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Full Length Article

Hypotheses behind the very rare cases of thrombosis with thrombocytopenia syndrome after SARS-CoV-2 vaccination

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ABSTRACT

As of 4 April 2021, a total of 169 cases of cerebral venous sinus thrombosis (CVST) and 53 cases of splanchnic vein thrombosis were reported to EudraVigilance among around 34 million people vaccinated in the European Economic Area and United Kingdom with COVID-19 Vaccine AstraZeneca, a chimpanzee adenoviral vector (ChAdOx1) encoding the spike protein antigen of the SARS-CoV-2 virus. The first report of the European Medicines Agency gathering data on 20 million people vaccinated with Vaxzevria® in the UK and the EEA concluded that the number of post-vaccination cases with thromboembolic events as a whole reported to EudraVigilance in relation to the number of people vaccinated was *lower* than the estimated rate of such events in the general population. However, the EMA's Pharmacovigilance Risk Assessment Committee concluded that unusual thromboses with low blood platelets should be listed as very rare side effects of Vaxzevria®, pointing to a possible link. The same issue was identified with the COVID-19 Vaccine Janssen (Ad26.COV2.S).

Currently, there is still a sharp contrast between the clinical or experimental data reported in the literature on COVID-19 and the scarcity of data on the unusual thrombotic events observed after the vaccination with these vaccines. Different hypotheses might support these observations and should trigger further in vitro and ex vivo investigations. Specialized studies were needed to fully understand the potential relationship between vaccination and possible risk factors in order to implement risk minimization strategies.

1. Introduction

As of 4 April 2021, a total of 169 cases of cerebral venous sinus thrombosis (CVST) and 53 cases of splanchnic vein thrombosis were reported to EudraVigilance among around 34 million people vaccinated in the European Economic Area (EEA) and United Kingdom (UK) with COVID-19 Vaccine AstraZeneca (AZD1222, Vaxzevria®, AstraZeneca, Cambridge, United Kingdom), a chimpanzee adenoviral vector

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(ChAdOx1) encoding the spike protein antigen of the SARS-CoV-2 virus [1]. The first report of the European Medicines Agency (EMA) gathering data on 20 million people vaccinated with Vaxzevria® in the UK and the EEA concluded that the number of post-vaccination cases with thromboembolic events as a whole reported to EudraVigilance in relation to the number of people vaccinated was *lower* than the estimated rate of such events in the general population [2]. However, the EMA's Pharmacovigilance Risk Assessment Committee (PRAC) concluded that unusual thromboses with low blood platelets should be listed as very rare side effects of Vaxzevria®, pointing to a possible link. The COVID-19 subcommittee of the WHO Global Advisory Committee on Vaccine Safety (GACVS) also reviewed reports of rare cases of thromboses with low platelets following vaccination with the AstraZeneca COVID-19 vaccine and concluded in a public statement that based on available information, a causal relationship between the vaccine and the occurrence of thromboses with low platelets was considered plausible but not yet confirmed [3]. Specialized studies were needed to fully understand the potential relationship between vaccination and possible risk factors. The EMA's Committee for Medicinal Products for Human use (CHMP) has further analyzed available data to put the risk of these very rare thromboses in the context of the vaccine's benefits for different age groups and different rates of infection. Graphic representations of the findings assuming an 80% vaccine effectiveness over a period of four months, are available on the EMA's website [4].

Importantly, the same issue was identified for a human adenovirus vector vaccine (Ad26.COV2.S, COVID-19 Vaccine Janssen, Janssen-Cilag International NV, Beerse, Belgium). In the clinical trial program, a case of CVST with thrombocytopenia occurred in a Janssen vaccine recipient which led to a pause in the clinical program. No clear causality was established, and the data and safety monitoring board agreed that the study could restart. The vaccine recipient was subsequently found to have had antibodies against platelet factor 4 (PF4) at the time of the event. Venous thromboembolism was added as an important potential risk in its risk management plan due to an imbalance of thrombotic events in general during clinical trials [5]. The Food and Drug Administration (FDA) and the Centers for Disease Control and Prevention (CDC) recommended a pause in vaccination with COVID-19 Vaccine Janssen in the United States to allow further study. At that moment, six cases were reported among >7.2 million persons who had been vaccinated with COVID-19 Vaccine Janssen globally [6]. During the pause, the FDA and the CDC examined available data to assess the risk of CVST with thrombocytopenia (Table 1). During March 2–April 21, 2021, the Vaccine Adverse Event Reporting System (VAERS), the US vaccine safety monitoring system, had received 15 reports of thrombosis with thrombocytopenia syndrome (TTS) after Janssen COVID-19 vaccination, with clots located in the cerebral venous sinuses and other unusual locations, including in the portal vein and splenic vein, and a combination of venous and arterial thromboses. On April 23, 2021, the Advisory Committee on Immunization Practices (ACIP) voted in favour of using of the COVID-19 Vaccine Janssen in all persons aged above or equal to 18 years. The importance of providing education for vaccination providers and the public about the risk for TTS and availability of other COVID-19 vaccine options, particularly for women aged 18–49 years was emphasized [7]. The FDA and the CDC later lifted the recommended pause on COVID-19 Vaccine Janssen use and added a warning to the Janssen COVID-19 vaccine EUA and fact sheets regarding rare clotting events [8]. The EMA's PRAC also concluded that a warning about unusual thrombosis with low blood platelets should be listed as very rare side effects in the product information for COVID-19 Vaccine Janssen [9].

