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REVIEW

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Platelet factor 4 triggers thrombo-inflammation by bridging innate and adaptive immunity

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Abstract

Platelet factor 4 (PF4, synonym: CXCL4) is an evolutionary old chemokine with proposed roles in hemostasis and antimicrobial defense. In addition, PF4 has attracted considerable attention as a crucial mediator of one of the most prothrombotic adverse drug effects affecting blood cells, heparin-induced thrombocytopenia (HIT). Interest in PF4 substantially increased in 2021 when it was identified as the target antigen in the life-threatening adverse effect, vaccine-induced immune thrombotic thrombocytopenia (VITT). We address the concept that a major biological function of PF4—a strongly cationic chemokine—is to bind to negatively-charged prokaryotic microorganisms, resulting in structural changes in PF4 that trigger a danger signal recognized by the adaptive immune system. Application of biophysical tools has provided substantial insights into the molecular mechanisms by which PF4 becomes immunogenic, providing insights into a new mechanism of autoimmunity. Binding of autoantibodies with high affinity induces conformational change(s) in the endogenous protein, which are then recognized as foreign antigen, as exemplified by the prothrombotic disorders, autoimmune HIT and VITT. The final part of our review summarizes current assays for HIT and VITT, explaining how structural aspects of anti-PF4 pathobiology relate to assay design and performance characteristics. Currently, functional (platelet activation) assays using washed platelets detect HIT antibodies when heparin is added, and VITT antibodies when PF4 is added. Solid-phase PF4-dependent immunoassays using microtiter plates are sensitive for both HIT and VITT antibodies, while rapid immunoassays, in which the PF4/heparin antigen is coated on beads, are sensitive and specific for HIT, but not for VITT antibodies.

KEYWORDS
chemokine receptors, conformational changes, heparin, HIT, platelet factor 4, thrombocytopenia, thrombosis, VITT

1 | INTRODUCTION

Platelet factor 4 (synonym: CXCL4) is a 32 kDa positively-charged tetrameric chemokine protein primarily stored in, and released from, platelets but which can also be synthesized by monocytes.[1] A variety of functions have been attributed to PF4; for example, PF4 modulates thrombogenicity by binding to heparan sulfate of endothelial cells,[2] thereby interfering with vascular wall charge. PF4 also interacts with bacteria[3] and viruses and, in the presence of anti-PF4 antibodies, appears to have a role in anti-microbial defense. Opsonization of

gram-negative bacteria[4] by anti-PF4 antibodies augments their phagocytosis by leukocytes and even directs killing of *Escherichia coli* by platelets.

Besides these potential roles in vascular wall homeostasis and antimicrobial activity, PF4 has also been implicated in certain autoimmune conditions, especially systemic sclerosis.[5] However, the main biological function of PF4 remains unknown. This is remarkable given that the receptor for PF4 is one of the most preserved chemokine receptors in evolution.[6] This CXCR3B receptor is a homeostatic receptor, and shows constitutive G protein and β-arrestin activity[7,8]; homeostatic receptors act as sensors detecting changes in the internal or external environment, upon which they transduce a signal to maintain homeostasis.

PF4 has gained major biomedical attention as it is directly implicated in two highly prothrombotic adverse drug effects: heparin-induced thrombocytopenia (HIT) and vaccine-induced immune thrombotic thrombocytopenia (VITT). The theme of this review is that the biological—and pathobiological effects through HIT and VITT pathogenesis—depend on the structural presentation of PF4. We will address key features of HIT and VITT, including a summary of major biophysical findings on the pathobiologic role of anti-PF4 antibodies. The concepts have important implications for the development of laboratory assays to detect the pathogenic anti-PF4 antibodies underlying HIT and VITT.

1.1 | HIT and VITT

HIT is a well-known adverse effect of certain strongly negatively-charged drugs, most prominently, heparin.[9] Polyanionic heparin forms complexes with cationic PF4, resulting in conformational changes within PF4 that elicit an immune response. Moreover, the PF4/heparin complexes formed are multimolecular, which allows binding of several immunoglobulin G (IgG), producing large PF4/heparin-IgG immune complexes that activate platelets and monocytes via their FcγIIa receptors (FcγRIIa). This triggers a cascade of thromboinflammatory events, leading to thrombocytopenia, massive thrombin generation, and substantial absolute risk of new thromboembolic complications (HIT, ~30%–70%; VITT, >95%), with significant morbidity and mortality.

Risk factors for developing HIT include unfractionated heparin (versus low-molecular-weight heparin) and major (versus minor) surgery or medical/pregnant patients. Females are at slightly higher risk than males, and some genetic polymorphisms within FcγRIIa and related signaling proteins also increase risk. However, large genome-wide association studies did not show any major genetic risk factors other than blood group O.[10,11] Diagnostic laboratory testing is discussed later in this review; treatment recommendations are summarized in the 2018 guidelines of the American Society of Hematology.[12]

Vaccination against severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) represents a biomedical breakthrough against the Covid-19 pandemic that has prevented millions of fatalities. However, beginning in March 2021, cerebral venous sinus thrombosis (CVST), splanchnic vein thrombosis, and other often severe thrombotic events, in combination with thrombocytopenia, were reported in otherwise healthy individuals, beginning 5–20 days post-vaccination[13–17] with adenoviral vector-based vaccines encoding the spike glycoprotein of SARS-CoV-2 (Covid-19 Vaccine AstraZeneca [Vaxzevria]; Covid-19 Vaccine Janssen; Sputnik V).[18–20] This novel disorder, "vaccine-induced immune thrombotic thrombocytopenia" (VITT), is associated with high-titer IgG anti-PF4. These antibodies cluster PF4 without the need for heparin, forming multimolecular immune complexes that—as in HIT -- activate platelets, monocytes, and granulocytes via Fcγ receptors, with platelet activation greatly enhanced by PF4.[13]

1.2 | Strong negative charges are a danger signal for eukaryotic organisms

There is a potential role of microbial pathogens in explaining HIT/VITT pathogenesis. Most bacteria are strongly negatively charged. This provides them an evolutionary advantage, as the repellent forces between two bacteria (zeta potential) keep them away from potential (similarly strongly anionic) prokaryotic "predators." In contrast, eukaryotic cells are considerably less negatively charged (reduction of cell surface charge presumably was a prerequisite to form stable multicellular eukaryotic organisms) (Figure 1A). Thus, dissimilar charge densities represent a fundamental difference between prokaryotic and eukaryotic cells. This helps explain why negative charges are potent activators of the innate immune system: for example, polyanions activate the bradykinin/kininogen and complement systems, and activate granulocytes as zymogens. In contrast, the adaptive immune system does not recognize charge per se.

