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THE USE OF 1E12, A MONOCLONAL ANTI-PLATELET FACTOR 4 ANTIBODY TO IMPROVE THE DIAGNOSIS OF VACCINE-INDUCED IMMUNE THROMBOTIC THROMBOCYTOPENIA

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Running head: a new competitive immunoassay for the diagnosis of VITT

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ABSTRACT

Background. Vaccine-induced immune thrombotic thrombocytopenia (VITT) is a complication of adenoviral-based vaccine against SARS-COV-2 due to prothrombotic IgG antibodies to platelet factor 4 (PF4), and may be difficult to distinguish from heparin-induced thrombocytopenia (HIT) in patients treated with heparin.

Objectives. We assessed the usefulness of competitive anti-PF4 enzyme immunoassays (EIA) in this context.

Methods. The ability of F(ab')₂ fragments of 1E12, 1C12 and 2E1, 3 monoclonal anti-PF4 antibodies, to inhibit the binding of human VITT or HIT antibodies to PF4 was evaluated using EIAs. Alanine scanning mutagenesis was performed to define the amino acids (AA) involved in the interactions between the monoclonal antibodies and PF4.

Results. A strong inhibition of VITT IgG binding to PF4 was measured with 1E12 (median inhibition 93%, n=8), whereas it had no effect on the binding of HIT antibodies (median: 6%, n=8). In contrast, 1C12 and 2E1 inhibited VITT (median: 74 and 76%, respectively) and HIT antibodies (median: 68 and 53%, respectively) binding to PF4. When a competitive anti-PF4 EIA was performed with 1E12 for 19 additional VITT samples, it strongly inhibited IgG binding to PF4, except for one patient, who had actually developed HIT according to the clinical history. Epitope mapping showed that 1E12 interacts with 5 key AAs on PF4, of which 4 are also required for the binding of human VITT antibodies, thus explaining the competitive inhibition.

Conclusions. A simple competitive anti-PF4 EIA with 1E12 could help confirm VITT diagnosis and distinguish it from HIT in patients when both diagnoses are possible.

Keywords: Immunoassay, Platelet Factor 4, Thrombocytopenia, Thrombosis, Vaccines

INTRODUCTION

The COVID-19 pandemic emerged worldwide in the first months of 2020 and the global research community urgently developed efficient and safe vaccines against the SARS-CoV-2 virus. Among them, adenovirus-based vaccines were developed, including ChAdOx1 nCov-19 from AstraZeneca [1], which was the first validated, and then Ad26.CoV2.S from Johnson & Johnson. However, few months later, some severe and multiple thrombotic events, including splanchnic vein and cerebral venous sinus thrombosis (SVT and CVST, respectively) were reported [2,3,4]. All cases were associated with thrombocytopenia and occurred within 4 weeks following the first dose of COVID-19 adenoviral vaccines [5]. Although the pathophysiology of VITT is not fully understood, the main finding was a high titre of platelet factor 4 (PF4)-specific IgG antibodies, which were showed to strongly activate platelets *in vitro* without heparin and in the presence of PF4 [2,3,4]. This syndrome, called vaccine-induced immune thrombotic thrombocytopenia (VITT), shares similar characteristics with heparin-induced thrombocytopenia (HIT), another disease associated with thrombosis and thrombocytopenia [6]. HIT is also mediated by IgG PF4-specific antibodies, but most often directed toward PF4/heparin complexes (anti-PF4/H IgG). The similarities between HIT and VITT may explain why in some patients treated with heparin and having received an adenovirus vaccine, the distinction between the two entities is delicate, especially as biological assays used for the diagnosis of VITT are those initially developed for HIT [7]. However, the distinction between HIT and VITT is essential for the treatment of patients since the severity of VITT justifies systematic administration of intravenous immunoglobulin (IVIg) [8], which is not the case in HIT. In addition, VITT antibodies remain in the circulation for much longer than those in HIT [9], indicating the patient needs to be monitored differently. In addition, it is important to distinguish HIT from VITT to avoid re-exposure to heparin and a recurrence of HIT.

We had previously developed a PF4-sensitized serotonin release assay (PF4-SRA) to confirm the diagnosis of HIT [10], and this test is also capable of detecting platelet-activating VITT antibodies [11]. On the other hand, we have produced several monoclonal chimeric anti-PF4 IgG1 antibodies with a human Fc portion, called 1E12, 1C12 and 2E1, which mimic human PF4-specific antibodies [12]. 1E12 behaves like human VITT antibodies, and its F(ab')₂ fragment inhibits their binding to PF4 with a mechanism not fully understood [13]. On the other hand, the inhibitory effect of 1C12 and 2E1 was not assessed.

