralizing antibodies.[17,18] However, the risk of exacerbation of COVID-19-associated coagulation derangements with CCP as well as transfusion-related complications have also been expressed.[16] Therefore, investigation of the effect of CCP on platelets is of clinical importance. In this study, we showed that sera from CCP donors do not induce a procoagulant phenotype or platelet activation in healthy platelets.

GPVI, the platelet immunoreceptor tyrosine-activating motif receptor for collagen, has been shown to play a prominent role on vascular integrity during inflammation.[24] Bongiovanni and colleagues reported that enhanced GPVI levels during SARS-CoV-2 infection might hint toward a

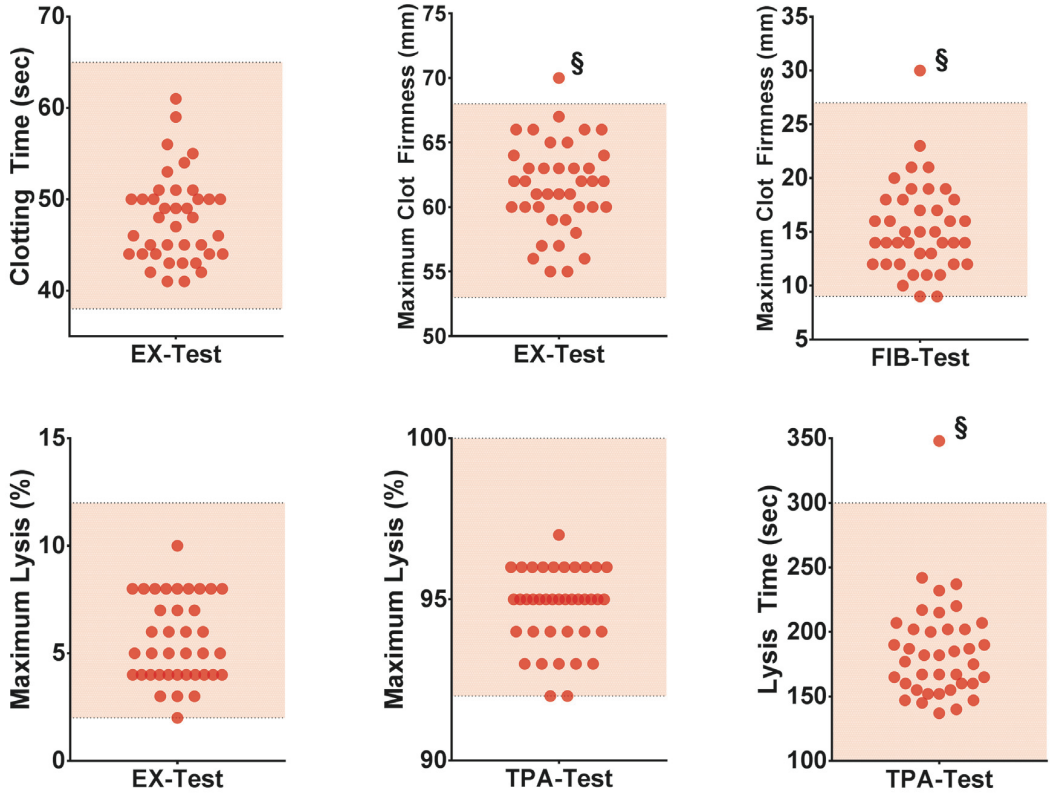

Fig. 5 Viscoelastic properties of clot formed in blood samples from COVID-19 convalescent plasma (CCP) donors. Citrated blood samples were collected and investigated within 2 hours using a viscoelastic test system (ClotPro Enicor GmbH, Munich, Germany). EX test, FIB test, and tPA test results in CCP donors. Pink area illustrates the reference ranges determined by the manufacturer; § denotes case no. 7.

hyperactivated phenotype of platelets during COVID-19, and this might play a role during hypercoagulopathy observed in COVID-19 and hence influence the patient outcome.[25] Apart from antibody-mediated procoagulant platelet generation, other mechanisms have also been reported previously. Costimulation of GPVI along with protease-activated receptors, PAR1 and PAR4, has been shown to increase PS exposure and subsequent procoagulant platelet formation.[26,27] During dual stimulation of GPVI and PAR1/4, a sustained increase in Ca^{2+} levels leads to PS exposure in platelets.[28] Thus, it still remains unclear whether procoagulant platelet formation in severe COVID-19 is caused solely by antibody-mediated mechanisms or also by increased thrombin generation and higher levels of inflammation factors. In our study, no significant differences were observed in GPVI levels of CCP donors as compared with plasma donors at baseline as well as after stimulation with CRP. Similarly, sera from CCP donors as well as from plasma donors induced similar levels of GPVI cleavage from healthy platelets.

As more people recover from COVID-19, discussions have begun over the possibility of post-COVID syndrome or the so-called long-COVID syndrome.[12] A recent epidemiological study demonstrated a significantly increased readmission rate (3.5-fold, 95% CI: 3.4–3.6) and post-discharge mortality rate (7.7-fold, 95% CI: 7.2–8.3) in COVID-19 patients compared with a non-COVID control group.[29] Several retrospective studies reported the rate of thromboembolic events after discharge in patients with COVID-19. The rates of venous thromboembolism and arterial thromboembolism in these studies are 0.2 to 2.6%[30–35] and 0 to 1.9%,[31–33] respectively. These studies are mostly retrospective and lack a comprehensive follow-up of the patients after discharge, which suggests that the true incidence of thromboembolic events could be even higher.

Very few studies investigated cellular and plasmatic components of the coagulation system after acute COVID-19 infection. Most recently, von Meijenfeldt and colleagues reported elevated plasma levels of factor VIII and PAI-1 in COVID-19 patients 4 months after discharge.[13] Townsend et al found increased D-dimer values in both hospitalized and nonhospitalized patients at median of 80.5 days after initial diagnosis.[14] In this study, we used thromboelastometry to evaluate the coagulation status in CCP donors. An increased maximum clot firmness and hypofibrinolysis in thromboelastometry have been reported in hospitalized COVID-19 patients. Hulshof et al reported an increased maximum clot firmness over 80% of all measurements in critically ill COVID-19 patients.[36] In the same study, a sufficient (>90%) clot breakdown was not achieved in more than half of the samples.[36] In a previous study from

our group, an increased maximum clot firmness and extended lysis time in COVID-19 patients admitted to normal wards or to the intensive care has been demonstrated.[37] The fibrin clots in the lungs of COVID-19 patients are more compact, consist of thin fibers, and have small pores compared with fibrin clots in patients with influenza infection.[38] Together with reduced fibrinolytic activity, this altered clot structure might cause thrombus in COVID-19 patients to be resistant to fibrinolysis. Two previous studies have investigated global coagulation status using rotational thromboelastometry after ICU discharge in COVID-19 patients.[15,39] Magomedov et al reported that maximum clot firmness reduced significantly within 12 weeks after discharge in COVID-19 patients.[39] Most recently, Hulshof et al have shown that maximum clot firmness was within normal range in the tissue-type plasminogen activator rotational thromboelastometry in COVID-19 patients 6 months after discharge from ICU.[15] However, although the lysis time in the same test overall significantly reduced 6 months after discharge, it remained over the normal range in 4 of 22 (18%) patients.[15] Similarly, von Meijenfeldt reported a prolonged clot lysis time in COVID-19 patients 4 months after hospital discharge, suggesting a sustained hypofibrinolytic state.[13] In this current study, we evaluated coagulation and fibrinolysis in CCP donors with rotational thromboelastometry. We demonstrated increased hypercoagulability and a hypofibrinolytic state in one donor (2%). This donor did not experience any thrombotic event during COVID-19 infection and thereafter. However, this donor had arterial hypertension and diabetes mellitus type 2. Yürekli et al found an increased maximum clot firmness in diabetic patients than in controls.[40] Comorbidities of this donor might be responsible for the abnormal findings in thromboelastometry. Further studies are needed to better define the risk of thrombosis after discharge and in the convalescent phase in COVID-19 patients. Of note, impaired fibrinolysis is not restricted to COVID-19 or sepsis. In an ongoing study, we observed an increased resistance to clot lysis in some patients with vascular occlusive disorder after stem cell transplantation (unpublished data). The clinical relevance of these findings has not been investigated yet.

Our study has several limitations. First, we did not have blood samples at the time of infection that would allow us to compare the changes in time. Second, this study is focused on platelets and we did not measure plasmatic coagulation factors in blood. Further studies should investigate the alterations in plasma components of coagulation and fibrinolytic system after acute COVID-19 infection. Finally, plasma donors undergo routine clinical examination as required by local regulations which may cause a selection bias since they are relatively younger and healthier compared with other COVID-19 convalescent individuals.

In conclusion, we could neither detect a procoagulant platelet phenotype or increased platelet activation nor a hypercoagulable or hypofibrinolytic state in CCP donors after primary infection. Moreover, sera from CCP donors did not induce significant changes in platelet activation or procoagulant status. These findings support data from clinical studies which indicate that transfusion of CCP to treat or prevent severe COVID-19 is not associated with increased risk of exacerbation of the coagulopathy in COVID-19.

"What Is Known About This Topic?"

- Platelets contribute to the hypercoagulable state in COVID-19 patients.
- Platelets of critically ill COVID-19 patients express a procoagulant phenotype.
- Immunoglobulin G fractions from severe COVID-19 patients induce a procoagulant phenotype in healthy platelets.

"What Does This Paper Add?"

- Procoagulant platelet phenotypes were not observed after mild COVID-19 infection.
- Sera from CCP donors do not activate healthy platelets or induce procoagulant phenotype.

Author Contributions

G.U., A.S., L.P., K.A., P.B., H.K., and T.B. designed the study. G. U. and S.N-H. collected and analyzed the clinical data. G.U., A.S., W.A-K., L.P., K.W., and K.A. performed the experiments. G.U., A.S., P.B., H.K., and T.B. analyzed the data, interpreted the results, and wrote the manuscript. All authors read and approved the manuscript.

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Conflict of Interest

T.B. has received research funding from CoaChrom Diagnostica GmbH, DFG, Robert Bosch GmbH, Stiftung Transfusionsmedizin und Immunhämatologie e.V.: Ergomed, DRK Blutspendedienst, Deutsche Herzstiftung, Ministerium für Wissenschaft, Forschung und Kunst Baden-Wuerttemberg; has received lecture honoraria from Aspen Germany GmbH, Bayer Vital GmbH, Bristol-Myers Squibb GmbH & Co., Doctrina Med AG, Meet The Experts Academy UG, Schoechl Medical Education GmbH, Stago GmbH, Mitsubishi Tanabe Pharma GmbH, Novo Nordisk Pharma GmbH, Leo Pharma GmbH, Swedish Orphan Biovitrum GmbH; has provided consulting services to Terumo; has provided expert witness testimony relating to heparin-induced thrombocytopenia (HIT) and non-HIT thrombocytopenic and coagulopathic disorders. All of these are outside the current work. Other authors declare no competing financial interests.

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3.2. No Correlation between Anti-PF4 and Anti-SARS-CoV-2 Antibodies after ChAdOx1 nCoV-19 Vaccination.

Authors: **Uzun G**, Althaus K, Bakchoul T.