It is our hypothesis that PF4 binds to negatively charged surfaces of microbial pathogens, thereby undergoing a conformational change which facilitates binding of anti-PF4 antibodies.[21] Since PF4 binds via charge–charge interactions with numerous types of bacteria, the intriguing implication—from an evolutionary perspective—is that an initial anti-PF4 immune response triggered by one type of bacterium will facilitate anti-PF4-mediated immune system recognition against different bacterial species that has not previously been encountered by the immune system (Figure 1B). This hypothesis is also consistent with the PF4 receptor being highly preserved, that is, an evolutionary ancient immune defense mechanism. Further consistent with this hypothesis, anti-PF4 antibody producing B-cells can be found in nearly all individuals,[9,22] including from cord blood of healthy newborns,[23] indicating that these antibodies belong to the repertoire of natural antibodies.[24,25]

Anti-PF4 antibodies are detectable in ~5% of the normal population.[26] However, these antibodies usually show low reactivity in immunoassays and typically do not cause platelet activation in functional assays. In contrast, a misdirected strong anti-PF4 antibody response, as seen in HIT and VITT, features high-avidity, platelet-activating anti-PF4 antibodies, some of them reacting even in the

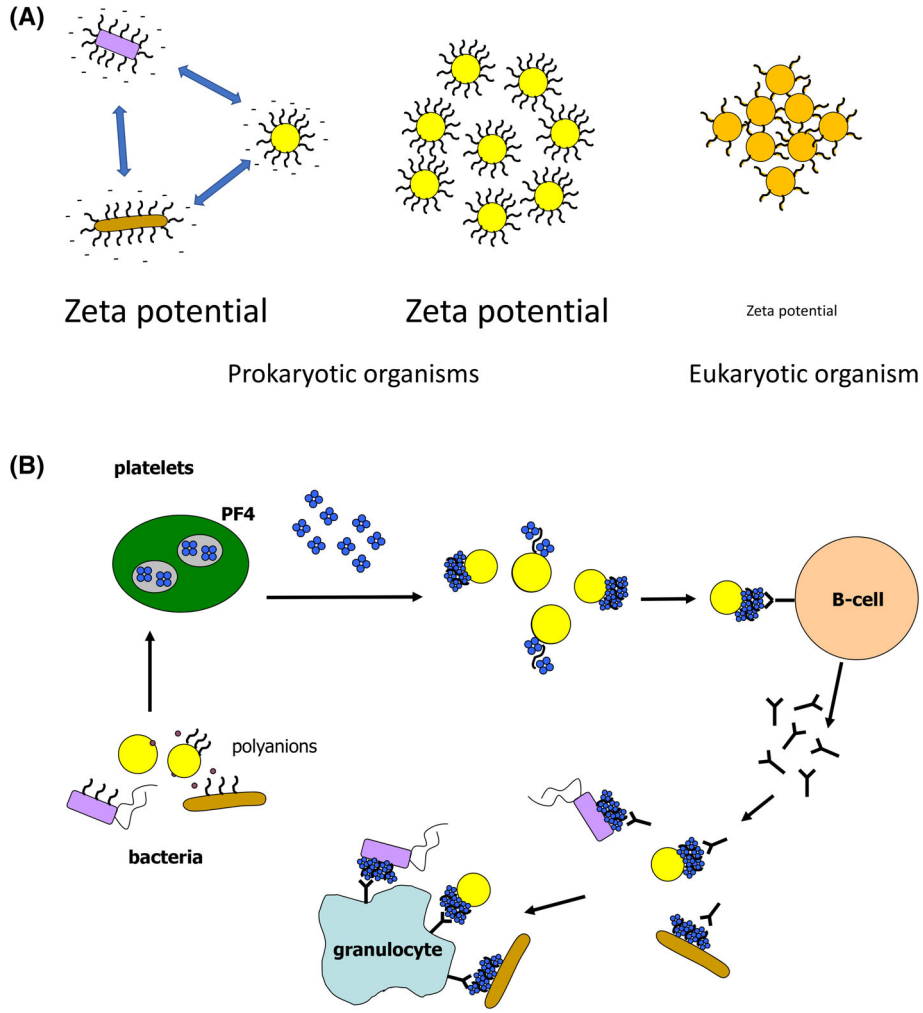

FIGURE 1 Negative charge density is a fundamental difference between prokaryotic and eukaryotic cells, with strong negative charge recognized by PF4. (A) Left and middle panel: prokaryotic cells are strongly negatively charged. The repellent forces result into a high zeta potential, which allows the microbes to maintain distance from each other without the need to consume energy. Right panel: for the switch from prokaryotic to eukaryotic organisms, reduction of the surface charge of individual cells became necessary to reduce the repellent forces between individual cells. (B) Bacteria directly or indirectly interact with platelets, with resulting platelet activation; activated platelets release PF4. PF4 is strongly positively charged and binds to the negatively charged polyanions on prokaryotic bacteria. The PF4/polyanion complexes are recognized by the cells expressing the cognate receptor. This leads to activation of B cells and production of anti-PF4/polyanion antibodies. The evolutionary interesting concept is that once antibodies against PF4 polyanion complexes are formed they can also opsonize other bacteria that the immune system has not previously encountered, by binding to complexes of conformationally altered PF4 on the bacterial surfaces

absence of heparin. Both HIT and VITT feature "pancellular" activation, typical for thrombo-inflammation, involving platelets, neutrophils, monocytes and endothelium.