In this context, the aim of the present project was to evaluate whether the F(ab')₂ fragment of 1E12, 1C12 and 2E1 could be used in an enzyme immunoassay (EIA) to confirm the diagnosis of VITT in a cohort of patients suspected of having developed VITT, and to potentially distinguish VITT from HIT.

PATIENTS AND METHODS

Patients with suspected VITT

As a reference laboratory for the diagnosis of VITT, we received samples from 105 consecutive patients with suspected VITT, referred to our laboratory by 36 different hospitals in France and Belgium from March 2021 to August 2021.

All patients had recently received one or two injections of COVID-19 vaccine based either on messenger RNA technology (MODERNA, mRNA-1273 and PFIZER Bio Tech, BNT 162b2) or on recombinant adenoviral vectors encoding the SARS-COV-2 spike protein (ChAdOx1 nCov-19, ASTRAZENECA and Ad26-COV2S Johnson and Johnson) (**Table I**).

Detailed clinical and biological information was gathered, including vaccination details, time to VITT suspicion, platelet count (PC) evolution, clinical course, especially thrombotic complications, or administered therapies.

We systematically detected platelet activating-anti-PF4 IgG using a platelet factor 4 (PF4)/polyanion EIA and a platelet functional assay to confirm the diagnosis of VITT.

This study was based on medical records, in strict compliance with the French reference methodology MR-004 established by French National Commission on Informatics and Liberties (CNIL), and approved by the Institutional data protection authority of CHU Lille (Number: DEC 21-128) and by the Ethics committee (ECH21/04).

Biological diagnosis of VITT

Antibodies to PF4 were detected using the LIFECODES PF4 IgG EIA (Immucor GTI Diagnostics, Waukesha, USA), which employs PF4–polyvinyl sulfonate (PVS) complexes as an antigen. This assay was performed according to the manufacturer's instructions, and an optical density (OD) ≥ 0.4 indicated significant levels of antibodies against PF4/PVS, whereas OD < 0.4 was considered as a negative result.

In order to detect platelet-activating VITT antibodies, a sensitive ^{14}C -serotonin release assay (PF4-SRA) developed in our laboratory [10] was also systematically performed on all samples. Briefly, PF4 (Hyphen BioMed) at 10 $\mu\text{g}/\text{mL}$ was preincubated for 10 minutes with washed platelets to allow its binding to the cell surface before samples from suspected patients were tested. Each sample was tested without and with 0.1, 0.5, 1 and 10 IU/mL of unfractionated heparin (UFH, Heparine Choay, Sanofi). The result was considered positive when a release of

^{14}C -serotonin $\geq 20\%$ was measured at 0, 0.1, or 0.5 IU/mL of UFH, with an inhibition at 10 IU/mL (^{14}C -serotonin release $< 20\%$ or 50% inhibition).

After careful analysis of clinical data from patients with suspected VITT, the diagnosis was considered as definite in case of both positive PF4/PVS EIA and PF4-SRA.

For patients with a clinical history highly suggestive of VITT and significant anti-PF4 IgG levels but with negative PF4-SRA, VITT diagnosis was considered as probable.

In the absence of anti-PF4 IgG antibodies, the diagnosis of VITT was excluded.

Diagnosis of HIT

Samples from patients with definite HIT diagnosis were also used. All patients had experienced HIT under heparin treatment, with both positive PF4/PVS EIA (LIFECODES PF4 IgG EIA, Immucor GTI Diagnostics) and conventional SRA. The blood collection was approved by the local Ethics Board (DC 2008-308).

Monoclonal antibodies 1E12, 1C12, and 2E1

The chimeric anti-PF4 monoclonal IgG1 antibodies, 1E12, 1C12, and 2E1 were produced and purified by ArkAb (Limoges) as previously described [12]. The F(ab')₂ fragments of these antibodies were obtained by using FragIT kit (Genovis), as previously described [13].

EIA competition assay with F(ab')₂ fragments of 1E12, 1C12 and 2E1

The ability of F(ab')₂ fragments of 1E12, 1C12 and 2E1 to inhibit the binding of antibodies to modified PF4 was evaluated by using PF4/PVS IgG assay (Immucor), as recently described [13], and plasma samples from patients considered as having developed definite/probable VITT or definite HIT diagnosis. Briefly, F(ab')₂ fragments of 1E12, 1C12 or 2E1 (10 $\mu\text{g}/\text{mL}$) were first incubated for 10 minutes at room temperature (RT) in PF4/PVS coated wells. Then, diluted

plasmas (1/50) were incubated for 50 minutes at RT. After washing, EIAs were performed in accordance to the manufacturer's instructions (HAT IgG, Immucor). Optical densities were measured at 450 nm using an Infinite M200 Pro microplate reader (Tecan). The percentages of inhibition of human VITT antibodies binding to modified PF4 were calculated using the following formula:

$$\text{Binding inhibition \%} = \frac{[(\text{Sample OD without Fab'2} - \text{Sample OD with Fab'2})]}{[\text{Sample OD without Fab'2} - \text{OD of Fab'2 alone}]} \times 100$$

Inhibition \geq 50% was considered as significant.