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Summary of the study:

Patients with VITT develop IgG antibodies reactive to PF4 within days after vaccination. Anti-PF4 antibodies are the drivers of the pathophysiology of VITT. However, the mechanism of the development of anti-PF4 antibodies after vaccination is yet to be defined. A non-replicating adenovirus carries DNA of spike protein of SARS-CoV-2 in vector vaccines. Spike protein will be produced by cells infected with adenovirus. Immune reaction to spike protein leads to production of antibodies against different antigenic points of the spike protein. A cross-reactivity between spike protein and PF4 is one of the proposed mechanisms to explain the development of anti-PF4 antibodies in patients with VITT.

To test this hypothesis, we measured anti-PF4/heparin antibodies and the antibodies against SARS-CoV-2 spike protein (Spike Trimer, Receptor Binding Domain [RBD], S1, S2) and nucleocapsid protein using a bead-based Luminex assay in healthcare workers (n=101) two weeks after the first vaccination with ChAdOx1 nCoV-19 and in patients (n=59) with clinically suspected VITT after ChAdOx1 nCoV-19. VITT diagnosis was confirmed with a modified heparin induced platelet aggregation assay (HIPA).

Of the 59 patients with clinically suspected VITT, 20 (34%) were diagnosed with VITT. As expected, the level of anti-PF4/heparin antibodies is higher in VITT patients compared to healthy controls and non-VITT patients. None of the patients with a negative anti-PF4/heparin ELISA had a positive HIPA. The levels of antibodies against Spike Trimer, RBD, S1, and nucleocapsid protein were similar in all groups. The level of anti-PF4/heparin antibodies did not correlate with anti-SARS-CoV-2 antibodies in all study groups.

As a conclusion, we did not find a correlation between antibodies against SARS-CoV-2 and against PF4. Anti-SARS-CoV-2 antibodies are not responsible for the development of VITT.

firmed, fulminant myocarditis that had developed within 2 weeks after Covid-19 vaccination, a direct causal relationship cannot be definitively established because we did not perform testing for viral genomes or autoantibodies in the tissue specimens. However, no other causes were identified by PCR assay or serologic examination.

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No Correlation betwen Anti-PF4 and Anti–SARS-CoV-2 Antibodies after ChAdOx1 nCoV-19 Vaccination

TO THE EDITOR: Vaccine-induced immune thrombotic thrombocytopenia (VITT), also known as thrombosis with thrombocytopenia syndrome, is a rare but potentially fatal complication of vector-based severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccines.[1-3] The clinical picture and the serologic findings in patients with VITT resemble heparin-induced thrombocytopenia.[1-3] Several groups have reported the presence of platelet factor 4 (PF4)–reactive antibodies in patients with VITT.[1-3] IgG from patients with VITT induces platelet activation and aggregation by cross-linking Fcγ receptor IIA on platelets.[1] PF4 is a tetrameric protein that is released from platelet alpha granules on activation. VITT antibodies bind to the heparin-binding site on PF4.[4] The link between vaccination and the formation of anti-PF4 antibodies is yet to be determined. A proposed mechanism includes cross-reactivity between anti–SARS-CoV-2 and anti-PF4 antibodies.[5] In the current study, we investigated the correlation between anti–PF4–heparin antibodies and anti–SARS-CoV-2 antibodies in vaccinated health care workers (healthy controls) and in vaccinated patients with clinically suspected VITT.

The level of anti–PF4–heparin antibodies was measured with the use of an enzyme-linked immunosorbent assay (ELISA), and the levels of antibodies against various antigenic sites of the SARS-CoV-2 spike protein (spike trimer, receptor-binding domain [RBD], subunit 1 [S1] domain, and subunit 2 [S2] domain) and against nucleocapsid protein were measured with the use of a bead-based assay (Luminex). Antibodies were measured in 101 healthy controls 2 weeks after the first dose of ChAdOx1 nCoV-19 (Oxford–AstraZeneca) had been administered and in 59 patients with clinically suspected VITT between 11 and 22 days after the first dose had been administered. The ability of the sera to activate platelets was tested with the use of a modified heparin-induced platelet aggregation assay. Details of the methods are provided in the Supplementary Appendix, available with the full text of this letter at NEJM.org.

VITT was confirmed in 20 of 59 patients (34%) on the basis of a positive PF4 ELISA and a positive modified heparin-induced platelet aggregation assay (Table S1 in the Supplementary Appendix). The level of anti–PF4–heparin antibodies was higher among the patients with confirmed VITT than among the healthy controls and the patients who did not have VITT (Fig. 1A and Table S1). The 95% confidence intervals for the differences between the groups are presented in Table S2; these confidence intervals were not adjusted for multiplicity and

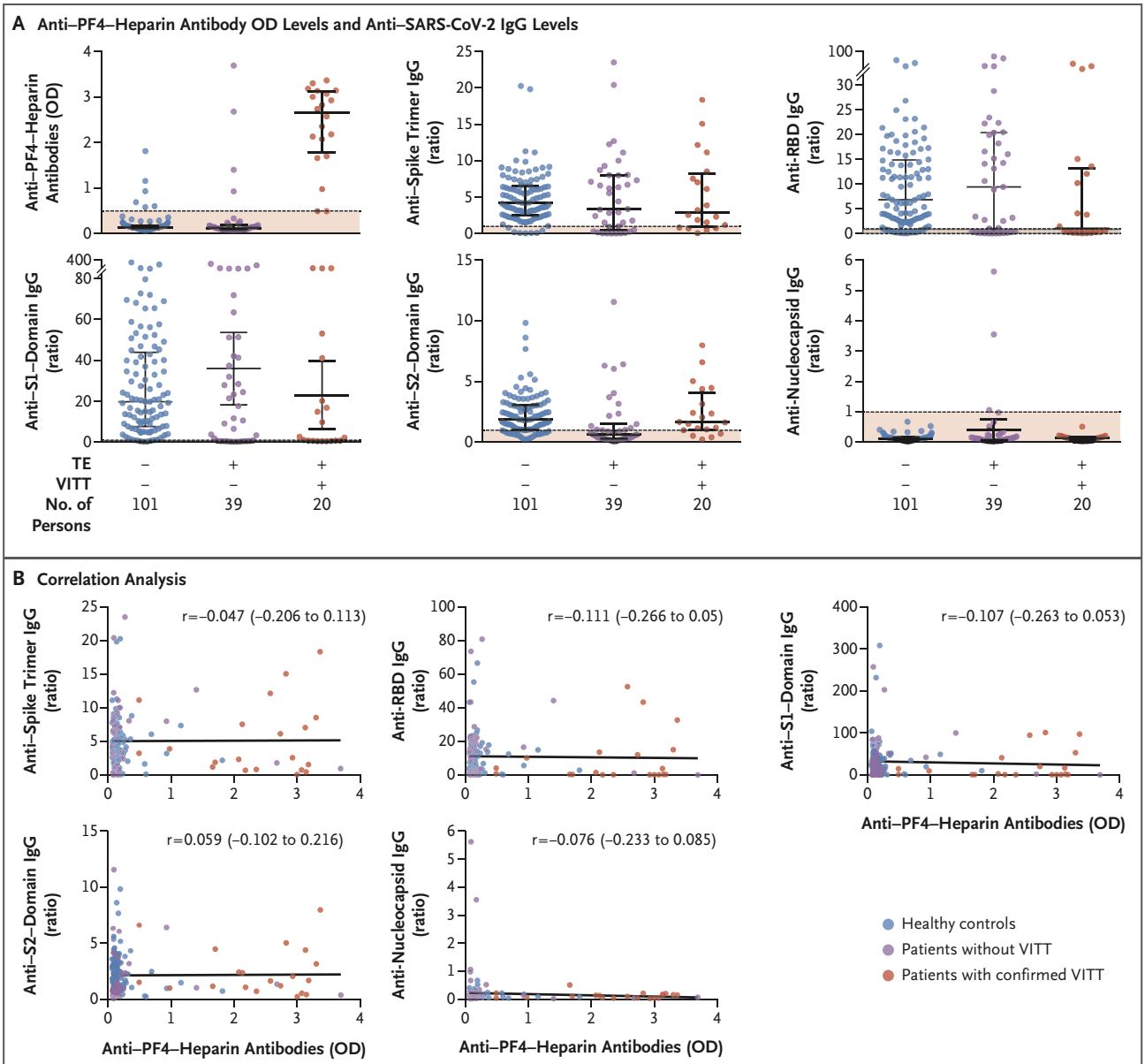

Figure 1. Antibody Levels and Correlation Analysis.

Panel A shows the anti–platelet factor 4 (PF4)–heparin antibody optical density (OD) levels and anti–SARS-CoV-2 IgG levels against spike trimer, receptor-binding domain (RBD), subunit 1 (S1) domain, subunit 2 (S2) domain, and nucleocapsid protein in the healthy controls, the patients without vaccine-induced immune thrombotic thrombocytopenia (VITT), and the patients with confirmed VITT. Panel B shows the correlation analysis between the level of anti–PF4–heparin antibodies and the level of anti–SARS-CoV-2 IgG antibodies against spike trimer, RBD, S1 domain, S2 domain, and nucleocapsid protein in the healthy controls, the patients without VITT, and the patients with confirmed VITT. Anti–PF4–heparin antibodies were quantified with the use of an enzyme-linked immunosorbent assay. Anti–SARS-CoV-2 IgG antibodies were quantified with the use of a bead-based assay (Luminex). Each dot in the figure represents an individual person, and the numbers of persons tested is shown in Panel A. The dashed lines in Panel A indicate the cutoff values, and the solid lines in Panel B indicate the correlation coefficient (r). TE denotes thrombotic event.

therefore cannot be used to infer effects. The levels of antibodies against spike trimer, RBD, S1 domain, and nucleocapsid protein were similar in the three groups. The levels of antibodies against S2 domain were lower among the patients who did not have VITT than among the persons in the other two groups. We did not find any correlation between the level of anti–

PF4–heparin antibodies and the level of anti–SARS-CoV-2 IgG antibodies in any of the three groups (Fig. 1B and Table S3).

Moreover, the levels of anti–SARS-CoV-2 antibodies did not differ substantially between vaccinated persons without complications (i.e., the healthy controls) and patients with VITT. Similarly, Scully et al.[2] reported that the levels of antibodies to spike protein and RBD in patients with VITT were in the same range as those of the recipients of one dose of ChAdOx1 nCoV-19. Furthermore, our study did not show a correlation between anti–PF4–heparin antibodies and anti–SARS-CoV-2 antibodies in patients with VITT. Although a preprint publication suggested that spike protein shares an immunogenic epitope with PF4, purified anti-PF4 and anti–PF4–heparin antibodies from patients with VITT did not show cross-reactivity to recombinant SARS-CoV-2 spike protein.[5]

Our results do not support the hypothesis that the immune response against SARS-CoV-2 proteins leads to the formation of anti-PF4 antibodies in patients with VITT. However, we cannot exclude the possibility of cross-reactivity between a subgroup of anti–SARS-CoV-2 antibodies and a subgroup of anti-PF4 antibodies. A better understanding of the link between vaccination and VITT is necessary for the development of more targeted therapies.

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Effectiveness of an Inactivated SARS-CoV-2 Vaccine

TO THE EDITOR: In the article by Jara and colleagues (Sept. 2 issue)[1] reporting a study of real-world efficacy of the CoronaVac vaccine against Covid-19, the authors provide a graph showing the incidences of infection among fully vaccinated, partially vaccinated, and unvaccinated participants. From this graph, the reader can conclude that partially vaccinated persons are especially vulnerable, since the incidence of infection is higher among them than among unvaccinated persons. This fact could have some causal explanation, but I suggest that it is purely a statistical artifact.