Major risk factors for forming pathogenic anti-PF4 antibodies are inflammation and tissue trauma, as these provide immunologic "danger signals" that increase the likelihood and intensity of forming an anti-PF4 immune response.[27,28] This is consistent with the concept of the anti-PF4 response as an evolutionary old antimicrobial immune defense mechanism. The requirement of a proinflammatory co-signal, indicating to the immune system that something is really going wrong, before triggering an anti-PF4 immune response, reduces risk of an unwanted prothrombotic immune response.

2 | BETTER UNDERSTANDING THE MOLECULAR MECHANISMS LEADING TO HIT

With the adaption of biophysical tools and techniques in physics, it became possible to identify features that make PF4 immunogenic and to characterize the structure and the thermal stability of complexes formed by PF4 and polyanions, which are relevant for HIT.[29] For VITT, experimental difficulties currently preclude definite conclusions. The technical difficulties are inherent to the super-strong antibody response in VITT as further explained at the end of the article.

2.1 | Clustering and conformational changes of PF4 are associated with expression of neoepitopes

The first indirect evidence that HIT antigens are formed by multimolecular PF4/heparin complexes was provided by experiments showing that high heparin concentrations release PF4 in a dose-dependent manner from PF4/heparin complexes covalently coated to microtiter plates using a linker, with a parallel decrease in anti-PF4/heparin antibody binding.[30] Later, it was shown that the size of the PF4/heparin complexes is governed by the PF4/heparin molar ratio, with maximal complex size occurring at equimolar ratios, at which particles carry a neutral charge.[31]

The first direct confirmation of multimolecular PF4/heparin complexes as binding sites for anti-PF4/heparin antibodies was achieved by visualization. Rauova and coworkers showed by electron microscopy that PF4 and UFH form complexes, which dissociate into smaller complexes upon addition of heparin.[32]

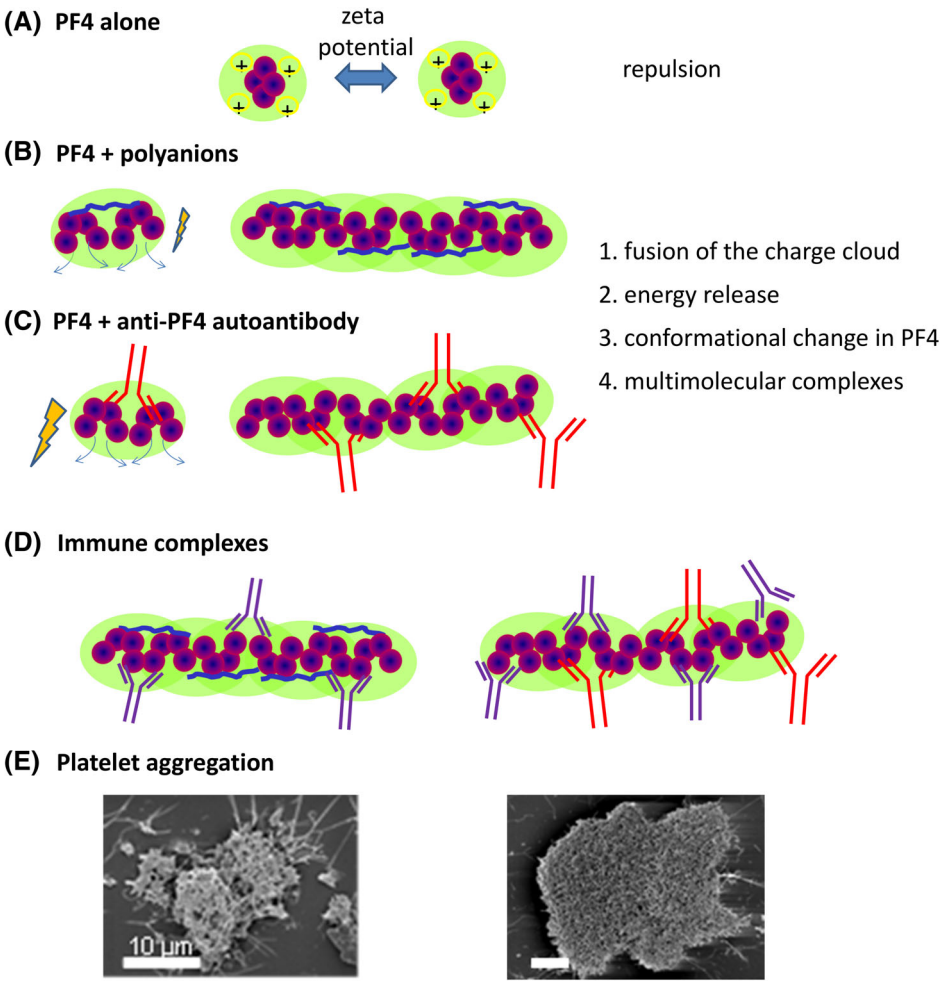

FIGURE 2 Generation of the heparin-induced thrombocytopenia (HIT) antigen(s) by polyanions and anti-PF4 autoantibodies (modified with permission from Reference 54). (A) PF4 is strong positively charged. The resulting zeta potential causes repulsion of single PF4 molecules. (B) Longer polyanion chains can bridge two PF4 molecules, resulting in fusion of the individual charge clouds of the single PF4 molecules; this is an exothermic reaction and the released energy is high enough to induce a conformational change in PF4 by which two monomers at the one side get closer together and the other two monomers open up. Large complexes can be formed between PF4 and polyanions. (C) This process can also be induced by some high avidity anti-PF4 aHIT autoantibodies (red IgG molecules). (D) Anti-PF4/H antibodies (blue IgG antibodies) bind to PF4/polyanion complexes. The same anti-PF4/H antibodies bind to the PF4-containing complexes formed by anti-PF4 aHIT autoantibodies (shown in red). These complexes contain more IgG, because both the heparin-dependent (blue IgG) antibodies as well as the aHIT autoantibodies (red IgG) bind. (E) This is likely the reason why aHIT autoantibodies cause stronger platelet activation and much larger and denser platelet aggregates, as shown by the electron photomicrographs. The white scale bar represents 10 µm. (F) The electron photomicrographs shown in (E) were taken from the supplementary material published with Reference 45

A very interesting finding is that HIT antigen sites are exposed on PF4 only when PF4 is present in its tetrameric form.[33] This is consistent with our hypothesis that PF4 labels pathogens, in essence, *translating charge into structure*, which is then recognized by the adaptive immune system. The necessity of a preserved tetrameric structure strongly reduces spontaneous expression of the epitope for anti-PF4 antibodies during degradation of PF4.