Epitope mapping the interactions of 1E12, 1C12 and 2E1 with PF4

Epitope mapping of 1E12, 1C12 and 2E1 with PF4 was performed using alanine-scanning mutagenesis to create 70 unique PF4 mutants by replacing non-alanine amino acids with alanine and alanine with valine as previously described [14]. We assessed binding patterns of 1E12, 1C12 and 2E1 against 70 PF4 mutants to identify the specific AA binding targets. The binding of anti-PF4 antibodies to wild-type recombinant PF4 and PF4 mutants was measured using a modified PF4/heparin IgG-specific EIA where wells were coated with 10 $\mu\text{g mL}^{-1}$ streptavidin and 1 IU/mL biotinylated-heparin. Wild-type recombinant PF4 or PF4 mutants at 5 $\mu\text{g/mL}$ were then added and incubated. Diluted patient plasma samples (1:50) were tested in technical duplicates then reacted with alkaline-phosphatase-conjugated goat anti-human IgG (γ -chain-specific; Jackson ImmunoResearch Laboratories) at a 1:3,000 dilution. Finally, p-Nitrophenyl phosphate substrate (Millipore-Sigma) dissolved in diethanolamine buffer was used for detection and OD was measured at 405 nm and 490 nm (as a reference) using a BioTek 800TS microplate reader (Agilent Technologies) to assess the binding of antibodies to wild-type

recombinant PF4 and PF4 mutants. A critical binding AA on PF4 was identified when the corresponding PF4 mutant caused a greater than 50% reduction in binding compared to wild-type PF4.

Statistical analysis

The statistical analysis was performed with GraphPad Prism software (version 9.0.1). The quantitative variables related to the cohort of patients were analyzed using the Student's t test, those related to the competitive EIAs using the Mann-Whitney test, while the qualitative variables were analyzed using the Chi-square test. A p-value of < 0.05 was considered significant.

RESULTS

Biological and clinical features of VITT cases

Among the 105 patients suspected of VITT, 89 patients had received only one dose of SARS-CoV-2 vaccine and the 16 others received two doses. A recombinant adenoviral vaccine had been injected in 79 patients while the 26 others had received an mRNA-based vaccine.

VITT was suspected after a median interval of 10 days (range: 1-120 d) following the last vaccine dose and patients presented with either isolated thrombocytopenia (n = 15, median platelet count: 12 G/L, range: 1 – 135 G/L) or thrombosis (n = 40) or both symptoms (n = 45, median platelet count: 46 G/L, range: 9 – 148 G/L). Five additional patients presented with other clinical manifestations (**Table I**).

Significant levels of IgG antibodies to PF4 were detected with PVS/PF4 EIA (median OD = 2.4; range 0.72-3.35) in 26 patients. Of note, all but one of the patients had anti-PF4 IgG OD >1.0. Since the 26 plasma samples were always able to induce strong platelet activation in the presence of PF4 (positive PF4/SRA), the diagnosis of VITT was therefore considered as definite in all these patients. In one additional case, high plasma levels of IgG antibodies to PF4 were also present (OD = 2.4), but PF4-SRA was negative, and the diagnosis of VITT was thus only considered as probable.

These 27 patients had received the ChAdOx1 nCov-19 adenoviral vaccine, a first injection in 26 cases and a second injection in one case. Of note, females were more represented in the group of patients with VITT (1 male for 1.7 female) than in those without (1 male for 0.83 female ($p < 0.001$)) (**Table I**).

VITT was suspected in these patients 7 to 22 days after vaccination, and all had developed thrombocytopenia (median 30 G/L, range 9-95 G/L) and thrombosis (T+T). Cerebral vein thrombosis (CVT) ($n = 10$, 37%) and splanchnic vein thrombosis (SVT) ($n = 7$, 26%) were the most frequent events. Deep venous thrombosis (DVT) and/or pulmonary embolism (PE) occurred in 4 patients and arterial thrombosis (AT) or ischemic stroke in 4 and 2 cases, respectively. In addition, in these patients with VITT, fibrinogen levels were consistently lower (median 1.79 g/L) than in those without VITT (median 3.97 g/L) ($p < 0.001$), and plasma levels of D-Dimers, when measured at the time of suspicion, were always high (median: 20 000 ng/mL).