The authors mention that vaccination status is a time-dependent variable, so for the efficacy calculations they correctly follow Thompson et al.,[2] using person-days, rather than numbers of persons, for the calculation of hazard ratios. A person's vaccination status may vary over time, so it is not possible to separate participants into groups according to vaccination status. Still, if one tries to do this, as the authors do, all the persons who become infected during the interval between receiving the first and second doses of vaccine will be labeled as partially vaccinated, which severely biases the incidence of infection in this "group." I think that the readers of the *Journal* would benefit from an explanation of this misleading and slightly frightening graph in this otherwise very important article.

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No potential conflict of interest relevant to this letter was reported.

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Uzun G, Althaus K, Bakchoul T. No correlation between anti-PF4 and anti–SARS-CoV-2 antibodies after ChAdOx1 nCoV-19 vaccination. N Engl J Med 2021;385:1334-6. DOI: 10.1056/NEJMc2111305

Supplementa**ry** Appendix

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Methods

Sera were collected from health care workers at the blood donation center in Tübingen two weeks after the vaccination with ChAdOx1 nCoV-19. Additionally, sera of the patients with clinically suspected vaccine-induced immune thrombotic thrombocytopenia (VITT) were also analyzed.

The diagnosis of VITT was serologically confirmed according to the recommendation of the International Society of Thrombosis and Hemostasis Scientific and Standardization Committee Platelet Immunology Subcommittee.[1]

We measured PF4/heparin antibodies using a commercial IgG- enzyme-linked immunosorbent assay (ELISA) to detect IgG antibodies against PF4/heparin (Hyphen Biomed, Neuville-sur-Oise, France). The ability of sera to activate platelets was tested using a modified functional assay, heparin induced platelet aggregation assay (HIPA), as previously described.[2] In brief, serum was tested with washed platelets from four different healthy donors in the absence (buffer alone) or in the presence of heparin (0.2 IU/mL and 100 IU/mL). In addition, platelets were preincubated with PF4 (50 µg/mL). Reactions were placed in microtiter wells containing spherical stir bars and stirred at approximately 500 revolutions per minute (rpm). Wells were examined optically at five-minutes interval for loss of turbidity. A serum was considered reactive (positive) if a shift from turbidity to transparency occurred within 30 min in at least two platelet suspensions. Observation time was 45 min. Each test included a diluted serum from a patient with heparin induced thrombocytopenia (HIT) as a weak positive control, collagen (5µg/mL) as strong positive control and a serum from a healthy donor as a negative control.

COVID-19 antibodies were measured with a multiplex assay (MULTICOV AB, NMI, Reutlingen, Germany) with the FLEXMAP 3D® system (Luminex Corporation, Austin, USA) according to manufacturer's recommendations.[3] In brief, 25 µL of the 1:200 diluted samples

were incubated with bead mix for 2 hours at 21 °C on a microplate shaker. Unbound antibodies were removed using a magnetic plate separator and the beads were washed three times with 100 µL of wash buffer (1x PBS, 0.05% (v/v) Tween20). R-phycoerythrin labeled goat-anti-human IgG (Dianova, Cat# 109-116-098, Lot#148837, used at 3 µg/mL) antibodies were added and the plate was incubated for 45 min at 21 °C. For each sample, a single measurement was performed. Quality control samples were also measured duplicate. Readout was done using a Luminex FLEXMAP 3D instrument and the Luminex xPONENT Software 4.3 (settings: sample size: 80 µL, 50 events, Gate: 7,500–15,000, Reporter Gain: Standard PMT). Antibody levels were calculated by dividing the mean fluorescence intensity (MFI) values of each sample by the mean MFI value of quality control samples for each antigen separately.

The study was conducted in accordance with the declaration of Helsinki. The study protocol was approved by the Institutional Review Board of the University of Tuebingen (236/2021BO1). We used GraphPad Prism, Version 7.0 (GraphPad, La Jolla, USA) for statistical analysis. Normality was tested using D'Agostino & Pearson normality test. The mean and 95% confidence interval of the difference between the groups were calculated using ANOVA with Tukey's multiple comparisons test and presented in Table S2. The confidence intervals have not been adjusted for multiplicity and cannot be used to infer effects. Correlation was analyzed using Spearman's rank correlation test. Data are presented as median (interquartile range) or as mean (95% confidence interval).

Results

We analyzed sera from health care workers (n=101) who were received their first vaccination with ChAdOx1 nCoV-19 (vaccinated control group). Sera from 59 patients have been referred to us during the study period (08 March 2021 to 01 June 2021) for the diagnosis of VITT. For patients in the suspected and confirmed VITT cohorts, the median duration

(interquartile range) after vaccination and symptom begin was 9 days (7-14 days). The median time (interquartile range) from vaccination to blood collection for testing was 15 days (11-22 days) in patients with clinically suspected VITT. We confirmed VITT in 20 (34%) patients and 39 (56%) patients did not have VITT.

A positive PF4/heparin ELISA was obtained in 6/101 (6%) vaccinated controls. Sera from convalescent COVID-19 patients who have anti-Spike antibodies revealed no reaction against PF4/heparin in ELISA (data not shown). Anti-PF4/heparin values was higher in positive VITT cases compared to both vaccinated controls and negative cases. After high dose heparin, PF4 reactivity diminished in all positive samples (data not shown). Although some patients had a strong reaction on ELISA, modified HIPA was negative in these cases. Antibodies to nucleocapsid protein of SARS-CoV-2 virus has been detected in four patients in the VITT negative group. It is possible that these patients had a recent COVID-19 infection. On the other hand, nucleocapsid antibodies were not detected in samples from vaccinated controls and in those from patients with VITT. The antibody levels against Spike Trimer, RBD, S1 domain and nucleocapsid protein were similar between all three groups (Table S1, Table S2, Figure 1A). However, anti-S2 antibodies were lower in VITT negative cases compared to vaccinated controls and positive VITT cases (Table S1, Figure 1A).

Table S1 Demographic characteristics and serological findings in study groups

	Vaccinated control		VITT			
			Negative		positive	
n	101		39		20	
	median (interquartile range)	mean (95% confidence interval)	median (interquartile range)	mean (95% confidence interval)	median (interquartile range)	mean (95% confidence interval)
Age (years)	44 (36-55,5)	44 (41.9-46.8)	55 (43-64)	54.3 (49.1-59.5)	42,5 (30,5-60,8)	43.2 (36.3-50)
Gender (m/f)	23/78		12/27		8/12	
Anti-PF4 (OD)	0.14 (0.1-0.18)	0.19 (0.15-0.24)	0.12 (0.09-0.19)	0.34 (0.11-0.57)	2.66 (1.79-3.12)	2.36 (1.94-2.78)
Anti-Spike Trimer (Ratio)	4.2 (2.5-6.5)	4.92 (4.24-5.6)	3.4 (0.5-8)	5.27 (3.49-7.06)	2.9 (0.9-8.3)	5.17 (3.49-2.77)
Anti-RBD (Ratio)	6.9 (2.8-14.8)	10.1 (7.98-12.2)	9.4 (0.3-20.4)	14.1 (8.03-20.3)	1.1 (0.2-13.2)	9.58 (2.33-16.8)
Anti-S1 domain (Ratio)	19.9 (7.7-44)	31.3 (23.1-39.5)	17.7 (0.7-51.2)	36 (18.3-53.7)	2.4 (0.7-35.9)	23 (6.52-39.5)
Anti-S2 domain (Ratio)	1.9 (1-3.1)	2.27 (1.93-2.6)	0.6 (0.3-1.5)	1.61 (0.84-2.38)	1.7 (1-4.1)	2.47 (1.47-3.48)
Anti-Nucleocapsid protein (Ratio)	0.11 (0.08-0.16)	0.14 (0.12-0-16)	0.12 (0.05-0.24)	0.42 (0.08-0.75)	0.12 (0.05-0.18)	0.13 (0.08-0.18)

Table S2 The mean and 95% confidence interval of the difference between the groups

	Vaccinated control vs. VITT negative		Vaccinated control vs. VITT positive		VITT negative vs. VITT positive	
	mean	95% Confidence interval	mean	95% Confidence interval	mean	95% confidence interval
Anti-PF4 (OD)	-0.146	-0.37 to 0.08	-2.17	-2.46 to -1.88	-2.02	-2.35 to -1.69
Anti-Spike Trimer	-0.35	-2.26 to 1.56	-0.349	-2.83 to 2.13	0.001	-2.79 to 2.79
Anti-RBD	-4.06	-10.2 to 2.06	0.508	-7.43 to 8.45	4.57	-4.36 to 13.5
Anti-S1 domain	-4.74	-24.5 to 15	8.26	-17.4 to 33.9	13	-15.9 to 41.9
Anti-S2 domain	0.659	-0.207 to 1.52	-0.204	-1.33 to 0.92	-0.863	-2.13 to 0.4
Anti-nucleocapsid	-0.277	-0.51 to -0.046	0.0075	-0.29 to 0.31	0.285	-0.05 to 0.62

Table S3 Analysis of correlation between anti-PF4 and anti-SARS-coV-2 antibody levels in each group.

	Vaccinated control		VITT			
			Negative		positive	
	r	95% confidence interval	r	95% confidence interval	r	95% confidence interval
Anti-Spike Trimer	-0.149	-0.34 to 0.054	0.218	-0.114 to 0.506	0.0165	-0.441 to 0.467
Anti-RBD	-0.115	-0.309 to 0.0877	0.13	-0.203 to 0.436	0.0571	-0.407 to 0.498
Anti-S1 domain	-0.155	-0.346 to 0.0473	0.172	-0.161 to 0.47	0.114	-0.359 to 0.54
Anti-S2 domain	-0.19	-0.377 to 0.0112	0.433	0.127 to 0.664	0.0316	-0.428 to 0.478
Anti-nucleocapsid	-0.035	-0.234 to 0.167	-0.112	-0.421 to 0.22	0.109	-0.363 to 0.536

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3.3. The use of IV immunoglobulin in the treatment of vaccine-induced immune thrombotic thrombocytopenia.

Authors: **Uzun G**, Althaus K, Singh A, Möller P, Ziemann U, Mengel A, Rosenberger P, Guthoff M, Petzold GC, Müller J, Büchsel M, Feil K, Henkes H, Heyne N, Maschke M, Limpach C, Nagel S, Sachs UJ, Fend F, Bakchoul T.

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Summary of the study:

Intravenous immunoglobulin (IVIG) is used in the treatment of immune mediated platelet disorders such as immune thrombocytopenia and heparin induced thrombocytopenia. IVIG blocks the Fc receptor on platelet surface. Platelet activation in VITT occurs via Fcgamma RIIA (FcγRIIa) receptors. It has been shown that IVIG can mitigate platelet activation in HIPA and flow cytometry induced by sera from VITT patients.. However, clinical experience on the use of IVIG in patients with VITT is limited. This study aimed to analyze the clinical response to IVIG therapy in patients with VITT. Furthermore, the effect of IVIG therapy on anti-PF4 antibody levels and platelet activation has been investigated.