Atomic force microscopy (AFM) further demonstrated that polyanions such as UFH and LMWH bring PF4 tetramers into close approximation[34] (Figure 2). In essence, the globular PF4 tetramers align along a heparin polysaccharide molecule like pearls in a chain, forming long, ridge-like clusters. The height of PF4 in PF4/heparin complexes is a fraction of a nanometer lower compared to non-complexed PF4, indicating that PF4 undergoes a conformational change. However, the challenging question remained, whether the antigenic sites recognized by anti-PF4/heparin antibodies are formed when two PF4 molecules come into close proximity to one another, or only after PF4 undergoes additional conformational change.

2.2 | Direct evidence for conformational changes of PF4 induced by polyanions

The first direct evidence for structural changes in PF4 during complex formation with heparin, relevant for HIT epitopes, was provided by circular dichroism (CD) spectroscopy.[35] The spectrum of PF4 changed

after addition of heparin, which can only be explained by conformational changes. This technique permits assessment of the risk of polyanions to induce potentially immunizing conformational changes in PF4, such as evaluation of nucleic acid-based drugs like aptamers. It might therefore be an interesting tool to be used during preclinical drug development.

Any conformational alterations are associated with a change in the energy status of a molecule. This can be measured by heat release or consumption using isothermal titration calorimetry (ITC). ITC showed that a threshold energy of ∼−4000 cal/mol PF4 is required to drive expression of (the) binding site(s) for anti-PF4/heparin antibodies.[36] Only heparin molecules with chain length ≥10 monosaccharides fulfill these requirements, which matches perfectly with previously reported findings of minimum polysaccharide chain length required to yield positive tests to detect HIT antibodies.

These observations raised a fundamental question. It is well accepted that the interaction of PF4 with polyanions is not dependent on the structure of polyanions and is entirely charge driven. How could it then be that (on the same weight basis) longer polyanions expose neoepitopes on PF4, while shorter polyanions do not, although both have the same grade of sulfation and, therefore, the same density of negative charges per carbohydrate unit? Single molecule force spectroscopy measurements provide an explanation. Each PF4 is surrounded by a single charge cloud. This causes a repellent force (∼50 pNewton) between two single PF4.[37] Short heparins bind to one PF4 tetramer only, whereas long heparins bind to at least two PF4 tetramers[37] (Figure 2). Longer polyanions bring two PF4 molecules sufficiently close to one another that the individual charge clouds of the single PF4 molecules fuse. This forms one larger charge cloud around the complexed PF4. This redistribution/fusion of the charge clouds provides the additional energy needed to induce the conformational changes that expose the neoantigens.

The final proof for this concept was provided by Cai et al.[38] They solved the crystal structure of PF4 in complex with fondaparinux and with Fab fragments of the HIT-like monoclonal antibody, KKO. The polyanion binds to and stabilizes PF4 tetramers, which then undergoes a conformational change and opens up at one side. Based on studies with different antibodies, it is well accepted that for HIT antibodies at least two antigenic sites exist[39,40] on PF4, which are located in the polar regions (if one imagines the PF4 tetramer as a globe[41]).

2.3 | Biophysical tools to characterize anti-PF4 antibody binding to PF4 in HIT and autoimmune HIT

After clarifying how antigens on PF4 are formed in HIT, the next important problem to address was why only about 50% of sera containing anti-PF4/heparin antibodies (per enzyme-immunoassays [EIAs] using immobilized PF4/heparin complexes) activate platelets in functional (platelet activation) assays. Even more challenging was the question why anti-PF4/polyanion antibodies identified in sera from patients with certain severe, atypical presentations of HIT (called "autoimmune HIT"[42]) are capable of activating platelets also in the absence of heparin.

Several groups have shown distinct binding patterns among nonpathogenic and pathogenic (platelet-activating) anti-PF4/heparin antibodies, with the general observation that platelet-activating antibodies bind stronger than nonpathogenic antibodies.[43,44] Using a combination of single molecule force spectroscopy and ITC studies, it became obvious that the biological effects of anti-PF4 antibodies are a composite of qualitative and quantitative characteristics[45] of the antibodies. These antibodies must have a minimal binding strength to the complexes to be able to activate platelets and they also need to be present at a minimal concentration. The higher their concentration and the stronger their binding forces, the more likely these antibodies activate platelets. However, in vitro data may not totally reflect the in vivo situation, as the binding strength of anti-PF4 antibodies also depends on the surface upon which PF4 is immobilized.[46] For example, the same monoclonal HIT-like antibody (KKO) binds much stronger to PF4 immobilized on platelets compared to PF4 immobilized on an artificial surface.[47]

In autoimmune HIT, the binding forces of the anti-PF4/polyanion autoantibodies exceed even the binding force of long heparin molecules to fuse together two PF4 tetramers. This raised the intriguing hypothesis that these anti-PF4 (/polyanion) autoantibodies may cluster two PF4 tetramers in the same way long heparin molecules do, hereby inducing similar conformational changes as polyanions (in essence, the highly pathogenic antibodies help to create their own target antigens).