Table 1
Summary of the risk of thrombosis with thrombocytopenia syndrome (TTS) according to age group with Vaxzevria® and COVID-19 Vaccine Janssen. Data were obtained from the report of the European Medicines Agency[a] and the Centers for Disease Control and Prevention (CDC)[b].

Age group	Absolute risk per 1,000,000 persons after first dose of Vaxzevria®	Age group	Absolute risk per 1,000,000 persons with COVID-19 Vaccine Janssen
20–29	19	18–29	5.2
30–39	18	30–39	11.8
40–49	21	40–49	4.3
50–59	11	50–64	1.5
60–69	10	65+	0.0
70–79	5.0		
80+	4.0		

[a] Source: https://www.ema.europa.eu/documents/chmp-annex/annex-vaxzevria-art53-visual-risk-contextualisation_en.pdf.
[b] Source: https://www.cdc.gov/vaccines/acip/meetings/downloads/slides-2021-04-23/03-COVID-Shimabukuro-508.pdf.

2. Description of the cases

Among the 40 cases reported in the literature presenting with the thrombosis with thrombocytopenia syndrome (TTS) after Vaxzevria® vaccination, the median age was 40 (range: 21 to 77 years of age) with a relative predominance of females (i.e. 70%). The onset was within 5 to 24 days after vaccination and reported initial symptoms were headache, backache, abdominal pain, visual disturbance and leg or arm weakness. Thromboses were found at cerebral, abdominal, deep vein, or pulmonary sites or were even arterial. Platelet nadir varied between 7000 and 113,000 platelets per cubic millimeter. The majority, but not all, of cases were positive for PF4-heparin antibodies and were reactive to functional platelet activation assays [10–13].

Among the 15 cases of TTS with COVID-19 Vaccine Janssen reported by the CDC [14] including the six cases reported by Muir et al. [15], the median age was 37 (range: 18 to 59 years of age). Thirteen occurred among women aged 18–49 years, and two occurred among women aged ≥50 years. All affected persons were women (i.e. 100%). The time to symptom onset ranged from 5 to 16 days (median: 8 days) and 12 cases presented with CVST. The platelet nadir ranged from 9000 to 127,000 per cubic millimeter. Eleven cases were positive for PF4-heparin antibodies and results were not available for the 4 remaining cases [14]. Among subgroups by age (18–29, 30–39, 40–49, 50–64, and ≥65 years), the reported rate was highest among women aged 30–39 years, with 11.8 TTS cases per 1 million Janssen COVID-19 doses administered [7] (Table 1). All 15 patients were hospitalized, and 12 were admitted to an intensive care unit. Three patients had died. Although there is disproportionality in the occurrence of these TTS events and the rare associated of thrombotic events compared to the normal population, it has to be noted that COVID-19 is also associated with a near 10-fold increased risk of developing CVST (Table 2).

With laboratory assays primarily used for detecting heparin-induced thrombocytopenia (HIT) antibodies and heparin-induced platelet aggregation (HIPA) tests with various experimental conditions, it has been concluded that the observed cases were resembling to a rare form of spontaneous HIT (i.e. autoimmune HIT without proximate heparin exposure) [16], and possibly due to an immune process where the inducer is not heparin but rather a component of the vaccine, through the exposure to a polyanionic macromolecule [17]. The vaccine or some of its components could also have been an inducer of a broad immune response, including the activation and expansion of autoreactive B-cells [17].