Five patients were included in this retrospective study. Laboratory parameters such as platelet count, D-dimer and anti-PF4 antibody levels were recorded. Additionally, sera of the patients were used to analyze platelet activation in HIPA and procoagulant platelets in flow cytometry. IVIG was administered at a dose of 1 g/KG body weight 2 to 5 days.

In this study, we observed rapid response (platelet count ≥ 100×10^9/L) in 4 patients within 96h. One patient had a platelet response (platelet count ≥ $30x10^9$/L and at least 2-fold increase the baseline count) within 72h after IVIG therapy. D-dimer levels decreased after IVIG therapy. Although anti-PF4 antibody levels did not change after IVIG therapy, procoagulant platelet generation was reduced.

In conclusion, IVIG interferes with the pathogenic anti-PF4 antibodies by competing with them to bind to FcγRIIa receptors which might be *in vivo* associated with reductions in platelet activation and disseminated intravascular coagulation. The adjunct use of IVIG together with anticoagulation is recommended in the acute treatment of VITT to mitigate the progression of the disease.

SF3B1 MT patients compared with 40% among those with WT.[5] Recent data from a phase 2 clinical study with imetelstat suggest potential preferential and disease modifying activity among small number of *SF3B1* MT patients.[6]

We reclassified our patients based on IWG *SF3B1* new proposal criteria.[1] Notably, 175 patients were classified as MDS-*SF3B1* by the new proposed criteria, 145 patients with *SF3B1* MT not meeting the new proposed criteria, and 1412 *SF3B1* WT MDS patients (Table 1). The median OS was 120 months (95% CI, 77-164 months), 55 months (95% CI, 42-69 months), and 31.5 months (95% CI, 28-35 months), respectively ($P < .005$; Figure 1C). The median leukemia-free survival was not reached among all *SF3B1* MT patients compared with 58 months among *SF3B1* WT patients ($P < .005$) (Figure 1D). The rate of AML transformation was 4.7%, 22%, and 38%, respectively ($P < .005$). t-MDS was observed in 10%, 9%, and 17% of the 3 above-mentioned groups. Excluding t-MDS, the median OS was 142, 57, and 36 months, respectively ($P < .005$). There was no difference in response rates to erythroid stimulating agents, hypomethylating agents, and lenalidomide between patients classified as *SF3B1* by IWG new criteria compared with other *SF3B1* MT MDS.

In summary, we confirm and validate the findings reported recently by Malcovati et al that *SF3B1* MT MDS should be classified as a unique disease entity based on the new proposal criteria. Furthermore, we demonstrate that *SF3B1* MT retained favorable prognostic value in the context of t-MDS and worse outcome among patients with isolated del(5q), and we compliment the IWG findings by reporting responses to current available therapies based on *SF3B1* MT status.

Authorship

Contribution : R.K. wrote the manuscript, designed the research study, and analyzed and interpreted data; V.V., O.C., N.A.A., and D.S. collected and analyzed data and approved the final manuscript; E.P. designed the research study and approved the final manuscript; and D.A.S. reviewed data, designed the research study, and approved the final manuscript.

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Footnote

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TO THE EDITOR:

The use of IV immunoglobulin in the treatment of vaccine-induced immune thrombotic thrombocytopenia

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The ChAdOx1 nCoV-19 is a recombinant chimpanzee adenoviral vector vaccine encoding the spike glycoprotein of severe acute respiratory syndrome coronavirus 2, which has a good efficacy rate and safety profile.[1] Over the past 2 months, concern has been raised over reported thrombotic events associated with thrombocytopenia after ChAdOx1 nCoV-19 vaccination, a complication called vaccine-induced immune thrombotic thrombocytopenia (VITT).[2–6] The pathophysiology of VITT is still unclear but seems to be similar to spontaneous autoimmune heparin-induced thrombocytopenia (aHIT).[2,7] In fact, as in aHIT, VITT patients develop platelet factor-4 (PF4) antibodies without any recent exposure to heparin. These antibodies are able to activate platelets and induce procoagulant platelet phenotype via cross-linking the Fc γ receptor IIA on platelet surface. IV immunoglobulin (IVIG) has been successfully used in the treatment of spontaneous aHIT.[8,9] We and others have recently shown that IVIG inhibits the in vitro induction of procoagulant platelet phenotype by sera from VITT patients.[2,7] Herein, we report our clinical experience on the use of IVIG in the management of VITT and present novel laboratory analysis of the effect of IVIG therapy on anti-PF4 antibody level and platelet activation in VITT patients.

The study cohort consisted of patients who were admitted to our hospitals between February 1 and May 5, 2021 with suspected VITT due to neurological or hematological symptoms after first immunization with ChAdOx1 nCoV-19 (Vaxzevria; AstraZeneca, London, United Kingdom). The diagnosis of VITT was serologically confirmed according to the recommendations of the International Society on Thrombosis and Haemostasis Scientific and Standardization Subcommittee on Platelet Immunology,[10] using an immunoglobulin G (IgG)-enzyme immune assay (EIA) to detect IgG antibodies against PF4 (Hyphen Biomed, Neuville-sur-Oise, France). The ability of the sera to activate platelets was tested using a modified heparin-induced platelet aggregation assay as previously described.[7] Sera-induced procoagulant platelets were analyzed using flow cytometer as previously described.[11] For more details, see the supplemental material, available on the

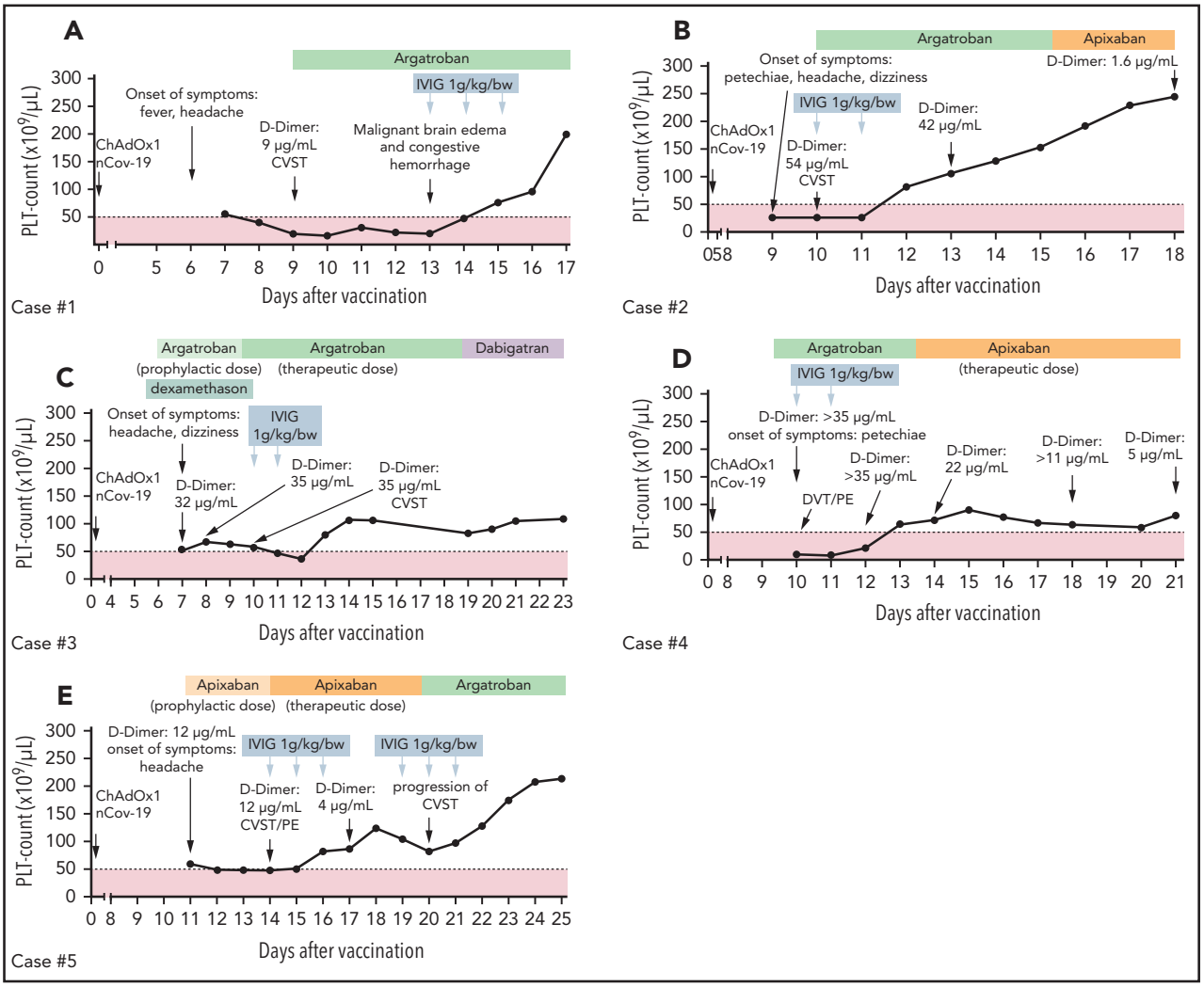

Figure 1. Individual course of the platelet counts and therapies. Five cases (A-E) of VITT after severe acute respiratory syndrome coronavirus 2 vaccination were identified. Patients were treated with nonheparin anticoagulation (argatroban, green blocks; danaparoid, lavender blocks; direct oral anticoagulants, orange blocks) combined with IVIG. Patients receiving therapeutic anticoagulation with platelet counts below 50 × 10^9/L (dashed line) were considered to be at enhanced risk for major hemorrhage. CSVT, cerebral sinus vein thrombosis; DVT, deep vein thrombosis; PE, pulmonary embolism; PLT, platelet.

Blood Web site. The study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Institutional Review Board of the University of Tuebingen (236/2021BO1). We used GraphPad Prism, version 7.0 (GraphPad, La Jolla, CA) for statistical analysis. A value of $P < .05$ was accepted as statistically significant.

Five patients (3 females) with a median age of 47 years (range, 20-57) were included in this study. The duration between vaccination and hospital admission was 7 to 9 days. All patients had severe thrombocytopenia ($41.2 \pm 9.7 \times 10^9$/L; range, 10-60; Figure 1A-E) and increased D-dimer (9 µg/mL or higher; range, 9-54). At admission, several thrombotic events, including cerebral venous sinus thrombosis (CVST; 4 patients, cases 1-3 and 5), pulmonary embolism (2 patients, cases 4 and 5), and deep vein thrombosis (case 4), were detected. Detailed case descriptions and patient characteristics are given in the supplemental methods and in supplemental Table 1A. VITT diagnosis was confirmed by detecting anti-PF4 IgG antibodies in EIA (optical density [OD] 2.98 ± 0.23; range, 2.07-3.36), platelet activation in the modified heparin-induced platelet aggregation assay (median time to platelet aggregation, 5 minutes; range, 5-5 minutes), and formation of procoagulant platelets ($CD62p/PS^+$ platelets mean: 45 ± 7; range, 23-66). Laboratory investigations at admission and after IVIG therapy are presented in supplemental Table 1B.

All patients received parenteral anticoagulation with argatroban (n = 4) or danaparoid (n = 1), and 1 patient (case 5) initially received apixaban. Two patients (cases 3 and 5 on a prophylactic dose of argatroban and apixapan, respectively) developed a new thromboembolic complication at day 4 and 3 of hospitalization (before IVIG administration), respectively. Anticoagulation was continued in these patients with argatroban in a therapeutic dosage (Figure 1C,E).