By a combination of classic EIAs, electron microscopy and single molecule force spectroscopy, we showed[45] that antibodies with binding forces of approximately 60–100 pN activate platelets in the presence of polyanions, while a subset of antibodies from autoimmune HIT patients with binding forces ≥100 pN binds to PF4 alone in the absence of polyanions. These antibodies with high binding forces cluster PF4-molecules forming antigenic complexes, which allow additional binding of polyanion-dependent anti-PF4/heparin antibodies. The resulting immune complexes induce massive platelet activation in the absence of heparin. Figure 3 summarizes the different platelet activation patterns and binding forces of anti-PF4 antibodies which do not activate platelets, anti-PF4 antibodies which activate platelets only in the presence of heparin (typical HIT antibodies), and anti-PF4 antibodies (as found in autoimmune HIT) that activate platelets without addition of heparin (or any other cofactor).

3 | TRANSLATING THE FINDINGS IN HIT TO VITT

We strongly assume that a similar phenomenon as described in detail for autoimmune HIT is also relevant for VITT. To recapitulate, in many cases of autoimmune HIT, formation of anti-PF4 antibodies is triggered by heparin, but then the antibodies recognize PF4 alone in the absence of any polyanion.

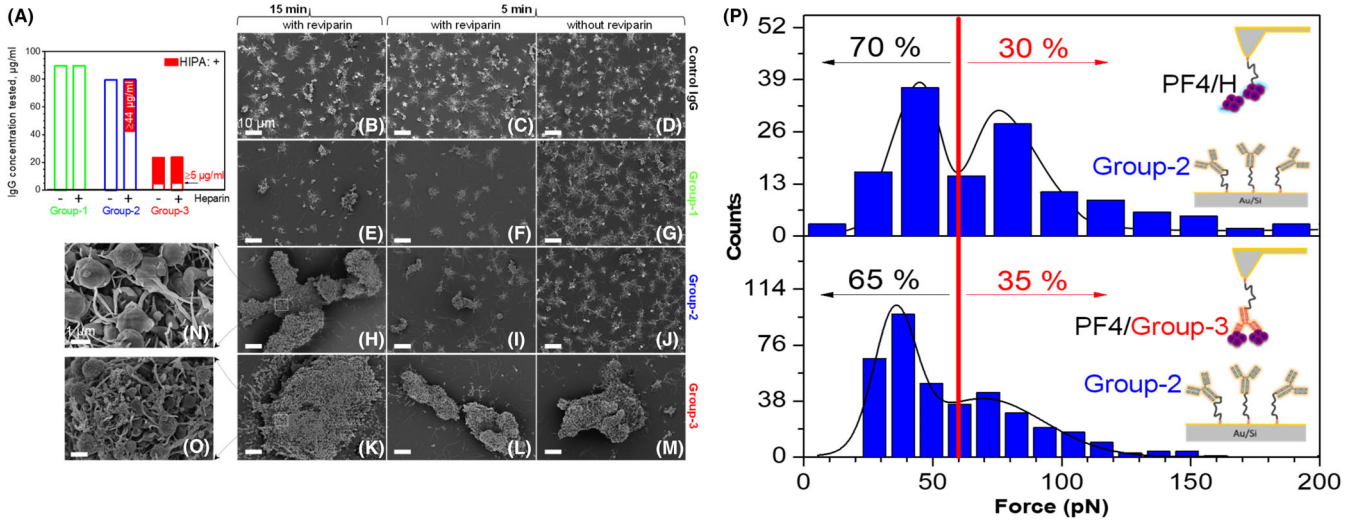

FIGURE 3 Interaction of different anti-PF4 antibodies with platelets and characterization of the binding forces by single molecule force spectroscopy. (Obtained from reference Reference 45). (A) Dependence of platelet aggregation on antibody (ABS) concentration: group-1 ABS ($n = 5$) did not activate platelets, either in the absence (−) or in the presence (+) of low molecular weight heparin (reviparin), up to a concentration of 89.7 µg/mL (green); group-2 ABS (blue) ($n = 5$) induced platelet activation (red part) at concentrations >44 µg/mL but only in the presence of reviparin. Group-3 ABS ($n = 5$) (red) activated platelets at much lower concentrations (≥5 µg/mL) either in the presence or absence of reviparin. (B–O) Scanning electron microscopic images show detailed platelet aggregates in the presence of different ABS. Only small aggregates occurred in the presence of control IgG (B–D) or group-1 ABS (E–G), whether or not reviparin was added. Group-2 ABS induced large platelet aggregates in the presence of reviparin after a lag-time of 15 min (H), but not earlier (I, J); while group-3 ABS activated platelets within 5 min independently of reviparin (K–M). Enlargements show looser platelet aggregates in the presence of group-2 ABS (N), but tighter and denser aggregates for group-3 ABS (O). Scale bar is 10 µm for images (B–M) and 1 µm for (N, O). (P) Two schematic inserts of either PF4/heparin complexes (upper part) or PF4/group-3 ABS complexes (lower part) bound to the tips interacting with group-2 ABS immobilized on the substrates. The columns show the binding forces when either PF4/heparin complexes bound to group-2 ABS (upper columns), or PF4/group-3 ABS bound to group-2 ABS (lower columns). Both presentations of PF4 allow the same binding forces between PF4 and group-2 ABS, confirming that PF4 bound to group-3 ABS expose the same epitope as PF4 within PF4/heparin complexes

3.1 | The binding site of VITT antibodies on PF4

Huynh et al[48] used alanine scanning mutagenesis to systematically modify single amino acids in the PF4 molecule. The resulting constructs allowed them to identify amino acids critical for anti-PF4 antibody binding. They showed that anti-PF4 antibodies identified in VITT patients bind to a different epitope than anti-PF4 antibodies found in HIT patients. The anti-PF4 antibodies in VITT recognize amino acids that are also relevant for heparin binding to PF4, which are located in the equatorial plane of the PF4 molecule (while classic heparin-dependent HIT antibodies bind to the poles). This has major implications for the treatment of VITT. As heparin often inhibits the binding of VITT antibodies, it could act as an antidote by blocking antibody/antigen binding.