Another recent report mentioned bilateral superior ophthalmic vein

Table 2
Summary of the risk of cerebral venous thrombosis associated or not with COVID-19 disease.[a]

Absolute risk per 1,000,000 within 2 weeks after COVID-19 diagnosis (hospitalized)	Highest baseline absolute risk per 1,000,000 over any 2 weeks period
39	0.41

[a] Source: Taquet et al. Open Science preprint (April 15, 2021): https://osf.io/a9jdq/.

thrombosis and immune thrombocytopenia along with an ischaemic stroke but did not observe the presence of PF4-specific antibodies [18]. Therefore, and importantly, the presence of any confounders in spontaneous cases is needed all cases since the etiology remains uncertain. Different hypotheses should be investigated, and adequate laboratory tests performed (Table 3).

3. Anti-PF4 antibodies in COVID-19

Anti-PF4 antibodies have been frequently detected in COVID-19 patients suggesting that heparin administration in severe COVID-19 patients could lead to classical HIT. However, many patients developed anti-PF4 antibodies that were not able to activate platelets in dedicated assays [19–22]. This confirms that, among patients who had received heparin, the distinction between non-HIT and HIT antibodies is not always evident and that functional assays are mandatory for HIT confirmation [23].

Immunoassays for anti-PF4 antibodies are not very specific to HIT, since for instance they detect such antibodies in up to 50% of patients after cardiac surgery without any thrombocytopenia or thrombosis [23,24]. These tests can also show cross reactivity with other types of IgG antibodies recognizing epitopes present on the test antigen sequence that resembles to PF4/heparin complex [25]. This is especially true if a low optical density result is obtained. In addition, due to the lack of standardization for HIT antibody testing, the inter-laboratory variations, and dependence on experimental conditions, it is mandatory to specify which technique has been used to better appreciate the true positivity of the cases [26,27].

Interestingly, Brodard et al. reported COVID-19 patients with high levels of PF4-specific antibodies as assessed with an enzyme immunoassay (EIA), but these were not platelet-activating antibodies. Among the twelve COVID-19 patients with suspected HIT studied, the EIA performed were positive in nine cases but functional assays in only three. The hypothesis that a factor present in the serum of COVID-19 patients, and inhibiting platelet activation induced by anti-PF4 antibodies, was evaluated and not confirmed [28]. In addition, another report also showed that among COVID-19 patients, the rate of positive EIA is significantly higher than in the general population [29].

Another study of ten COVID-19 patients with HIT suspicion revealed that samples were tested positive with the serotonin release assay (SRA), even in absence of heparin [30]. Since anti-PF4/heparin antibodies could not be detected in these patients, the diagnosis of HIT was ruled out [30]. The addition of IV.3 (anti-human CD32 (FcγRIIa), Clone IV.3) antibody inhibited platelet activation, indicating that immune complexes containing IgG likely triggered the platelet activation in vitro via FcγRIIa. Of note, convalescent plasma from non-critically ill COVID-19 subjects ($n = 8$) did not activate platelets in the SRA, despite having high levels of anti-SARS-CoV-2 antibodies, indicating that antibodies alone were insufficient for platelet activation [30]. The authors of this study concluded that a subset of critically ill COVID-19 patient sera contains immune complexes that mediate platelet activation via FcγRIIa. The antigen in the immune complexes remains elusive, but may be part of SARS-CoV-2, similar to what was reported with H1N1 viral infection [31]. This is further supported by the inhibition of platelet activation observed with therapeutic concentrations of heparin, which may bind the SARS-CoV-2 receptor binding domain (RBD) and induce a conformational change with altered binding specificity, potentially disrupting immune complexes [32]. Also, interaction with syndecan-1 and endocan, two polyanionic proteoglycans released by damaged endothelial cells, may also form complexes with PF-4 then stimulating the production of anti-PF4 antibodies.

Therefore, these results suggest that in COVID-19 patients, immune complexes unrelated to PF4 could also activate platelets via FcγRIIa.

Table 3
Summary of the different hypotheses for the risk of cerebral venous sinus thrombosis following vaccination with AZD1222. The following hypotheses all need to be supported by further pre-clinical and clinical laboratory investigations. Additional clinical information on potential confounders is also needed, underlying the need for complete and documented reporting of serious adverse events in pharmacovigilance databases (see text).