IVIG was administered at a dose of 1 g/kg body weight for 2 to 5 days. Median of total IVIG dose was 140 g (range, 95-600 g). Absolute platelet increment was $32.6 \pm 17.1 \times 10^9$/L within 48 hours (*P* vs baseline, .12) and $94.2 \pm 23.3 \times 10^9$/L within 72 hours after IVIG (*P* vs baseline, .01; Figure 2A). A complete platelet response (platelet count $\geq 100 \times 10^9$/L) was achieved in 4 patients within 96 hours. One patient had a platelet response (platelet count $\geq 30 \times 10^9$/L and at least twofold increase the baseline count) within 72 hours after IVIG therapy. From a clinical perspective, increasing the platelet count is important in thrombocytopenic patients requiring therapeutic anticoagulation. This is most critical when thrombosis occurs at unusual sites, such as CVST, because of the increased mortality risk due to hemorrhagic transformation after an arterial stroke or CVST. In our cohort, 1 patient (case 1) suffering from VITT-associated CVST had postthrombotic hemorrhage during the thrombocytopenic period, prior to receiving IVIG.

Successful use of IVIG in the treatment of aHIT has been reported.[8,9,12] Mainly because of similarities between aHIT and VITT, recent societal guidelines recommend the use of IVIG in VITT with the assumption that IVIG could mitigate the platelet activation induced by anti-PF4 antibodies and thus reduce the platelet consumption and the development of new thrombosis.[10,13,14] However, concerns of increased new thrombotic events limit its use.[15] Serial D-dimer levels were available from 3 cases, and all of them showed a decrease within 72 hours after IVIG therapy (Figure 1). We observed progression of CVST in 1 patient (case 5). Other patients receiving nonheparin anticoagulation at therapeutic doses combined by IVIG did not develop new thrombosis, indicating sufficient antithrombotic efficacy. Similarly, Thaler et al successfully used IVIG (1 g/kg for 2 consecutive days) and argatroban in a patient with VITT.[16] Tiede et al reported a positive platelet response in 3 VITT patients after IVIG therapy (1 g/kg for 2 consecutive days).[5] However, 2 patients developed new thromboembolic events (extensive splanchnic vein thrombosis and popliteal artery occlusion).[5] New thromboembolic events are common in patients with VITT.[5-7] Therefore, clinicians should pay attention to dynamic changes in clinical as well as laboratory parameters and exercise extra vigilance in VITT patients in order to detect new thromboembolic events in a timely manner.

To assess the mechanism by which high-dose IVIG downregulates hypercoagulability in VITT, we analyzed the ability of VITT patients' sera to generate procoagulant platelets before and after IVIG therapy. The reactivity in PF4 EIA did not change significantly after IVIG administration (n = 4, 3.21 ± 0.06 OD vs 3.18 ± 0.08 OD; *P*: .798; supplemental Table 1B). On the other hand, the

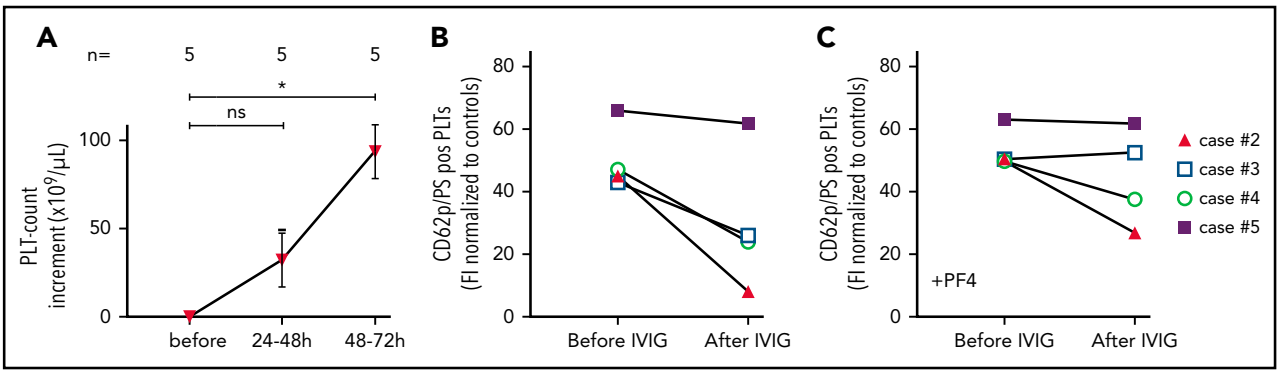

Figure 2. Effect of IVIG therapy on PLT count and procoagulant platelets. Platelet count increment (A) and procoagulant platelets after IVIG therapy (B-C). Procoagulant platelets (CD62P/Phosphatidylserine $[PS]^+$) were analyzed in patients before and after IVIG therapy via Annexin V-FITC and CD62p-APC antibody staining. Where indicated, PLTs were pretreated with PF4 (C). Data are presented as fold increase compared with healthy control. ns, not significant. $*P < .05$. The number of sera tested is reported in each graphic.

ability of the sera from VITT patients to induce procoagulant platelets reduced after IVIG therapy in 3 cases in the absence and in 2 out of 4 cases in the presence of PF4 (Figure 2B-C; supplemental Table 1B). Noteworthy, diluted sera showed specific platelet activation only in the presence of PF4 (supplemental Figure 1A-D). These data suggest that IVIG interferes with the pathogenic anti-PF4 antibodies by competing with them to bind to Fc γ receptor IIA receptors, which might be in vivo associated with reductions in platelet activation and disseminated intravascular coagulation. The later ones are confirmed in our study by the rapid response in platelet count and decrease in D-dimer levels.[7,9] However, the effect of IVIG on other cells cannot be ruled out as another explanation for the observed therapeutic benefit.

In summary, we showed that high-dose IVIG inhibits antibody-mediated procoagulant platelet generation, rapidly increases the platelet count, and finally, deescalates the hypercoagulable state in VITT. Adjunct use of IVIG can be recommended as a therapeutic option to prevent disease progression.

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Authorship

Contribution: G.U., K.A., and T.B. designed the study; P.M., U.Z., A.M., P.R., M.G., G.C.P., J.M., M.B., K.F., H.H., N.H., M.M., C.L., and S.N. were responsible for the treatment of the patients and collected and analyzed the clinical data; G.U., K.A., U.J.S., F.F., and T.B. reviewed medical reports; G.U., K.A., and A.S. performed the experiments; G.U., K.A., A.S., and T.B. analyzed the data, interpreted the results, and wrote the manuscript; and all authors read and approved the manuscript.

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Footnotes

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Data may be requested for academic collaboration from the corresponding author.

The online version of this article contains a data supplement.

There is a *Blood* Commentary on this article in this issue.

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TO THE EDITOR:

Immune thrombocytopenic purpura after vaccination with COVID-19 vaccine (ChAdOx1 nCov-19)

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Coronavirus disease 2019 (COVID-19) is an ongoing pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).[1] Newly developed vaccines are powerful tools for interrupting the ongoing dissemination of SARS-CoV-2. Because the first vaccines were approved for clinical use within a short period of time, the available data on adverse effects in relation to vaccination for SARS-CoV-2 are still limited.[2] One available vaccine is the adenovirus vector–based ChAdOx1 nCov-19 (also known as AZD122) from AstraZeneca.[3,4] As this vaccine is considered to be safe,[2-4] a new condition named vaccine-induced immune thrombotic thrombocytopenia (VITT) syndrome was reported in relation to previous administration of ChAdOx1 nCov-19[5–7] and Ad26.COV2.S (Janssen Pharmaceuticals).[8]

VITT is associated with thrombocytopenia accompanied by thrombosis and antibodies against platelet factor 4 (PF4) in the serum, but it differs from postvaccination immune thrombocytopenic purpura (ITP), a phenomenon associated with both live and inactivated vaccines.[9-11] To our knowledge, however, ITP has not yet been described as being associated with administration of ChAdOx1 nCov-19 vaccine. Here, we report our findings in a cohort of 4 patients who presented with severe thrombocytopenia in the absence of thrombosis a short time after receiving a ChAdOx1 nCoV-19 adenoviral vector vaccine at our (single-center) institution.

We conducted retrospective and prospective analyses of patients who received treatment in our institution for ITP associated with ChAdOx1 nCoV19 vaccination within a 19-day period in May 2021. We evaluated patients' records and confirmed the diagnosis of ITP. Patients' demographic and clinical characteristics are presented in Table 1. Detailed case descriptions are provided in the supplemental information (available on the *Blood* Web site). Informed consent was provided by each patient, and monocentric data acquisition was in line with local requirements according to the Hamburg Hospital Act (HmbKHG) §12 and in accordance with the Declaration of Helsinki.

The patients were White women and men between 64 and 72 years of age from Germany. They presented 2 to 15 days after receiving the first dose of ChAdOx1 nCov-19 with severe symptomatic thrombocytopenia of $\leq 6 \times 10^9$/L cells. Patients 1 and 2 had a medical history of thyroid disorders (autoimmune thyroiditis and latent autoimmune hypothyroidism, respectively), patient 3 was previously diagnosed with minor thrombocytopenia ($\sim 60 \times 10^9$/L), and patient 4 reported preexisting conditions, including chronic obstructive pulmonary disease and arterial hypertension. Initial symptoms included petechiae (patients 1, 3, and 4), hematomas (patient 1), headaches (patient 2), hyposphagma (patient 3), and epistaxis (patient 4). All patients reported that prior vaccinations against seasonal influenza (all patients), pneumococcus, and rubella (patients 2 and 3) were well tolerated. At admission, all patients were SARS-CoV2 negative according to a polymerase chain reaction test. In addition, patient 2 had a serologic test that was positive for SARS-CoV-2 spike receptor-binding domain and SARS-CoV-2 spike trimer immunoglobulin G (IgG) or IgM antibodies and negative for SARS-CoV2 nucleocapsid IgG or IgM antibodies (Elecsys Anti-SARS-CoV2, electrochemiluminescence immunoassay [ECLIA], Roche), indicating active immune response after vaccination.