An additional tool to characterize binding sites of patient antibodies are monoclonal antibodies, which compete with patient-derived anti-PF4 antibodies for the same binding site. Vayne et al[49] characterized the antibody 1E12, the first VITT-like monoclonal antibody. 1E12 is a chimeric anti-PF4 antibody with a human Fc-fragment that fully mimics the effects of human VITT antibodies. In summary, the binding epitopes of VITT antibodies largely overlap with the binding site of heparin on PF4 and the binding site of the monoclonal antibody, 1E12.

3.2 | The search for binding partners of PF4 in VITT

PF4 binds to adenoviruses,[50,51] including the vaccine vectors Ad26, Ad5 and ChAdOx1. The major adenovirus hexon proteins provide most of the negative charge, and interestingly, the hexon protein of ChAdOx1 is more negatively-charged than the hexon proteins of the other adenoviruses tested. However, the binding force of PF4 to adenovirus seems to be low affinity (Kd 300 nmol), which is much lower than the interaction forces of PF4 with polyanions exposing HIT epitopes. Besides hexon proteins on viral surfaces, Michalik et al identified free hexon proteins in both vaccines,[52] and super-resolution microscopy and electron microscopy of the PF4 complexes with the ChAdOx1 vaccine supports the notion that complex formation between PF4 and vaccine constituents requires more than virus/PF4 interaction (Figure 4). It seems that rather amorphous constituents of the vaccine than the complete virus are included into the complexes. However, the definite binding partner of PF4 within vaccine constituents has not yet been identified.[53] A major challenge is created by the many proteins of the cell lines in which the virus has been propagated during vaccine preparation. In the ChAdOx1 vaccine preparation more than 2000 proteins of the

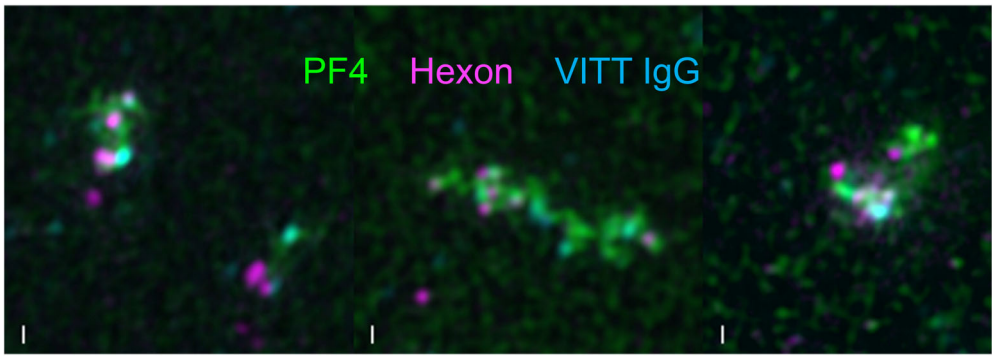

FIGURE 4 3D-super-resolution microscopy of complexes following incubation of PF4 with AstraZeneca Covid-19 vaccine and anti-PF4 VITT antibodies. 3D-super-resolution microscopy of PF4, adenovirus hexon proteins and affinity purified anti-PF4 antibodies (blue, obtained from VITT patients) reveals complex formation. PF4 (green) and adenoviral hexon protein (purple). PF4 forms multimolecular complexes, which stain for PF4, unassembled adenovirus hexon protein and to which IgG binds. However, beside the hexon proteins, the complexes seem to contain further material, which remains unidentified

HEK cell line, in which the adenovirus has been propagated, have been identified.[52]

3.3 | The dilemma of epitope spreading in strong (auto-)antibody responses

The usual straightforward approach to identify the binding partner of PF4 that causes the conformational changes inducing the immune reaction is to incubate PF4 in the presence and absence of different potential binding partners and measure whether anti-PF4 antibodies bind to putative complexes. This approach, however, cannot be used in VITT because the antibodies became autoantibodies that bind to and cluster PF4 by themselves with very high affinity. Broadened reactivity of antibodies in a boosted immune response is a hallmark of certain disorders. Above we have described as one example, autoimmune HIT, which features heparin-dependent reactivity that extends to include heparin-independent reactivity.[54]

However, there are other examples. Post-transfusion purpura (PTP) reflects a strong alloimmune response that progresses to include autoreactive properties.[55,56] PTP is an autoimmune disease induced by boosting of an alloimmune response to a platelet antigen (blood group). Typically, PTP occurs in women who do not express the common human platelet antigen (HPA) 1a. They usually became immunized during pregnancy. When these women later in life receive a blood transfusion, the HPA 1a positive platelets in the blood bag boost the immune response toward this platelet antigen. After approximately 7–10 days after transfusion, patients present with severe thrombocytopenia caused by high-titer anti-HPA 1a antibodies which also bind to the patient's own HPA-1a negative platelets, causing platelet destruction. The theory is that the boosted immune response breaks tolerance and allows—by epitope spreading—binding of these antibodies to HPA-1a negative platelets. Although life-threatening in the acute phase, the autoreactive antibodies wane within a few weeks.

Another example is thrombocytopenia caused by autoreactive antibodies induced by small molecule inhibitors of the platelet fibrinogen receptor GPIIbIIIa used during complicated coronary artery interventions. The antibodies recognize a neoepitope on the platelet fibrinogen receptor, which is exposed after binding[57] of the drug to the receptor. In most cases, the patient has preformed circulating natural antibodies and thrombocytopenia occurs soon (hours) after drug administration. However, in a small subset of patients, thrombocytopenia occurs about 7–10 days after treatment, when the small molecule drug (which is usually given for maximal 72 h) is definitely no longer present in the circulation. In vitro these antibodies show specificity for the complex of GPIIbIIIa and the small molecule inhibitor (eptifibatide or tirofiban), but in vivo the antibodies also bind to the receptor in the absence of the drug. Thus, high-titer drug-induced antibodies become autoantibodies again by epitope spreading, which bind to platelets in the absence of the reaction-initiating drug.[58]

In summary, the region on PF4 to which antibodies in VITT bind is well characterized but why tolerance is broken after vaccination, and why antibodies are formed against this epitope region remains unclear. The lessons learned from HIT strongly indicate that the crucial preceding step must include conformational changes in PF4, presumably caused by direct or indirect effects of vaccination and most likely a constituent present in adenoviral vector-based vaccines. However, given that highly reactive VITT antibodies exhibit features of autoantibodies created by epitope spreading, it is challenging to identify what vaccine constituents might induce conformational change of PF4. By analogy, if only autoimmune HIT sera were available to HIT researchers, it would not have been possible to identify heparin as the causative agent.