Hypothesis	Rationale	Current limitations
VIPIT	- IgG Anti PF4/heparin antibodies present - No proximate exposure to heparin - "Severe" thrombocytopenia - Temporal association (events observed 14 days after vaccination) - FcγRIIa dependent platelet activation (resembles 'spontaneous' – 'autoimmune' HIT)	- Multiple confounders not investigated like the presence of recent infection - Insufficient laboratory confirmation - Platelet activation already observed with the buffer condition and addition of human PF4 had clearly a potentiating effect - Laboratory data only available in some cases
Previous or current SARS-CoV-2 infection[b]	- COVID-19 patients may develop high levels of IgG anti-PF4 antibodies (no polyanionic macromolecule). - IgG anti-PF4/heparin antibodies not detected. - COVID-19 patients were reported positive with SRA even in absence of heparin. - PF4 can bind to polyanionic structures like DNA/RNA, which are released from viruses during infections. - Some IgG antibodies directed against the spike protein may be able to interact with FcγRIIa. - Sera from COVID-19 patients caused platelet apoptosis via cross-linking with FcγRIIa. - immune complexes containing IgG might trigger platelet activation in vitro. - NETosis can be triggered via the interaction between SARS-CoV-2 and platelets leading to P-selectin secretion and neutrophils activation.	- Majority of cases reported negative results for SARS-CoV-2 PCR and serological investigations.[a] - Insufficient or no laboratory information on the serological status of the patients (i.e. antibodies targeting the nucleocapsid protein of the virus or measurement of viremia).
Reactivation of previous immune response	- Certain COVID-19 patients have developed antibodies directed against the RBD of the spike protein and able to activate platelet in vitro without addition of heparin. - Vaccinated patients could have developed such an immune response before the vaccination (due to e.g. pregnancy, blood transfusion) - The vaccine has reactivated a previous immunization against PF4 or other integrins on the surface of platelet, activating them.	- Insufficient or no laboratory information on the serological status of the patients (i.e. antibodies targeting the nucleocapsid protein of the virus or measurement of the viremia). - The same event rate with the different COVID-19 vaccines currently on the market is not observed.
Platelet activation by the viral vector	- Adenoviruses interact with platelets through CAR, αIIbβ3 and α5β3 receptors among others, possibly leading to platelet activation	- Pharmacokinetics investigations of Vaxzevria® and COVID-19 Janssen Vaccine need to support this hypothesis (important: intravenous administration of COVID-19

(continued on next page)

Table 3 (*continued*)

Hypothesis	Rationale	Current limitations
	- Initiation of apoptosis-like markers, including caspase activation and mitochondrial permeability changes. - Activated platelets release ADP, polyphosphate or serotonin. - Polyphosphates/PF4 complexes can mediate heparin independent platelet activation in HIT. - Other cases of thromboembolic events have been observed with another adenovirus vector COVID-19 vaccine.	vaccine cannot be excluded and should be part of pharmacokinetic investigations).
Activation of other cell types	- Monocytes, macrophages and endothelial cells express FcγRIIa - FcγRIIa can be activated by immune complexes formed after Vaxzevria® vaccination triggering thrombin generation or a proinflammatory state. - Thrombin generation is less regulated in extracerebral vessels due to very low level of thrombomodulin contributing to the particular clinical expression of the thrombotic events observed. - Eculizumab was successfully used in two patients. Activation/consumption/dysregulation of the complement system cannot be excluded and deserve further laboratory investigations.	- Lack of laboratory investigations.

Abbreviations: CAR, coxsackievirus and adenovirus receptor; FcγRIIa, Fc γ receptor IIa; IgG, immunoglobulin G; PCR, polymerase chain reaction; PF4, platelet factor 4; RBD, receptor binding domain.

[a] The type of serological testing is not always reported. In the presence of vaccination, testing should target the nucleocapsid protein of the virus to avoid cross-reactivity with the antibodies generated after vaccination. Importantly the temporal association between serological positivity against SARS-CoV-2 (i.e. +14 days) and the appearance of the event may generate false negative results. Testing should be done at least two weeks after the appearance of symptoms.

[b] Different findings are listed in the rationale column, sometimes contradictory, or pointing to mechanistic heterogeneity.

4. Anti-PF4 antibodies and spontaneous HIT

HIT antibodies are typically induced by heparin exposure, which causes the formation of macromolecular complexes associated with a conformational change in PF4, thereby creating neoantigens [33]. HIT IgG antibodies activate platelets via FcγRIIa, leading to the release of platelet-derived procoagulant microvesicles [34].

Even if it is confirmed that recent (i.e. 3 months) heparin exposure cannot be involved in the advent of TTS after vaccination, we have to be cautious about possible confounders regarding the trigger of the immune response toward PF4. Indeed, it has already been reported that so-called 'spontaneous' HIT may occur in patients who had proximate episodes of infection, or major surgery such as total knee or hip replacement surgery [35–42]. Although the nature and pathogenesis of spontaneous HIT is still a matter of debate, Nguyen et al. showed that some patients with autoimmune HIT developed two types of antibodies, with different specificities: anti-PF4/H antibodies, with moderate affinity for modified PF4, and high affinity antibodies, capable of recognizing PF4 whether complexed or not with heparin [43]. In vitro data showed that high affinity anti-PF4 antibodies were able to promote an approximation between adjacent PF4 tetramers allowing the binding of anti-PF4/heparin antibodies to PF4 even in the absence of heparin. This could permit inducing conformational changes on PF4 like those induced by heparin [43]. However, this immune response varies widely according to the clinical context. Indeed, non-activating anti-PF4 antibodies can be found in the general population, but also in up to 50% of non-HIT patients after cardiac surgery as well as in COVID-19 patients [19,44]. In addition, while heparin-dependent platelet-activating IgG antibodies are typically evidenced in classical HIT, they are found in up to 20% of non-HIT patients after major surgery. Atypical anti-PF4 IgG antibodies able to strongly activate platelets in the absence of heparin but in a PF4-dependent manner are also sometimes observed [24].