The diagnosis of VITT was excluded in the remaining 78 patients, since anti-PF4 IgG antibodies were absent, or detected in 6 cases with low levels (median OD 0.52, range 0.42-0.88), and PF4-SRA was always consistently negative.

Usefulness of competitive EIA with the F(ab')₂ of 1E12, 1C12, and 2E1

Sixteen plasma samples from patients with definite VITT (n=8) or HIT diagnosis (n=8) were tested in the presence of F(ab')₂ fragments of 1E12, 1C12 and 2E1. A strong inhibition of VITT IgG binding to PF4 was measured with 1E12 F(ab')₂ (median inhibition: 93%, range: 73-99%) while it was not effective at all in inhibiting the binding of HIT IgG (median: 7.5%, range: 2-21%) (**Figure 1**). The F(ab')₂ fragment of 1C12 equally inhibited VITT and HIT IgG antibodies binding to PF4 (median: 76.5%, range: 49-92% and median: 77.5, range: 2-88, respectively), while 2E1 F(ab')₂ slightly better inhibited HIT than VITT antibodies binding to PF4 (median: 67, range: 4-76% and median: 53%, range: 19-67%, respectively). These results therefore clearly indicated that the F(ab')₂ fragment of 1E12 was considerably better at discriminating the binding of VITT and HIT antibodies to PF4 than 1C12 and 2E1 F(ab')₂.

Epitope mapping the interactions of 1E12, 1C12, and 2E1 with PF4

To better understand the results obtained in competitive EIAs, which suggested that 1E12 displayed a specificity toward PF4 more similar to human VITT antibodies than 1C12 and 2E1, we performed epitope mapping using alanine scanning mutagenesis of the key interactions of the 3 monoclonal antibodies 1E12, 1C12 and 2E1 with PF4 (**Figure 2**). The data obtained supported that 1E12 interacts with 5 AAs on PF4 (Arg22, His23, Leu45, Lys50, Lys66), distributed in 3 distinct areas of its primary sequence. Of these 5 AAs, 4 have previously been showed to be required in the binding of VITT antibodies to PF4 (Arg22, His23, Lys50, Lys66), whereas none were involved in HIT antibody binding [15]. These results confirmed that 1E12 recognizes the same epitope on the surface of PF4 as human VITT antibodies, which explains why the F(ab')₂ of 1E12 inhibits their binding to PF4 in this competitive EIA, but not that of HIT antibodies.

On the other hand, the epitope mapping analysis performed with 1C12 and 2E1 showed that 1C12 likely binds to only 1 key AA (Asparagine 47) required in the binding to PF4 of VITT antibodies, while 2E1 doesn't bind to any key AA. In contrast, both antibodies recognize key AAs for the binding of HIT antibodies (cysteine 52 for 1C12 and cysteine 10 for 2E1), which may explain why they inhibit both HIT and VITT antibody binding to PF4, albeit more or less depending on the human sample tested.

The competitive EIA with the F(ab')₂ of 1E12 can distinguish VITT from HIT

Results obtained in competitive EIA with 1E12, 1C12 and 2E1, as well as epitope mapping data prompted us to select 1E12 for further testing using 19 additional plasma samples from VITT patients (**Figure 3**). The data obtained confirmed that 1E12 efficiently inhibited the binding of IgG antibodies to PF4 in 18 samples, including the one from the patient with « probable » VITT (% of inhibition by 1E12: 70%). Therefore, this sample likely contained anti-PF4 IgG antibodies sharing similar specificity toward PF4 than VITT antibodies. In contrast, the inhibitory effect of 1E12 was not significant with one plasma sample (% of inhibition by 1E12: 17%), suggesting that the specificity of anti-PF4 IgG antibodies in this patient was different from those of other VITT samples tested.

This result therefore prompted us to analyse further the clinical history of this patient, who had been referred to our laboratory for suspected VITT. Careful examination of his file revealed that he had also been exposed to heparin before VITT suspicion (**Figure 4**). This 62-year-old man with a previous history of arteriopathy of the lower limbs had received 2 doses of ChAdOx1 nCov-19, the first in April 2021, and the second on June 4th, 2021. Nineteen days later on June 23th, he was admitted for an acute left femoropopliteal arterial thrombosis necessitating an angioplasty with multiple stentings. A treatment with UFH was initiated (day