4 | TRANSLATING UNDERSTANDING OF THE MOLECULAR MECHANISMS OF HIT AND VITT INTO LABORATORY ASSAYS

Here, we briefly discuss diagnostic assays for HIT and VITT, emphasizing key points from the perspective of the various pathophysiological aspects of HIT and VITT summarized in the first part of this review. We will focus on the following concepts:

1. High sensitivity of certain diagnostic assays for HIT and VITT (emphasizes role of plasma IgG antibodies in forming in situ platelet-activating immune complexes on platelet surfaces);
2. Addition of PF4 and/or heparin, as well as serum/plasma dilution, in optimizing platelet activation assay performance (emphasizes role of stoichiometric concentrations of antigen components and immune complex formation); indeed, the key requirement for stoichiometric concentrations of PF4 and polyanion helps to explain the invariable inhibition of antibody reactivity in the presence of very high (~10–100 U/mL) heparin.
3. Low to moderate specificity of PF4-dependent immunoassays versus platelet activation assays (emphasizes relatively high frequency of forming nonpathogenic anti-PF4 antibodies, particularly in HIT, with reference to "iceberg model" of HIT);
4. Differences in specific antigen targets on PF4 between HIT and VITT (emphasizes differences in test sensitivity between PF4-dependent solid-phase enzyme-immunoassays and certain "rapid immunoassays").

Our comments will also be organized with respect to the historical order of assay development for HIT, that is, platelet activation assays, PF4-dependent enzyme-immunoassays, and PF4-dependent rapid immunoassays.

5 | HIGH SENSITIVITY OF DIAGNOSTIC ASSAYS FOR HIT AND VITT

A striking feature of diagnostic testing for HIT and VITT is the high sensitivity of certain assays, namely platelet activation assays (using washed platelets) and PF4-dependent solid-phase enzyme-immunoassays. This differs from many other platelet immunology disorders, such as drug-induced immune thrombocytopenia (D-ITP) and autoimmune thrombocytopenia (AITP), in which it can be difficult to demonstrate platelet-reactive antibodies within serum or plasma of many patient samples.

There are two major reasons why tests have high sensitivity. First, HIT and VITT antigens reside on nondegraded complexes of PF4 (in contrast, D-ITP antigens sometimes reflect drug metabolites that are not present in diagnostic test systems). Thus, provided that stoichiometrically appropriate PF4/polyanion complexes are present (discussed in more detail subsequently), anti-PF4 antibodies are readily detectable. Second, free, non-antigen bound anti-PF4 antibodies of IgG class must be present in serum/plasma in order to be able to assemble multimolecular PF4/polyanion/IgG complexes on platelet surfaces (by way of contrast, high-affinity autoantibodies in AITP might result in minimal residual "free" IgG in patient serum/plasma).

6 | PLATELET ACTIVATION ASSAYS FOR HIT AND VITT

6.1 | Washed platelet assays: SRA, HIPA

Two groups of investigators[59,60] showed that "washing" platelets in the presence of apyrase and resuspending the platelets in divalent cation-containing buffer, yields platelets highly sensitive to activation by HIT antibodies. Whereas the McMaster group[59] quantitated release of radiolabeled serotonin as the platelet activation endpoint marker (serotonin-release assay [SRA]), the German group employed platelet aggregation as activation endpoint (heparin-induced platelet activation [HIPA] test).[61] In both assays, a narrow heparin concentration range (peak reactivity, ~0.2 U/mL) optimizes platelet reactivity. The narrow range of heparin is explained by the necessity to create optimal stoichiometric ratios between PF4 and polyanion. Both assays have high diagnostic sensitivity (~95%) and specificity (~95%) for HIT,[62] although much lower sensitivity for VITT (~50%).

6.2 | PF4-enhanced washed platelet activation assays: PF4-SRA, PF4/H-SRA, PEA, PIPA

Despite the high (~95%) sensitivity of washed platelet activation assays for HIT, it became apparent that occasional HIT samples tested SRA-negative, that is, so-called "SRA-negative HIT."[63–65] In 2015, two groups reported that addition of PF4 enhances detectability of HIT antibodies in washed platelet activation assays. Two resulting PF4-enhanced assays include the "PF4-SRA" (McMaster)[66] and the PF4-dependent P-selectin expression assay (PEA) (Versiti).[67] A further modification of the SRA, reported by French investigators (Tours), adds fixed amounts of heparin (0.5 IU/mL) and PF4 (10 μg/mL), which we refer to here as the PF4/H-SRA.[68] This observation already indicated that there is a subgroup of anti-PF4 antibodies, which show enhanced reactivity if the assay is supplemented with PF4. Preliminary data indicate that these antibodies do not only recognize PF4/polyanion complexes but also PF4 alone.[69]

In sharp contrast to HIT testing, one of the initial reports[13] describing VITT found that most sera tested negative in the standard HIT functional assay (HIPA). This prompted development of the HIPA-modified assay, the "PIPA."[13] Here, 10 μg/mL PF4 is added in vitro to optimize antibody detectability. Most VITT sera exhibit greater platelet activation (i.e., shorter lag time to platelet aggregation) with added PF4 versus buffer control; in contrast to

HIT, addition of low-dose heparin or LMWH usually *inhibited* antibody-induced platelet activation, with heparin-induced enhancement of platelet activation seen with relatively few (~5%) VITT sera.[70] Finally, inhibition of platelet activation by high concentrations of heparin (100 U/mL), characteristic of HIT serum reactivity, is also observed with VITT sera. These high heparin concentrations detach PF4 from the platelet surface.