The patients did not present with signs or symptoms of thrombotic events, and no antibodies to PF4-polyanion complexes were detected in enzyme-linked immunosorbent assay (Asserachrom HPIA-IgG, Stago), so the patients discussed were not associated with VITT. In addition, a magnetic resonance imaging scan was conducted in patient 2 to rule out intracranial bleeding and cerebral vein or sinus thrombosis because she presented with headaches. A bone marrow biopsy from patient 2 revealed an increased megakaryocyte count with no signs of malignancy. No clinical signs or symptoms of infection were present in any of the patients. On the basis of presentation and after ruling out differential diagnoses, ITP was diagnosed in all 4 patients.[12]

The patients received corticosteroids (prednisolone 100 mg/day; initial bolus of 250 mg in patient 1) as initial treatment (Figure 1). No further treatment was initiated in patients 3 and 4 because of a quick recovery of the platelet counts that increased to 23×10^9/L and 98×10^9/L after 4 and 6 days, respectively. Patients 3 and 4 were discharged for outpatient follow-up with a steroid reduction plan. Intravenous immunoglobulin (IVIG) was administered to patients 1 (0.4 g/kg) and 2 (1 g/kg) (Figure 1). Patient 2 did not respond, so dexamethasone (40 mg) was initiated for 7 more days. Platelet counts increased in patients 1 (142×10^9/L)

Supplemental material

Methods:

Preparation of washed platelets

Whole blood from healthy donors was centrifuged at 120g for 20 minutes (min*) without break at room temperature (RT). The supernatant platelet rich plasma (PRP) was gently collected and immediately supplemented with apyrase (5 µL/mL PRP, [Sigma-Aldrich, St. Louis, USA]) and prewarmed anticoagulant-citrate dextrose solution A (ACD-A)(111 µL/mL PRP). Subsequently, platelets were separated from PRP via centrifugation (650g, 7 min*, RT, without brake), resuspended in 5 mL of wash-solution (modified Tyrode buffer: 5 mL bicarbonate buffer, 20 percent (%) bovine serum albumin, 10% glucose solution, 2.5 U/mL apyrase, 1 U/mL hirudin [Pentapharm, Basel, Swiss], pH 6.3) and allowed to rest for 15 min* at 37°C. Following a final centrifugation step (650g, 7 min*, RT, without brake) platelets were resuspended in 2 mL of resuspension-buffer (50 mL of modified Tyrode buffer, 0.5 mL of 1 mM MgCl2, 1 mL of 2 mM CaCl2, pH 7.2) and adjusted to $300x10^9$ /L after cell count measurement at a Cell-Dyn Ruby hematological analyzer (Abbott Park, Illinois, USA).

Testing for anti-platelet factor 4 (PF4)/heparin antibodies

A commercially available IgG-Enzym Immune assay (EIA) was used in accordance to manufacturer's instructions (Hyphen Biomed, Neuville-sur-Oise, France). Per manufacturer's recommendation, a sample was considered reactive if the optical density (OD) was ≥ 0.500. The ability of sera to activate platelets was tested using the functional assay heparin induced platelet aggregation assay (HIPA). In brief, serum was tested with washed platelets from four different healthy donors in the absence (buffer alone), in the presence of heparin (0.2 IU/mL and 100 IU/mL) and PF4 (25µg/mL [Chromatec, Greifswald, Germany]). Reactions were placed in microtiter wells containing spherical stir bars and stirred at approximately 500 revolutions per minute (rpm). Wells were examined optically at five-minutes interval for loss of turbidity. A serum was considered reactive (positive) if a shift from turbidity to transparency occurred within 30 min in at least two platelet suspensions. Observation time was 45 min. Each test included a diluted serum from a patient with heparin induced thrombocytopenia (HIT) as a weak positive control, collagen (5µg/mL [Collagen Horn, Takeda, Linz, Austria]) as strong positive control and a serum from a healthy donor as a negative control.

Assessment of antibody-mediated procoagulant platelets

To exclude non-specific effects like the activation of platelets via complement or non-specific immune complexes, all sera were heat-inactivated (56°C for 30 min*), followed by a sharp centrifugation step at 5,000g. The supernatant was collected. All experiments involving patients' sera were performed after incubation of 5 µL serum with 25 µL washed platelets ($7.5x10^6$) for 1.5 h* under rotating conditions at RT. When indicated, cell suspensions were preincubated in presence of PF4 (25 µg/ml). Afterwards, samples were washed once (7 min*, 650g, RT, without brake) and gently resuspended in 75 µL of phosphate-buffered saline (PBS, Biochrom, Berlin, Germany). Platelets were then stained with Annexin V-FITC and CD62-APC (Immunotools, Friesoythe Germany) and directly analyzed by flow cytometry (FC). As positive control, washed platelets were incubated with ionomycin (5µM, 15 min* at RT [Sigma-Aldrich, St. Louis, USA]) and thrombin receptor activating peptide TRAP-6 (10 µM, 30 min at RT [Hart Biologicals, Hartlepool, UK]). Test results were determined as fold increase of the percentage of double CD62p/Phosphatidylserine (PS) positive events in platelets upon incubation with patients' sera compared to cells incubated with healthy donors tested in parallel.

Results

Clinical and laboratory features of vaccine induced immune thrombotic thrombocytopenia (VITT)

We report 5 patients (3 female, 2 male) with a median age of 47 years (range: 20 and 57 years) who were referred to our hospitals with suspected thrombotic complications after first vaccination with ChAdOx1 nCoV-19.

Case #1: female, 47 y., Cerebral venous sinus thrombosis (CVST)

A 47-year old female patient presented with headache, dizziness, and nausea 7 days after the first vaccination with ChAdOx1 nCoV-19. Initial platelet count revealed 56x10^9/L. Because of sustaining vertigo and increasing headache patient was referred to the neurology department. Coagulation parameters on the following days revealed a slight increased INR with 1.3, aPTT was also prolonged with 35s. Fibrinogen was in a normal range with 263 mg/dL and D-dimer was increased with 9 µg/ml. After further decrease of platelet count, a severe thrombosis of the sagittal superior sinus and right transverse sinus was detected in computed tomography (CT) and magnetic resonance (MR) imaging. HIT testing revealed positive results in the EIA (OD 2.07) and in HIPA. Patient was treated with argatroban (1.5-2 aPTT prolongation) with IVIG for 3 days (total dose: 140 g). After IVIG therapy the platelet count increased to 201x10^9/L.´The patient deteriorated 4 days after initiation of agatroban, directly at the day of first infusion of IVIG and revealed clinical and radiological signs of malignant brain edema and congestive hemorrhage. Therefore, bilateral hemicraniectomy has to be performed to decrease intracranial pressure. Neurological symptoms began to recover and the sagittal superior sinus showed radiological signs of recanalization. At the time of the writing of this manuscript (4 weeks after hospital admission), the patient was still in rehabilitation with persisting global aphasia.

Case #2: female, 57 y., CSVT, hematoma

A 57-year old female patient admitted to her family doctor because of continuing headache 9 days after the first vaccination with ChAdOx1 nCoV-19. Blood analysis showed thrombocytopenia (27x10x^9/µL) and the patient was transferred to a university hospital. At admission, she had petechiae in the extremities and hematoma. Platelet count was 25 x10^9/L and D-dimer was 54 µg/ml. Fibrinogen decreased (50mg/dl). Cranial CT revealed a thrombosis of the left sigmoid sinus and bleeding into right occipitotemporal region. HIT testing revealed positive result in EIA (OD: 3.23) and in HIPA (positive buffer reaction and

positive in presence of low molecular weight heparin). Anticoagulation was initiated with argatroban and IVIG was administered (2x1 g/kg body weight). After the therapy, platelet count steadily increased and D-dimer reduced. The patient was discharged with a platelet count of 237 x 10^9/L and D-dimer of 1.6 µg/ml after 7 days of hospitalization. Anticoagulation was changed to apixaban at discharge. No further thrombosis was observed during hospitalization and three weeks of follow-up.

Case #3: female, 29y., CVST

A 29-year old female patient had recurrent headache 9 days after the first vaccination with ChAdOx1 nCoV-19. At admission, MRI showed no signs of CVST. Platelet count at admission was 53 x10^9/L. Coagulation parameters were abnormal (fibrinogen 274 mg/dL, INR 1.2, aPTT 23 s). D-dimer was strongly elevated (32 µg/ml). Initial HIT-Testing revealed a positive rapid assay. Anticoagulation was then initiated with argatroban in prophylactic doses combined by dexamethasone (40 mg). After four days, the patient suffered from imminent headache and aphasia. High titer PF4/heparin IgG antibodies were detected in the EIA (OD 3.07). HIPA was positive with a positive buffer reaction and positive in presence of low molecular weight heparin. A second MRI showed CVST and a large parenchymal hemorrhage in the left hemisphere. The patient was then treated with a combination of argatroban at therapeutic doses and IVIG (2x1 g/kg body weight; total dose: 95 g). Platelet count recovered within four days to 114x10^9/L. At day nine after CVST diagnosis and stable hemorrhage, anticoagulation was changed to dabigatran 2x150 mg. The patient had no risk factor for thromboembolism except oral contraception. She had also suffered from chronic autoimmune thyroiditis with normal thyroid function under replacement therapy. Follow-up MRI showed beginning of the resorption of the hematoma and partial recanalization of the CVST. The patient was discharged after 22 days of hospitalization.

Case #4: male, 53 y., pulmonary embolism (PE), deep vein thrombosis (DVT)

A 53-year old male was admitted to hospital 7 days after the first vaccination with ChAdOx1 nCoV-19. He introduced himself because of slight shortness of breath, petechiae on the legs. Initial platelet count after admission was 8x10^9/L. D-dimer (>35µg/mL) were elevated and with a fibrinogen of 126 mg/dL with aPTT and INR in a normal range, patient presented beginning signs of consumption. Physical examination and ultrasound revealed a thrombosis in the right thigh (femoral vein). A CT-scan revealed thrombosis in both femoral veins and central embolism in the right lung. HIT-antibodies revealed strong positive result in the EIA (3.18 OD) and a positive HIPA-result (activation with buffer and low molecular weight

heparin). IVIG therapy (1 g/kg body weight for two consecutive days; total dose: 200 g) and alternative anticoagulation with argatroban was initiated immediately after diagnosis of VITT. Anticoagulation was switched to apixaban PO when the platelet count had reached $50x10^9$/L. The patient was discharged on day 11 after admission with a platelet count of $80x10^9$/L. In a follow-up interview 1 months later, he did not report any signs or symptoms of bleeding or thrombosis. His platelet count was stable at 180.000/µL.

Case #5: male, 20 y, CVST, PE

A-20-years old male patient with a body mass index of 37 admitted to the emergency room with severe headache 8 days after vaccination with ChAdOx1 nCoV-19. At admission, platelet count was $60x10^9$/L and D-dimer level was 12.4 µg/mL. As there was no evidence of thrombosis in a cranial CT scan, prophylactic anticoagulation with apixaban (2x2.5 mg) was initiated. On day 4 he was referred to university hospital where cMRI revealed thrombosis of the right transverse sinus. Additionally he complained of new onset thoracic pain on admission. CT of the thorax showed bilateral pulmonary artery embolism and infarct pneumonia. The dose of apixaban was increased to 2x10 mg and IVIG (1g/kg continuous administration on two consecutive days) was administered. High titer PF4/heparin IgG antibodies were detected in the EIA (OD 3.36). Platelet count increased to 124 $x10^9$/L. After 3 days, platelet count dropped again to 81 $x10^9$/L. Anticoagulation was changed to argatroban and a second dose of IVIG (1g/kg continuous administration on three consecutive days) was administered. A control MRI on the day of second IVIG administration showed progression of thrombus in size in right transverse sinus. After one week, MRI showed the regression of thrombosis in the transverse sinus. The patient received a total of 600 g IVIG. After IVIG therapy, platelet count increased to 175 $x10^9$/L. On 20^{th} day of the hospitalization, the anticoagulation was changed to dabigatran (2x150 mg). The patient was discharged after 22 days of hospitalization. The patient did not develop any further thrombosis during hospitalization and 3 weeks follow-up.