Moreover, even in the general population not treated with heparin during the 12 months preceding blood sampling, anti-PF4/heparin IgG antibodies can be detected with a relatively high incidence rate of 6.1% [36]. This is consistent with the concept that the general population is frequently exposed to PF4/heparin-like antigen complexes, resulting in a continuum of the immune response. PF4 can bind to the surface of various bacteria that cause infections in humans, such as *S. aureus*, *S. pneumonia*, and *E. coli*. This interaction is mediated by polyanionic structures present on their surface, and particularly for Gram-negative bacteria, the phosphate residues of lipid A of their lipopolysaccharide [45]. In addition, circulating DNA, potentially released from bacteria or viruses during infections, also induces conformational changes on the surface of PF4, which are identical to those observed in the presence of heparin and make it immunogenic inducing, at least in mice, the synthesis of anti-PF4/DNA antibodies that cross-react with PF4/heparin complexes [46]. These notions support the understanding that anti-PF4 immunization can occur without exposure to heparin, since other molecules can modify the structure of this chemokine and potentially its immunogenicity leading to false positive EIA assays directed against anti-PF4/heparin IgG antibodies.

Antibodies found in the general population can result from repeated challenges with bacteria, which elicit production of anti-PF4/heparin antibodies. While the epitopes involved in the binding of classical HIT antibodies to PF4 are partially known [47,48], those involved in autoimmune HIT syndromes have not yet been documented, and might be very different [49]. However, it is not excluded that these IgG antibodies directed against other epitopes are also able to activate platelets. In addition, the fact that these cases were mainly reported in young women should question on the possibility that we are facing previously immunized women. Such immunization, i.e. human platelet alloantibodies (HPA) or anti-PF4, could be observed in women during pregnancy or having received platelet transfusions [50], possibly exposing them to an increased risk of the vaccine –associated thrombotic complications.

On the other hand, recent data from Greinacher et al., may support the fact that some constituents from the ChAdOx1 nCov-19 vaccine can form antigenic complexes with PF4 as biophysical analyses allowed to visualize complexes between PF4 and the vaccine components, including virus proteins. Importantly, those complexes were recognized by antibodies from TTS patients [17]. However one need to substantiate these findings by the fact that these cases manifest as early as five days post vaccination. Thus, it is unlikely that the vaccine itself generates an independent immune reaction but rather reactivate a previous immune response. Indeed, there is typically not enough time for immunological tolerance to break and to generate high titers and class-switched, high affinity IgG antibodies to trigger the proposed mechanism. However, it is possible that anti-PF4 is a byproduct of an initial mechanism which in turn can eventually lead to thrombocytopenia and amplify a vicious cycle [51].

5. The SARS-CoV-2 infection status of the patients should be considered

It is important to note that only Norwegian Vaxzevria® cases and 2 US cases have reported the information on the previous nucleocapsid serological status (i.e. a serological marker which becomes positive 12–14 days after SARS-CoV-2 infection) of the patients. Among the 7 cases, none were positive for antibodies directed against the nucleocapsid protein [11,52]. In the other case series, the presence of antibodies directed against the nucleocapsid protein is unknown or the type of serological testing is not reported, precluding the exclusion of concurrent COVID-19 disease [5,10,14,18]. We have to keep in mind that we have to wait for ±14 days for nucleocapsid test to become positive and in light of the temporal occurrence of thrombotic event, it is possible that these patients have not yet become seropositive against the nucleocapsid protein [53].

It has already been proven that viruses like dengue [54], influenza [55], and HIV [56] can invade platelets, and there is preliminary evidence of the presence of SARS-CoV-2 in platelets of COVID-19 patients either through ACE-2 receptor, although controversial, or via other receptors like the αIIbβ3 integrin [57–61]. It might also be suggested that pre-infection with SARS-CoV-2 could generate, in certain patients, IgG antibodies, probably directed against the spike protein, which are able to interact with FcγRIIa on the platelet surface possibly leading to platelet activation and subsequent thrombocytopenia.