0), but on day 7, a new angioplasty with stentings was performed due to stent rupture and thrombosis. On day 8, recurrent thrombocytopenia (60 G/L) was evidenced and HIT was suspected (4Ts score = 5). UFH was therefore replaced by danaparoid sodium, but a rapid immunoassay (HemosIL AcuStar HIT-IgG) was negative (result: 0.03 AU/mL; cut-off: 1.0 AU/mL) and the diagnosis of HIT was ruled out. Since this patient had received a second dose of ChAdOx1 nCov-19 less than 30 days before, VITT was then suspected. High titres of PF4-specific IgG antibodies were detected using the PF4/PVS EIA (OD: 2.80). The PF4-SRA was also positive, with strong platelet activation without heparin, this pattern also in agreement with the diagnosis of VITT. Anticoagulation with danaparoid sodium was then maintained and combined with intravenous immunoglobulin (IVIG) for 3 days. The short-term clinical evolution was characterized by platelet count recovery (>100 G/L), although the following 15 days were marked by a recurrence of a PC drop and new thrombotic events, requiring further infusions of IVIG. The outcome was finally favorable with complete and stable PC normalization, allowing to prescribe a direct oral anticoagulant (apixaban).

This complex clinical history, especially due to the use of heparin together with the injection of ChAdOx1 nCov-19 vaccine, explained the difficulty to distinguish HIT from VITT in this particular patient. However, the result obtained with our competitive EIA using 1E12 F(ab')₂, supports the diagnosis of HIT.

DISCUSSION

In this study, we showed after evaluating a prospective cohort of patients with suspected VITT that functional assays could be avoided for confirming VITT, as a sensitive and specific EIA is often sufficient for achieving this objective. In this respect, it is essential to use a heparin-free assay, and we had previously showed that PVS has a lower impact than heparin on the binding

of human VITT antibodies to PF4 [11]. Therefore, using a commercial anti-PF4/PVS EIA that is easily available and sensitive, we also developed a simple competitive EIA based on the use of the F(ab')₂ fragment of 1E12, a monoclonal anti-PF4 IgG exhibiting similar specificity toward PF4 than human VITT antibodies [13]. Using this assay, 1E12 F(ab')₂ was shown to strongly inhibit the binding of human VITT antibodies to PF4 in 25 of the 26 (96.2%) definite VITT cases tested, but also in a patient with « probable » VITT but negative PF4-SRA. In contrast 1C12 as well as 2E1, 2 other monoclonal anti-PF4 antibodies were ineffective in distinguishing between VITT and HIT antibodies. However, these results were logical considering the data obtained after studying the epitope mapping of our 3 monoclonal anti-PF4 antibodies. Indeed, 1E12 did not appear to bind any AA previously identified as being crucial for the binding of human HIT antibodies to PF4 [15], while 1C12 and 2E1 bound to 1 key AA. Using a docking model, we previously suggested that the 3 antibodies had close epitopes on the surface of PF4 [12], but this conclusion is not supported by the present study. The docking analysis used bioinformatics models for statistical binding predictions, whereas the EIA using single PF4 mutants evaluated the importance of each AA in PF4 antibody binding. While this latter approach may also have limitations, it seemed particularly suitable here as PF4 was coated in wells during the epitope mapping process, similar to the competitive EIA.

Our data confirm and suggest that a simple 1E12-based competitive EIA could be useful for VITT confirmation in case of doubtful or unavailable functional assays.

The ISTH SSC Subcommittee on Platelet Immunology recommended for the diagnosis of VITT to perform platelet functional assays [16], and several authors proposed PF4-sensitized assays [2,8,11,17,18]. However, these assays also yielded false negative results in VITT [19], as evidenced in one of our patients with « probable » VITT, for whom PF4-SRA was negative. Schönborn *et al.* recently demonstrated using PF4-induced platelet activation (PIPA) tests that such failures might be due to an inappropriate PF4/anti-PF4 IgG ratio *in vitro*, which can be

overcome by diluting patients' samples [20]. Another potential pitfall can result from the intravenous injection of polyclonal immunoglobulins in the patient, which can inhibit VITT IgG-induced platelet activation in SRA [8,21]. Moreover, platelet functional assays cannot be used in many centers for a rapid diagnosis of VITT, reinforcing the crucial contribution of sensitive and specific anti-PF4 immunoassays in the diagnostic strategy of VITT [22].