Supplemental Tables

Table 1A Demographic and clinical data of cases with vaccine induced immune thrombotic thrombocytopenia

Case #	Age	Sex	First symptoms after vaccination (days)	Thrombosis /Bleeding	Laboratory investigations at admission				
					PLT, (150-450 x109/L)	D-Dimer, (<0.5 µg/ml)	Fibrinogen, (170-410 mg/dl)	INR	aPTT, (>40s)
1	47	f	7	CVST	56	9	263	1.3	35
2	57	f	9	CVST, hematoma	27	54	50	1.5	21
3	29	f	9	CVST	53	32	274	1.2	23
4	53	m	7	DVT, PE	8	>35	126	1.0	25
5	20	m	8	CVST, PE	60	12.4	n.a.	1.1	33

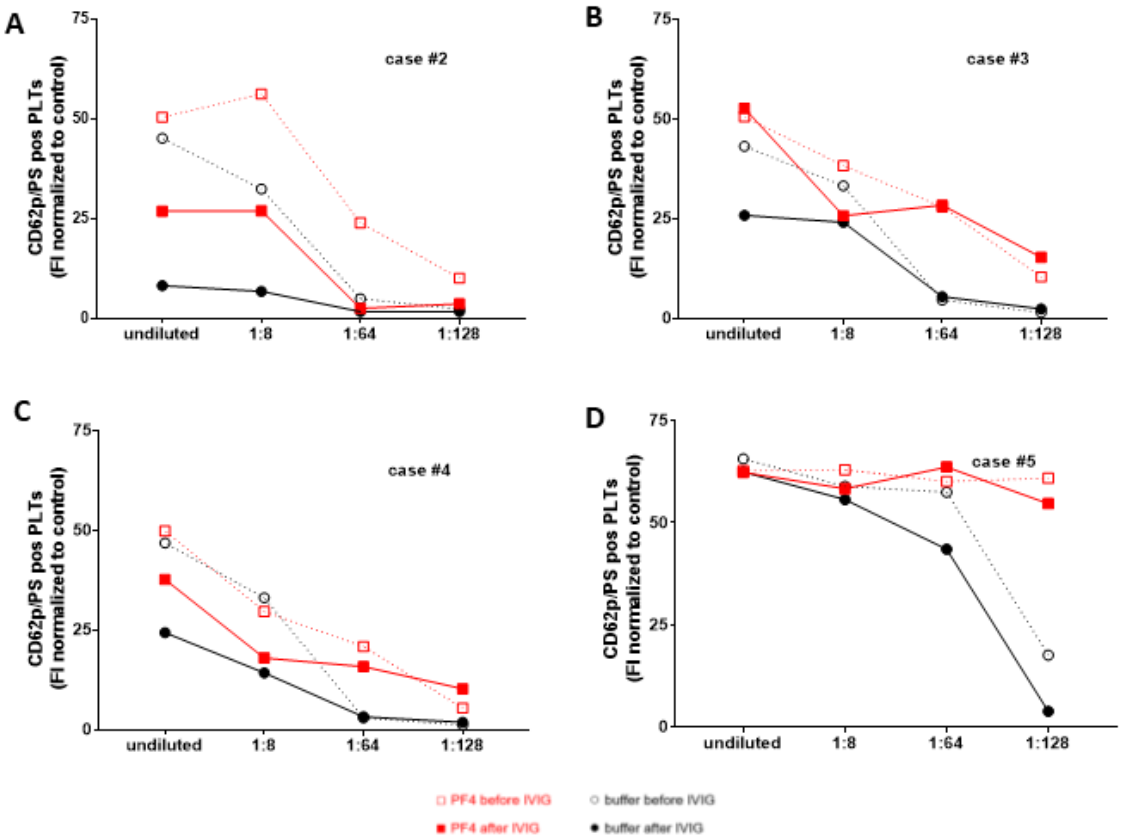
CVST indicates Cerebral venous sinus thrombosis; DVT, deep vein thrombosis; PE, pulmonary embolism; PLT, platelet, *upper limit of the D-dimer test; n.a., not available;

Table 1B Serological response to IVIG in VITT patients

Case #	Findings at admission			Time after IVIG therapy (day)	Findings after IVIG		
	Anti-PF4/heparin EIA (OD)	Modified HIPA (PF4) [time to aggregation, cutoff 30 min]	Procoagulant PLT [CD62p/PS pos PLTs, FI normalized to control, cut off 1.0]		Anti-PF4/heparin EIA (OD)	Modified HIPA (PF4) [time to aggregation, cutoff 30 min]	Procoagulant PLT [CD62p/PS pos PLTs, FI normalized to control, cut off 1.0]
1	2.07	5	23	/	n.a.	n.a.	n.a.
2	3.23	5	45	7	3.10	15	8
3	3.07	5	43	2	3.08	30	26
4	3.18	5	47	5	3.43	5	24
5	3.36	5	66	1	3.11	5	62

PF4 indicates platelet factor 4; EIA, enzyme immunoassay; OD, optical density; HIPA, heparin induced platelet activation assay; PLT, platelet; IVIG, intravenous immunoglobulin; n.a., not available

Supplemental Figure 1



Supplemental Figure 1: Effect of intravenous immunoglobulin (IVIG) therapy on procoagulant platelets

Procoagulant platelets (CD62P/Phosphatidylserine (PS) positive) were analysed in patients before and after IVIG therapy via Annexin V-FITC and CD62p-APC antibody staining. Where indicated, sera were serially diluted and PLTs were pre-treated with PF4 (Panel A-D). Data are presented as fold increase compared to healthy control.

4. Discussion

The COVID-19 pandemic, which began in late 2019, has caused significant morbidity and mortality worldwide. Critically ill patients with COVID-19 may have marked coagulopathy, including thrombocytopenia and diffuse arterial and venous thrombosis. The pathophysiology of COVID-19 focuses on the interplay between inflammation and coagulation. Despite tremendous research efforts over the past two years, there are still questions about the role of platelets in the pathophysiology and long-term consequences of the disease.

Given the importance of platelets in acute COVID-19, we were interested in the changes in platelet phenotype and activation status in the convalescent phase of COVID-19. In addition, one of our goals was to investigate whether sera from COVID-19 convalescent subjects would also induce platelet activation, which might be important for the safety of COVID-19 convalescent plasma transfusions in COVID-19 patients. In our study, we did not detect procoagulant platelet phenotype or increased platelet activation in COVID-19 convalescent subjects [133]. Furthermore, sera from COVID-19 convalescent plasma donors did not result in significant changes in platelet activation or procoagulant status. We did not observe persistent hyperecoagulopathy in our cohort. It should be noted that our study cohort consisted of routine plasma donors who underwent clinical examination according to local regulations, which may introduce selection bias because they are relatively younger and healthier compared with other COVID-19 convalescents. Looking forward, it would be also useful to measure plasmatic coagulation markers to better understand the coagulation status of convalescents and the correlation between post-COVID-19 syndrome and coagulation.

Vaccination efforts have been negatively impacted by the emergence of a rare complication, called VITT, which is also an immune-mediated coagulation problem. VITT occurs after a vaccination with adenoviral vector vaccines against COVID-19. Adenoviral vector vaccines carry a gene sequence encoding the spike protein of SARS-CoV-2. The gene sequence is transported into cells by adenoviral vectors at the injection site. The gene is translated into mRNA in the cells, and copies of the spike protein are assembled in the cytoplasm. Fragments of the spike protein are presented on the cell surface, which is subsequently recognized by immune cells that produce antibodies against the spike protein. In an attempt to understand the development of anti-PF4 antibodies in VITT, we measured anti-spike antibodies and anti-PF4 antibodies in vaccinated healthy controls and VITT patients. We did not find a correlation between immune response generated by vaccination and anti-PF4 antibodies [134]. The pathophysiology of VITT is yet to be understood. Other proposed triggers of PF4 immunity include vaccine components such as EDTA (Ethylenediaminetetraacetic acid) or other human proteins in the vaccine, spike splice variant transcripts, or the adenoviral vector itself [103].

Although vaccines based on adenoviral vectors are now less commonly used in the Western world, more than one billion vaccines have been donated to underdeveloped countries under a World Health Organization vaccine program. New cases of VITT can be expected in these countries. As medical professionals, we will continue to seek answers to the question of how to deal with such cases in the coming months. We urgently need data on the effectiveness of currently proposed treatments.

Antibody mediated platelet activation via FcγRIIa is the central pathological mechanism in VITT. Our study have demonstrated that IVIG can interfere in this step and mitigate hypercoagulable state in patients with VITT [135]. Similar findings have been reported by others also [128,136–138]. Due to the rarity of VITT, it would not be possible to conduct a randomized controlled trial on the use of IVIG in VITT. We recommend the use of IVIG in cases without waiting for confirmatory test results.

In conclusion, our results do not confirm sustained platelet activation in the convalescent phase of COVID-19. Moreover, antibodies against SARS-CoV-2 are not responsible for the development of anti-PF4 antibodies in patients with VITT. Finally yet importantly, high-dose IVIG therapy is an effective means to cool down the hypercoagulable state in patients with VITT and could be lifesaving. Investigating the pathophysiology of VITT and effectiveness of treatments will help us reduce mortality and increase public confidence in the vaccine.

5. Summary

Thromboembolic complications are a hallmark of COVID-19 and are usually associated with multiple organ failure and high mortality. Activated platelets contribute to COVID-19 coagulopathy. Recent evidence suggests a persistent hypercoagulable and/or hypofibrinolytic state in convalescent COVID-19 patients. However, platelets in the convalescent period have not been studied to date. In addition, a rare thrombotic and thrombocytopenic complication, vaccine-induced immune thrombotic thrombocytopenia (VITT), has been described after vaccination with adenoviral vector based COVID-19 vaccines. In VITT, antibodies reacting to platelet factor 4 (PF4) activate platelets via the Fcγ receptor IIa (FcγRIIa) and lead to thrombus formation as well as platelet destruction. The mechanism of anti-PF4 antibody development is not clear. In this work, we aimed to understand antibody–mediated platelet activation in COVID-19 patients in the convalescent phase and the pathophysiology of VITT.

In a prospective study, we investigated platelet phenotype by flow cytometry and global coagulation and fibrinolysis by thromboelastometry in convalescent COVID-19 patients and control subjects. Phosphatidylserine (PS) externalization, CD62P expression, and Glycoprotein VI (GPVI) shedding were measured by flow cytometry. Platelets from COVID-19 convalescents did not exhibit an increased procoagulant phenotype. In addition, GPVI shedding did not differ from that of the control group. Sera from COVID-19 convalescents did not induce significantly higher PS externalization or GPVI shedding in healthy platelets. In thromboelastometry, only one COVID-19 convalescent showed increased maximum clotting strength and prolonged lysis time. Consequently, we did not observe an increased platelet

activation/procoagulant phenotype in the convalescent phase after mild COVID-19 treatment.

We then focused on understanding the development of anti-PF4 antibodies in patients with VITT. One of the proposed mechanisms is cross-reactivity between SARS-CoV-2 and PF4. To investigate the correlation between anti-PF4 and anti-SARS-CoV-2 antibodies, we measured antibody levels in vaccinated controls and in patients with clinical suspicion of VITT after vaccination with ChAdOx1 nCoV-19. VITT patients had higher anti-PF4 antibody levels than healthy controls and non-VITT patients. Levels of anti-PF4 did not correlate anti-SARS-CoV-2 antibodies in any of the study groups. These results refute the assumption that the development of anti-SARS-CoV-2 antibodies contributes to the formation of anti-PF4 antibodies in VITT patients.