It has been reported by Althaus et al. that severe COVID-19 was associated with antibody-mediated upregulation of platelet apoptosis and that IgG from severe COVID-19 patients induces procoagulant platelets and thus contribute to the increased risk for thromboembolic complications [62]. In particular, sera from COVID-19 patients in the ICU cause IgG-mediated platelet apoptosis via cross-linking FcγRIIa [62]. To explore the mechanism of platelet apoptosis in COVID-19, patients' sera were incubated with washed platelets from healthy donors, and these samples induced a significantly higher mitochondrial inner membrane potential depolarization compared with sera from healthy donors [62]. More importantly, IgG-mediated platelet apoptosis was inhibited by blocking FcγRIIa with the IV.3 monoclonal antibody [62]. These data also indicate that IgG contribute to the increased phosphatidylserine expression on platelets of patients with severe COVID-19 infection.

Although Althaus et al. interestingly found an association between the IgG binding to the spike protein of SARS-CoV-2 and the phosphatidylserine externalization after incubation with patient sera [62], it remains unclear whether this results from a direct effect of IgG–virus complex such as previously reported in H1N1 infection and dengue fever [31,63]. Interestingly, blockade of the FcγRIIa by infusion of a large amount of immunoglobulins (IVIg) has been proposed as a therapeutic option for inhibiting excessive platelet activation in COVID-19 patients similarly to the attitude suggested for severe cases of HIT and autoimmune HIT with life-threatening complications [64]. .Targeting the signaling pathway downstream platelet FcγRIIa by inhibitors of Bruton Tyrosine Kinase (BTK) or Splenic Tyrosine Kinase (SYK), as suggested for HIT and more recently for cerebral venous thrombosis after SARS-CoV-2 vaccination [65,66], could be an alternative or complementary therapeutic option.

Moreover, the work from Nazy et al. showed that some severely affected COVID-19 patients, in whom HIT had been suspected due to the occurrence of thrombocytopenia and thrombosis, developed antibodies able to strongly activate platelets without addition of heparin in vitro. Interestingly, when studying the specificity of these antibodies, they found that they were not directed against PF4/heparin but rather against the RBD domain of the SARS-CoV-2 spike protein [30]. However, as mentioned above, convalescent plasma from non-critically ill COVID-19 subjects did not activate platelets despite having high titers of anti-SARS-CoV-2 antibodies, but platelet activation was independent of heparin which even inhibited platelet activation at both therapeutic and high doses. The development of such antibodies after vaccination may potentially contribute to the rare cases of thrombotic events observed.

Additional data through RNA sequencing coming from severely SARS-CoV2 infected patients have further demonstrated, distinct changes in the gene-expression profile of circulating platelets of COVID-19 patients [58]. Pathway analysis revealed differential gene-expression changes in pathways associated with protein ubiquitination, antigen presentation, and mitochondrial dysfunction [58]. In addition, mRNA from the SARS-CoV-2 N1 gene was detected in platelets from two of 25 COVID-19 patients of this study, suggesting that platelets may take up SARS-COV-2 mRNA [58]. However, among these studies, there was no clear correlation between the presence of SARS-CoV-2 mRNA in platelets and thrombocytopenia. If SARS-CoV-2 enters platelets, this seems to be a rare phenomenon, but this cannot be excluded.

One alternative hypothesis could be that, in those patients, administration of the vaccine would reactivate previous immune response to certain antigens such as PF4, leading to massive platelet activation and thrombotic state. The reasons for the unusual location of these thrombosis are not yet elucidated but these types of thrombosis shared similar risk factors like inflammation or autoimmune disease [67,68], It cannot be excluded that adenoviruses have a tropism for cell types encountered in these areas or for CSF therefore stimulating a pro-inflammatory state in these sites. The spike protein produced locally may then disturb the tight equilibrium between ACE-1 and ACE-2 (by inhibiting/occupying ACE-2) thus increasing locally the production of angiotensin leading a localized hypertensive area.

One cannot exclude that some patients suffering from TTS could have developed an immune response before the vaccination and that the vaccine itself has just reactivated a previous immunization against PF4. Indeed, one issue is that these cases manifest as early as 4 days post vaccination. This is typically short for immunological tolerance to generate high levels [51]. It is therefore possible that anti-PF4 is a byproduct of an initial mechanism.

Active or recent infection (infection since <14 days) should also be excluded. However, in this case scenario, one would expect to observe the same event rate with the different COVID-19 vaccines currently on the market.