Importantly, our 1E12-based competitive EIA could be of particular interest in patients for whom VITT and HIT may be difficult to distinguish. In our study, one patient had been administered both an adenoviral vector SARS-CoV-2 vaccine and heparin, and using our competitive EIA, we demonstrated that 1E12 failed to inhibit the binding of patient's IgG antibodies to PF4/PVS, who likely had developed HIT after carefully analyzing the clinical history. Of note, false negative HemosIL AcuStar HIT-IgG results have been described in patients with a strong clinical suspicion of HIT [23,24], which was probably the case for the patient described here. The 1E12-based competitive EIA therefore allowed us to highlight a probable misdiagnosed HIT case, but its usefulness deserves to be confirmed in a larger cohort of patients suspected of VITT and HIT. In particular, Huynh *et al.*, suggested that two different types of anti-PF4 IgG antibodies may be present in VITT patients, with either PF4-dependent or independent ability to activate platelets [25]. This could have an impact on the sensitivity of our competitive EIA, but will require further evaluation to ensure that 1E12 is capable of competing with both types of VITT antibodies.

Beyond the small number of samples tested, our study has limitations, as we suspected VITT in patients who experienced thrombocytopenia but didn't consider platelet count fall, while recent reports indicated that normal or subnormal platelet count may represent an early stage of the disease [5,26]. Likewise, a period of 5-30 days after vaccination has been considered in our study, but later cases occurring up to 42 days post-vaccination have been reported in few patients with less severe symptoms (i.e isolated DVT/PEs) [5]. Finally, we used a commercial

PVS/PF4 EIA for the development of our competitive assay because of its availability, but we cannot exclude that PVS may have caused some partial inhibition of VITT antibody binding to PF4 even before addition of 1E12 and that an in-house native anti-PF4 EIA could be more appropriate in the future.

In conclusion, we demonstrated that an anti-PF4/PVS EIA can be modified by using the monoclonal anti-PF4 IgG 1E12 to distinguish between VITT and HIT in specific patients, and this assay may also be useful to confirm VITT if functional assays are not easily available or doubtful. This is of particular importance because, beyond the fact that new adenovirus-based vaccine candidates are actually under development, several cases of VITT-like syndrome occurring at distance of any vaccination but after adenovirus infections have recently been detected [27,28], suggesting that VITT is still a topical issue.

AUTHOR CONTRIBUTION

C. Vayne performed the research, analysed data and wrote the paper, J. Rollin, R. Clare, M. Daka, M. Atsouawe, E.A. Guéry and I. Nazy performed the research and analysed data, P. Cauchie, P. Cuisenier, C. Cordonnier, E. De Maistre, M. Donnard, N. Drillaud, D. Faille, H. Galinat, I. Gouin-Thibault, S. Lemoine, G. Mourey, F. Mullier, V. Siguret, S. Susen, and A. Godon contributed to patient recruitment, clinical data collection and analysis, Y. Gruel and C. Pouplard designed the research, analyzed data, and wrote the paper. All authors reviewed and approved the final version of the manuscript.

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CONFLICTS OF INTEREST

C.V reports honorarium from Viatris, J.R reports research grant from Stago. HG reports transport fees from STAGO. F.M reports speaker fees from Fresenius, Technoclone and Werfen all outside the submitted work Y.G reports research grant and symposium fees from Stago. C.P reports research grant from Stago. All other authors of this paper have no conflicts of interest.

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LEGENDS OF FIGURES**Figure 1: Inhibition of human VITT and HIT antibodies binding to PF4 by 1E12, 1C12 and 2E1 in competitive immunoassay.**

Percentage (%) of inhibition of anti-PF4 IgG antibodies binding to PF4/PVS in EIA after pre-incubation of the F(ab')₂ fragment of the monoclonal anti-PF4 IgG 1E12, 1C12 and 2E1. Each symbol represents one definite VITT or HIT patient's sample (n=8 for each group). The solid lines indicate the median % inhibition for each condition.

Figure 2: Epitope mapping of the interactions of 1E12, 1C12 and 2E1 with PF4

The epitopes of the anti-PF4 IgG monoclonals 1E12, 1C12 and 2E1 on PF4 are indicated by colored areas using 3-D modelling (A) or the amino-acids primary sequence of PF4 (B). The empty black boxes on the primary sequence of PF4 indicate the 3 areas previously identified as involved in the binding of human VITT antibodies and is also the heparin-binding region on PF4 [15].

Figure 3: Inhibition of human VITT antibodies binding to PF4 by 1E12 in competitive immunoassay.

Percentage (%) of inhibition of anti-PF4 IgG antibodies binding to PF4/PVS in an EIA after pre-incubation of the F(ab')₂ fragment of the monoclonal anti-PF4 IgG 1E12. Each circle represents one definite or probable VITT sample distinct from the 8 samples already tested and described in figure 1 (n=19). The solid line indicates the median % of inhibition. The empty

circle indicates the patient for whom VITT diagnosis was considered probable although PF4-SRA was found negative. The red arrow indicates the patient initially diagnosed as VITT, and for whom HIT was more likely.