Finally, we investigated the effects of intravenous immunoglobulin therapy (IVIG) on platelet count and platelet activation in patients with VITT. All patients received a non-heparin anticoagulant. In addition, IVIG was administered at a dose of 1 g/KG body weight for 2 to 5 days. Four patients showed a complete platelet response (platelet count ≥ 100×10^9/L) and one patient a partial response (platelet count ≥ 30×10^9/L and at least 2-fold increase in baseline count) after IVIG therapy. D-dimer levels decreased after IVIG therapy, indicating a reduction in thrombus burden. After IVIG therapy, the ability of sera from VITT patients to induce procoagulant platelets in flow cytometry was reduced. However, anti-PF4 antibody levels were not affected by IVIG therapy. These data suggest that IVIG attenuates the activity of pathogenic

anti-PF4 antibodies by competing with them for binding to FcγRIIa receptors, which may be associated with a reduction in platelet activation in vivo.

In conclusion, our results do not confirm sustained platelet activation in the convalescent phase of COVID-19. Moreover, antibodies against SARS-CoV-2 are not responsible for the development of anti-PF4 antibodies in patients with VITT. Finally yet importantly, high-dose IVIG therapy is an effective means to cool down the hypercoagulable state in patients with VITT.

6. Zusammenfassung

Thromboembolische Komplikationen sind eine häufige Manifestation von COVID-19 und gehen in der Regel mit einem multiplen Organversagen und einer hohen Sterblichkeit einher. Aktivierte Blutplättchen tragen zur COVID-19-Koagulopathie bei. Neuere Erkenntnisse deuten auf einen anhaltenden hyperkoagulierbaren und/oder hypofibrinolytischen Zustand bei rekonvaleszenten COVID-19-Patienten hin. Die Thrombozyten in der Rekonvaleszenzphase wurden jedoch bisher nicht untersucht. Darüber hinaus wurde eine seltene thrombotische und thrombozytopenische Komplikation, die Vakzin-induzierte immunthrombotische Thrombozytopenie (VITT), bei Adenovirus-basierten Vektorimpfstoffen gegen COVID-19 beschrieben. Bei der VITT aktivieren Antikörper, die auf den Plättchenfaktor 4 (PF4) reagieren, die Thrombozyten über den Fcγ-Rezeptor IIa (FcγRIIa) und führen zur Thrombusbildung sowie zur Abbau der Thrombozyten. Der Mechanismus der Entwicklung von Anti-PF4-Antikörpern ist nicht klar. In dieser Arbeit wollten wir die Antikörper-vermittelte Thrombozytenaktivierung bei COVID-19-Patienten in der Rekonvaleszenzphase und die Pathophysiologie der VITT untersuchen.

In einer prospektiven Studie untersuchten wir den Phänotyp der Blutplättchen mittels Durchflusszytometrie und die globale Gerinnung und Fibrinolyse mittels Thromboelastometrie bei rekonvaleszenten COVID-19-Patienten und Kontrollpersonen. Die Externalisierung von Phosphatidylserin (PS), die Expression von CD62P und die Ausschüttung von Glykoprotein VI (GPVI) wurden mittels Durchflusszytometrie gemessen. Thrombozyten von COVID-19-Rekonvaleszenten wiesen keinen erhöhten prokoagulierenden Phänotyp auf. Darüber hinaus unterschied sich die GPVI-Abgabe nicht von der der Kontrollgruppe. Seren von

COVID-19-Rekonvaleszenten induzierten bei gesunden Thrombozyten keine signifikant höhere PS-Externalisierung oder GPVI-Ausschüttung. In der Thrombelastometrie zeigte nur ein COVID-19-Rekonvaleszent eine erhöhte maximale Gerinnungsstärke und eine verlängerte Lysezeit. Folglich konnten wir in der Rekonvaleszenzphase nach einer milden COVID-19-Behandlung keine erhöhte Thrombozytenaktivierung/prokoagulierenden Phänotyp beobachten.

Anschließend konzentrierten wir uns auf das Verständnis der Entwicklung von Anti-PF4-Antikörpern bei Patienten mit einer VITT. Einer der möglichen Mechanismen ist die Kreuzreaktivität zwischen SARS-CoV-2 und PF4. Um die Korrelation zwischen Anti-PF4- und Anti-SARS-CoV-2-Antikörpern zu untersuchen, wurden die Antikörperspiegel bei geimpften Kontrollen und bei Patienten mit klinischem Verdacht auf VITT nach der Impfung mit ChAdOx1 nCoV-19 gemessen. VITT-Patienten hatten höhere Anti-PF4-Antikörperspiegel als gesunde Kontrollpersonen und Nicht-VITT-Patienten. Die Anti-PF4-Werte korrelierten in keiner der Studiengruppen mit den Anti-SARS-CoV-2-Antikörpern. Diese Ergebnisse widerlegen die Vermutung, dass die Entwicklung von Anti-SARS-CoV-2-Antikörpern zur Bildung von Anti-PF4-Antikörpern bei VITT-Patienten beiträgt.

Schließlich untersuchten wir die Auswirkungen einer intravenösen Immunglobulintherapie (IVIG) auf die Thrombozytenzahl und die Thrombozytenaktivierung bei VITT-Patienten. Alle Patienten erhielten ein alternatives Antikoagulans. Darüber hinaus wurde IVIG in einer Dosis von 1 g/KG Körpergewicht über 2 bis 5 Tage verabreicht. Vier Patienten zeigten nach der IVIG-Therapie ein vollständiges Ansprechen der Thrombozyten (Thrombozytenzahl ≥

100×109/L) und ein Patient ein teilweises Ansprechen (Thrombozytenzahl ≥ 30×109/L und mindestens 2-facher Anstieg der Ausgangszahl). Die D-Dimer-Werte sanken nach der IVIG-Therapie, was auf eine Verringerung der Thrombuslast hinweist. Nach der IVIG-Therapie war die Fähigkeit der Seren von VITT-Patienten, in der Durchflusszytometrie prokoagulierende Thrombozyten zu induzieren, reduziert. Die Anti-PF4-Antikörperspiegel wurden durch die IVIG-Therapie jedoch nicht beeinflusst. Diese Daten deuten darauf hin, dass IVIG die Aktivität pathogener Anti-PF4-Antikörper abschwächt, indem es mit ihnen um die Bindung an FcγRIIa-Rezeptoren konkurriert, was mit einer Verringerung der Thrombozytenaktivierung in vivo verbunden sein könnte.

Zusammenfassend lässt sich sagen, dass unsere Ergebnisse keine anhaltende Thrombozytenaktivierung in der Rekonvaleszenzphase von COVID-19 bestätigen. Außerdem sind die Antikörper gegen SARS-CoV-2 nicht für die Entwicklung von Anti-PF4-Antikörpern bei Patienten mit VITT verantwortlich. Zu guter Letzt ist eine hochdosierte IVIG-Therapie ein wirksames Mittel zur Abkühlung des hyperkoagulierbaren Zustands bei VITT-Patienten.

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7. Declaration of Contributions

The work was carried out at the Institute for Clinical and Experimental Transfusion Medicine under the supervision of Prof. Dr Tamam Bakchoul.

The contributions of coauthors for each publication is given below.

Uzun G, Singh A, Abou-Khalel W, Pelzl L, Weich K, Nowak-Harnau S, Althaus K, Bugert P, Klüter H, Bakchoul T. Platelets and sera from donors of convalescent plasma after mild COVID-19 show no procoagulant phenotype. Haemostasiologie, Accepted on 12.03.2022

The authors contributed to the publications as indicated in the following table (indicated in %):

		Contributions						
	Authors	Research concept	Selection of methods	Recruitment of patients	Data acquisition	Data analysis	Interpretation of results	Preparation of Manuscript
1	Günalp Uzun	45%	30%	80%	60%	60%	60%	60%
2	Anurag Singh	5%	5%	-	5%	5%	5%	10%
3	Wissam, Abou-Khalel	-	10%	-	15%	10%	-	2%
4	Lisann Pelzl	-	10%	-	5%	5%	-	2%
5	Karoline Weich	-	-	-	10%	5%	-	2%
6	Stefanie Nowak-Harnau	5%	5%	10%	-	-	-	2%
7	Karina Althaus	5%	-	10%	-	-	5%	2%
8	Peter Bugert	5%	-	-	-	-	-	2%
9	Harald Klüter	5%	-	-	-	-	-	2%
10	Tamam Bakchoul	30%	40%	´-	-	10%	30%	16%

Uzun G, Althaus K, Bakchoul T. No Correlation between Anti-PF4 and Anti-SARS-CoV-2 Antibodies after ChAdOx1 nCoV-19 Vaccination. N Engl J Med. 2021 Sep 30;385(14):1334-1336.

		Contributions						
	Authors	Research concept	Selection of methods	Recruitment of patients	Data acquisition	Data analysis	Interpretation of results	Preparation of
1	Günalp Uzun	40%	50%	50%	50%	60%	50%	60%
2	Karina Althaus	10%	20%	25%	25%	20%	20%	20%
3	Tamam Bakchoul	50%	30%	25%	25%	20%	30%	20%

Uzun G, Althaus K, Singh A, Möller P, Ziemann U, Mengel A, Rosenberger P, Guthoff M, Petzold GC, Müller J, Büchsel M, Feil K, Henkes H, Heyne N, Maschke M, Limpach C, Nagel S, Sachs UJ, Fend F, Bakchoul T. The use of IV immunoglobulin in the treatment of vaccine-induced immune thrombotic thrombocytopenia. Blood. 2021 Sep 16;138(11):992-996.

		Contributions						
	Authors	Research concept	Selection of methods	Recruitment of patients	Data acquisition	Data analysis	Interpretation of results	Preparation of Manuscript
1	Günalp Uzun	36%	32%	5%	5%	60%	40%	50%
2	Karina Althaus	6%	10%	5%	5%	10%	5%	5%
3	Anurag Singh	1%	10%	5%	5%	10%	5%	5%
4	Peter Möller	1%	1%	5%	5%	-	1%	1%
5	Ulf Ziemann	1%	1%	5%	5%	-	1%	1%
6	Annerose Mengel	1%	1%	5%	5%	-	1%	1%
7	Peter Rosenberger	1%	1%	5%	5%	-	1%	1%
8	Martina Guthoff	1%	1%	5%	5%	-	1%	1%
9	Gabor C Petzold	1%	1%	5%	5%	-	1%	1%
10	Jens Müller	1%	1%	5%	5%	-	1%	1%
11	Martin Büchsel	1%	1%	5%	5%	-	1%	1%
12	Katharina Feil	1%	1%	5%	5%	-	1%	1%

13	Hans Henkes	1%	1%	5%	5%	-	1%	1%
14	Nils Heyne	1%	1%	5%	5%	-	1%	1%
15	Matthias Maschke	1%	1%	5%	5%	-	1%	1%
16	Caroline Limpach	1%	1%	5%	5%	-	1%	1%
17	Simon Nagel	1%	1%	5%	5%	-	1%	1%
18	Ulrich J Sachs	1%	1%	5%	5%	-	1%	1%
19	Falko Fend	6%	1%	5%	5%	-	1%	1%
20	Tamam Bakchoul	20%	32%	5%	5%	20%	34%	24%

I confirm that I wrote the dissertation myself (under the supervision of Prof. Dr. Tamam Bakchoul) and the publications with assistance from co-authors of the publications. I also confirm that any additional sources of information have been duly cited.

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