6. Other platelet activation hypotheses? A link between SARS-CoV-2 or adenovirus and possible platelet infection

Platelets express only one class of receptors for the Fc domain of IgG antibodies, the FcγRIIa. Once virus-directed antibodies are generated by the adaptive immune response, “bridged” interactions can be facilitated by platelet FcγRIIa, as demonstrated for influenza A virus [31]. Although antibody-bridged binding to platelets has not been specifically documented for many viruses, it is reasonable to speculate that such viral immune complexes and interactions with platelets are common. Similar to engagement of other receptors on the platelet surface, these multivalent adducts could crosslink FcγRIIa, causing platelet activation [57]. Many receptors on platelets that associate with viruses have been identified and include α5β1, αIIbβ3, and α5β3 integrins; the lectin, dendritic cell-specific intercellular adhesion molecule-3-grapping non-integrin (DC-SIGN); Toll-like receptors (TLRs) 2 and 4; coxsackie-adenovirus receptor (CAR) of note, ChAdOx1nCoV-19 is from adenovirus species E and uses the CAR while Ad26.COV2.S is from adenovirus species D and can engage CD46 as its cellular receptor -; complement receptor 2 (CR2); C-X-C chemokine receptor type 4 (CXCR4); C-type lectin domain family 2 (CLEC-2); chemokine C—C motif ligand (CCL); and glycoprotein VI [57]. Interestingly, the SARS-CoV-2 spike protein exhibits the tripeptide Arg-Gly-Asp (RGD) motif that is necessary for interaction with integrins [69,70]. The conservation of the motif and its location in the receptor-binding region of the SARS-CoV-2 spike protein suggests that integrins may be alternative receptors for this virus [70]. Thus, if patients presenting with TTS are currently infected by SARS-CoV-2 or another viral infection, platelet activation induced by SARS-

CoV-2 or this other virus cannot be excluded.

Integrins are critical in platelets–leukocytes interactions. In addition to their role in facilitating numerous direct platelet–virus interactions, they are also important in processes leading to immune clearance of blood-borne viruses. One example is through the recruitment of dendritic cells by the interaction of platelet surface junctional adhesion molecule-C (JAM-C) and dendritic cell integrin αMβ2 [71]. Trafficking of cytotoxic T lymphocytes (CTLs) to sites of infection can be mediated by platelet activation [72]. Also, some viruses induce platelet exposure of CD40 ligand, which promotes platelet–monocyte aggregates via platelet P-selectin and monocyte P-selectin glycoprotein ligand-1 (PSGL-1) [73]. Regarding adenoviruses, identified receptors on platelets concerns CAR, αIIbβ3 and α5β3 among others. The adenoviruses are correspondingly responsible for thrombocytopenia, also seen when adenovirus gene therapy vectors are intravenously administered to rhesus macaques and mice [74,75]. Current literature indicates that CAR mediates the binding of adenovirus to platelets, enabling subsequent entry [74,76]. The virus–platelet interaction is predominantly localized to sites of intercellular complex formation, implying that CAR expression is enhanced in response to platelet activation [77]. Vaccination with chimpanzee adenoviruses is quite recent and the evaluation of the risk of interaction with platelets or other cell types involved in hemostasis regulation is so far incomplete. The experience with Ebola vaccination (i.e. ChAd3-EBO-Z, a chimpanzee adenovirus type-3 (ChAd3)-vectored vaccine) has not reported to date a risk of thrombocytopenia, but the number of patients included is limited and therefore, further investigations are required [78,79].

Platelets circulate in a resting state and are stimulated by ligand–receptor engagement. Protein and carbohydrate receptors on the surface of platelets bind viruses from many distinct families and it is not surprising that the interactions known to exist between viruses and platelet receptors can facilitate platelet activation. For example, dengue virus has been demonstrated to cause the exposure of P-selectin and procoagulant phospholipid on the platelet surface [54]. It finally initiates apoptosis-like markers, including caspase activation and mitochondrial permeability changes [80]. Through their activation, platelets may also release several other components like ADP, polyphosphate or serotonin via their α-granule [81–83]. It has further been shown that polyphosphate/PF4 complexes can mediate heparin-independent platelet activation in HIT [84,85].

Thus, such alternate components could be part of thrombogenic mechanisms if platelets are activated by the interaction between the adenovirus and the receptor CAR, αIIbβ3 and α5β3 among others. However, pharmacokinetics investigations of these vaccines need to support this hypothesis since it implies that the adenovirus should spread into blood circulation at a sufficient concentration to interact with platelets and activate them sufficiently to generate massive platelet activation. Nevertheless, the presence of EDTA as excipients of the vaccine composition could lead to microvascular leakage as demonstrated by experiments done on endothelial cells and mouses [17,86]. This should be seen in relation to the level of calcium in the microenvironment [86].