Figure 4: Clinical course of a patient for whom the distinction between HIT and VITT was tricky

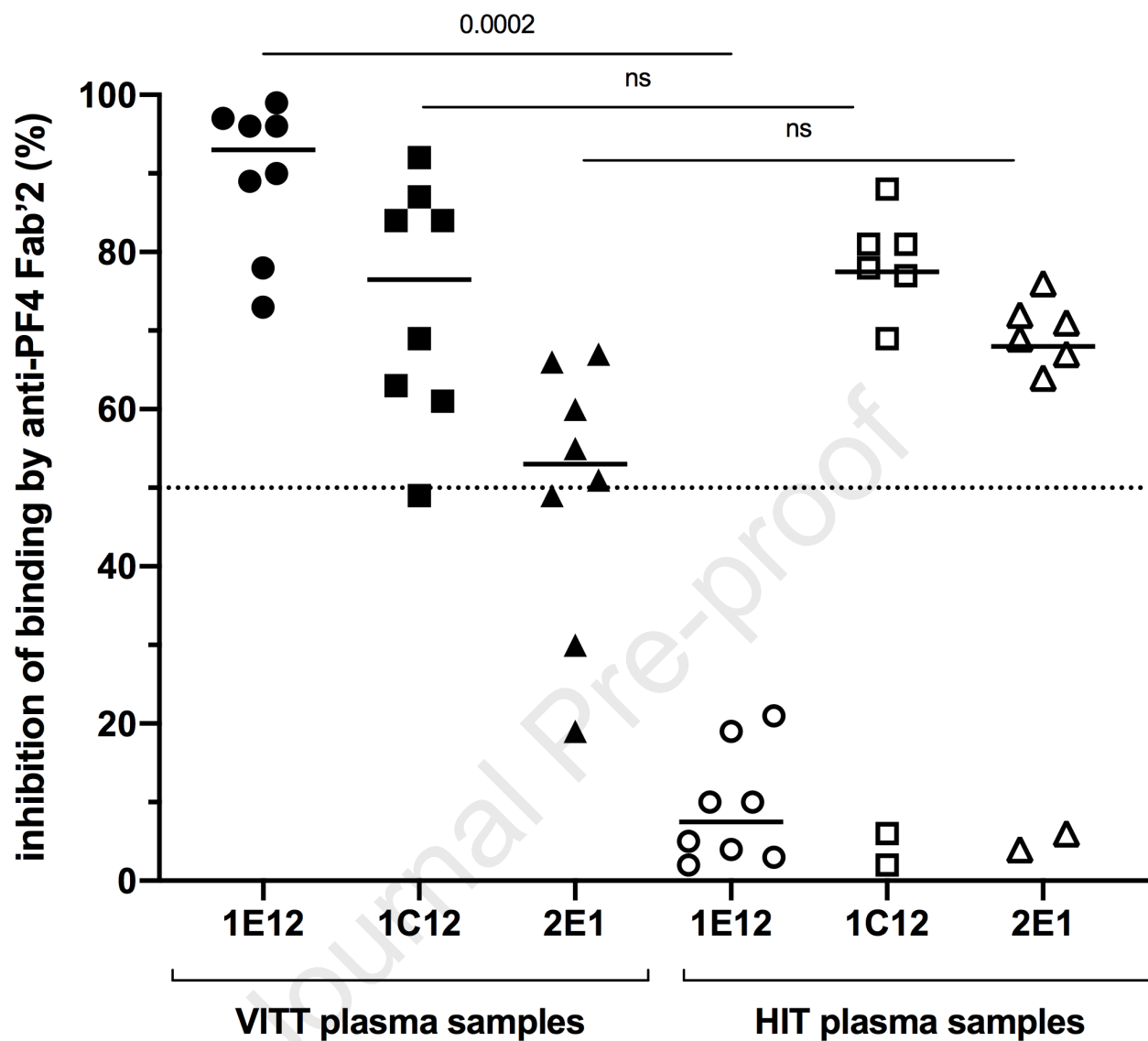
Events in red indicate thrombotic events, and blue ones, therapeutic procedures. Anticoagulants administered to the patient are indicated at the bottom of the figure. Results of the anti-PF4/PVS EIA and PFA-SRA at diagnosis, 3 and 6 months later are also reported.

HIT: heparin-induced thrombocytopenia, IVIG: intravenous immunoglobulins, PF4: platelet factor 4, UFH: unfractionated heparin, VITT: vaccine-induced immune thrombotic thrombocytopenia.

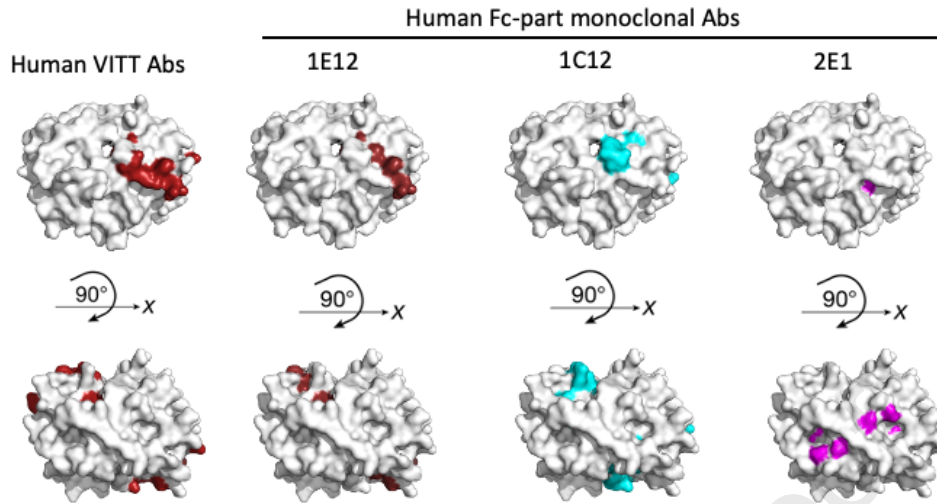
Table I: Biological and clinical features of the 105 enrolled patients suspected of VITT

n	All Patients	Non VITT (n= 78)		VITT 1 probable 26 definite
	105	mRNA SARS-CoV-2 vaccine 26	adenovirus SARS-CoV-2 vaccine 52	adenovirus SARS-CoV-2 vaccine 27
Age years	62 (21-93)	58.5 (24-93)	65 (21-84)	62 (22-79)
Sex (ratio M/F)	53/52	11/15	32/20	10/17
Suspicion after the 1st dose	88	18	44	26
Suspicion after the 2nd dose	17	8	8	1
Delay of symptoms	10 days (1-120 d)	9.5 days (2-120 d)	10 days (1-90 d)	10 days (7-22 d)
Classification of symptoms				
- Isolated Thromboses	40	10	30	0
- Platelet count (G/L)		196 (170-351)	221 (90-435)	
- DDi (ng/ml)		(2930-3600) *	2485 (370-23708)	
- Fibrinogen (g/L)		4.92 (4.35-5.44)	5.00 (2.54-6.95)	
- PVS/PF4 EIA (OD)		0.05 (0.00 - 0.25)	0.07 (0.01 - 0.34)	
- Thromboses + thrombocytopenia	45	9	9	27
- Platelet count (G/L)		92 (16 - 147)	114 (28 - 148)	30 (9 - 95)
- DDi (ng/ml)		1280 (1011 -1300)	9560 (270 -20000)	20000 (4000 - 20000)
- Fibrinogen (g/L)		4.30 (3.40 - 4.90)	2.96 (1.77 - 3.29)	1.79 (0.45 - 4.1)
- PVS/PF4 EIA (OD)		0.10 (0.02 - 0.88)	0.04 (0.02 - 0.54)	2.2 (0.72 - 3.5)
- Thrombocytopenia	15	5	10	0
- Platelet count (G/L)		7 (1 - 50)	36 (1 - 135)	
- DDi (ng/ml)		600 (330 - 3090)	623 (464 - 57000)	
- Fibrinogen (g/L)		3.21 (2.99 - 4.81)	3.00 (2.70 - 6.50)	
- PVS/PF4 EIA (OD)		0.16 (0.03 - 0.16)	0.03 (0.03 - 0.16)	
- Others	5	2	3	0
Positive PF4-SRA	26	0	0	26
Death	11	2	0	9

The values indicated regarding the age, the delay of symptoms and the classification of symptoms are medians, with the ranges in brackets.
* no median calculated as only two patients had available DDimers levels, indicated in the brackets



A



B

human VITT: EAEEDGDLQCLCVKTTTSQVRPRHITSLEVIKAGPHCPTAQLIATLKNGRKICLDLQAPLYKKIIKLLES

1E12: EAEEDGDLQCLCVKTTTSQVRPRHITSLEVIKAGPHCPTAQLIATLKNGRKICLDLQAPLYKKIIKLLES

1C12: EAEEDGDLQCLCVKTTTSQVRPRHITSLEVIKAGPHCPTAQLIATLKNGRKICLDLQAPLYKKIIKLLES

2E1: EAEEDGDLQCLCVKTTTSQVRPRHITSLEVIKAGPHCPTAQLIATLKNGRKICLDLQAPLYKKIIKLLES