It can also be hypothesized that other cells such as monocytes, macrophages and endothelial cells which also express many Fc receptors, including the FcγRIIa, can be activated by immune complexes formed after adenovirus vector vaccination and trigger thrombin generation [87]. Thrombin generation is probably less regulated in extra cerebral vessels due to the very low level of thrombomodulin [88–90], and this could contribute to the particular clinical expression observed in these cases. A recent report also suggests that the spike protein stimulates the phosphorylation/activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) through the CD147 receptor in cardiac pericytes potentially leading to endothelial disturbance [91]. Although interesting, this last hypothesis implies that these thrombotic events should have been observed with mRNA vaccines as well.

Another group has also hypothesized that immune complexes present in the pulmonary circulation of severely ill patients might activate platelets and contribute to high rates of thrombosis by a similar mechanism that has been identified in severe H1N1 infection, whereby immune complexes present in the lungs activate platelets via FcγRIIa [31,92]. It has also been suggested that afucosylated IgG may be common to immune responses against all enveloped viruses [93] and it has been demonstrated that immune complexes containing recombinant SARS-CoV-2 spike protein and anti-spike IgG are able to enhance platelet-mediated thrombosis on Von Willebrand Factor (VWF) in vitro, but only if the Fc domain of the IgG had an aberrant glycosylation state, similar to that previously observed in patients with severe COVID-19 [92,94–96]. Finally, two patient which has been vaccinated with Vaxzevria® and presenting with TTS improved after eculizumab administration, while other treatments, i.e. anticoagulation and IVIg, failed [97]. The first patient presented with the full picture of thrombotic microngiopathy, reminiscent of atypical haemolytic uremic syndrome. In the second case, evidence of complement consumption was noted. This suggests that it was due to uncontrolled complement activation supporting the hypothesis that FcγRIIA-dependent platelet activation may not be the only prothrombotic mechanism in this syndrome [98,99].

7. Concluding remarks

SARS-CoV-2 infection may lead to COVID-19 which is associated with an important risk of hospitalization and death. The overall benefits of Vaxzevria® and COVID-19 Janssen Vaccine in preventing COVID-19 is well established. Thrombosis with thrombocytopenia syndrome is a very rare, but clinically serious and potentially life-threatening adverse event that has been observed in association with these vaccines. This strongly argues for pathogenesis shared by the two adenoviral vector-based vaccines, although important differences exist between the two vaccines. Currently, there is still a sharp contrast between the clinical or experimental data reported in the literature on COVID-19 and the scarcity of data on the unusual thrombotic events observed after the vaccination with these vaccines. Different hypotheses might support these observations and should trigger further in vitro and ex vivo investigations. The work currently done by the hematologists and the clinical pathologists is of upmost importance to try to elucidate the mechanisms behind the observed TTS in those patients and to potentially identify risk factors and allow adequate risk minimization. Detailed clinical reports with fully documented time-related data (including platelet count changes after diagnosis and anticoagulant management) are eagerly awaited. The role of IVIG infusion also deserves to be fully investigated.

Authors' contribution

JD designed the document. JD wrote the first draft. JF, JMD, TL, SS, CC, AL, RG, PS, GP, YG, PN, CV, FM provided comments and provided reviews for the final version of the manuscript. JD finalized the document.

Declaration of competing interest

Among the authors, JD is the CEO and founder of QUALIblood s.a., a contract research organization manufacturing the DP-Filter, is a co-inventor of the DP-Filter (patent application number: PCT/ET2019/052903) and reports personal fees from Daiichi-Sankyo, Mithra Pharmaceuticals, Stago, Roche and Roche Diagnostics outside the submitted work; TL reports non-personal fees from IRIS and Stago. SS received research support for her institution from LFB and CSL-Behring, and performed consultancy for LFB, Roche, Sobi, Takeda: fees go to the institution. She is co-founder and owner of Laelaps Therapeutics. CC reports speaker fees from Boerhinger-Ingelheim, participated to scientific boards for Astrazeneca and Biogen, and is a member of steering

committee of international clinical trials for BMS and Biogen. CV reports non-financial support from Shire, Sobi, CSL-Behring, Roche, Takeda, outside the submitted work; FM reports institutional fees from Stago, Werfen, Nodia, Roche Sysmex and Bayer. He also reports speaker fees from Boehringer Ingelheim, Bayer Healthcare, Bristol-Myers Squibb-Pfizer, Stago, Sysmex and Aspen all outside the submitted work. The other authors have no conflict of interest to disclose.

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