In summary, three PF4-enhanced washed platelet assays—PF4-SRA, PIPA, PEA—appear to have high sensitivity for detecting VITT antibodies (in contrast, the PF4/H-SRA is preferred for detecting HIT rather than VITT antibodies[62]). An important caveat is that a false-negative platelet activation assay can result if the patient was treated with high-dose IVIG,[16] through inhibition of VITT antibody-induced platelet activation.[71,72]

6.3 | Serum dilution to optimize detection of VITT antibodies

An interesting finding reported by Schönborn and colleagues was that false-negative PIPA reactivity could be avoided by diluting patient serum by 1/4 to 1/10.[73] The counterintuitive explanation is the very high levels of VITT antibodies found in some sera. In VITT, the PF4 complexes are not generated by a PF4-reactive polyanion (like in HIT), but by the anti-PF4 antibodies themselves. In essence, by diluting patient serum, the stoichiometrically "correct" quantities of VITT antibodies and PF4 molecules are attained, thus enabling formation of large, platelet-activating PF4-IgG complexes.

7 | PF4-DEPENDENT ENZYME-IMMUNOASSAYS

The major discovery that PF4 bound to heparin represents the target of HIT antibodies led in the early 1990s to the development of solid-phase EIAs, with PF4 coated with stoichiometric concentrations of heparin or another suitable polyanion (e.g., polyvinylsulfonate), onto microtiter plates.[74] Both IgG-specific and IgG/IgA/IgM (polyspecific) assays were developed. Certain inherent characteristics of EIAs explain their two fundamental properties, namely, their high sensitivity but also their relatively low (vis-à-vis platelet activation assays) diagnostic specificity. For production of solid-phase EIAs it is critical how PF4 is purified. The production process must maintain the tetrameric integrity of PF4 and has to avoid spontaneous complex formation of PF4 molecules. Finally, the optimal ratio between PF4 and polyanions must be determined for each PF4 lot to obtain maximal sensitivity.

The high sensitivity of PF4/polyanion EIAs for both HIT and VITT antibodies can be explained by the diverse expression of PF4 antigens on the solid-phase. Besides the heparin-dependent PF4 antigens expressed by the complexes of PF4 and polyanion (to which classic HIT antibodies bind), also so-called "uncomplexed" PF4 is coated on the solid-phase (i.e., recognized by VITT antibodies). Thus, sensitivity of the microtiter plate-based EIAs for both HIT and VITT is so high because PF4 molecules in many different conformations are coated. The diagnostic sensitivity of PF4-dependent EIAs appears to be at least 99% for detecting both HIT and VITT antibodies (this high value assumes that an occasional negative assay in one type of EIA will yield a positive result with another EIA, presumably because coating of PF4 differs somewhat among different assays[75,76]). An advantage of EIAs (vs. platelet activation assay) is that testing a suspected VITT serum/plasma after the patient has received high-dose IVIG will not compromise test sensitivity.[71]

The relatively low diagnostic specificity of EIAs for platelet-activating anti-PF4 disorders is explained by the inability of EIAs to discriminate between pathogenic (platelet-activating) and nonpathogenic (non-platelet-activating) anti-PF4 antibodies. This is conceptualized by the "iceberg model" of HIT which posits that only a subset of anti-PF4 antibodies are platelet-activating and hence capable of causing HIT (analogous to the "tip of the iceberg" that extends above the waterline). As discussed above, PF4 is coated in different conformations on the microtiter plate. In case of detection of VITT antibodies this is an advantage, but not in respect to specificity for platelet-activating antibodies. Despite more than three decades of research, it remains unresolved which presentations of PF4 and PF4/polyanion complexes preferentially are recognized by platelet-activating antibodies.

There are several ways that EIA diagnostic specificity can be enhanced. First, IgG-specific assays (vs. polyspecific) have higher diagnostic specificity.[77–79] Similarly, the clinician can take note of the *magnitude* of the positive EIA test result: for both HIT and VITT,[73] the greater the strength of the positive EIA test result (expressed in optical density units), the greater the probability that the serum/plasma contains platelet-activating antibodies, and hence a higher probability of HIT.

8 | RAPID IMMUNOASSAYS

Rapid immunoassays (RIAs) for HIT aim to provide a result within an hour after preparation of plasma (or serum), in an "on-demand" basis. Unlike standard EIAs, where multiple conformational presentations of PF4 are formed on the solid-phase, in RIAs, the repertoire of PF4 antigen targets is more constrained (discussed below). The practical consequence is that there are major differences in operating characteristics of the RIAs for detection of HIT versus VITT antibodies. These differences reflect the distinct antigen sites of heparin-dependent HIT antigens (located on the "poles" of PF4, when heparin binds to the "equator") versus VITT antigens (located at the "equator" heparin-binding site). Since the RIAs were designed for detecting HIT antibodies, the unexpected observation that VITT antibodies are usually negative in RIAs is a striking finding that underscores differences in distinct PF4 locations of HIT versus VITT antigen sites, as first shown by Huynh et al.[48]

Four RIAs are marketed for HIT, with all showing sensitivities that exceed 95% (estimated sensitivity shown in parentheses): (a) particle

gel immunoassay (PaGIA; ~98%)[80]; (b) lateral-flow assay (LFA; ~97%)[81]; latex-enhanced immunoturbidimetric assay (LIA; ~97%)[82]; and chemiluminescence immunoassay (CLIA; ~96%–98%).[83,84] In contrast, none of these 4 RIAs exhibits a sensitivity for VITT antibodies >50%, with the CLIA notably yielding positive results against <10% of VITT sera tested.[75,76] Clearly, the method by which the manufacturer has bound PF4/heparin complexes to the beads does not permit expression of the VITT epitopes. Preliminary studies using the platform of a PF4-based CLIA indicate that all VITT samples yielded positive results in a new prototype VITT assay, with only few reacting weakly positive in the conventional (HIT) CLIA. On the other hand, most of the HIT samples showed positive results in the conventional assay, but some of them also tested positive in the prototype VITT CLIA. Negative control samples where all nonreactive in the both CLIAs. These findings open the perspective for an automatable rapid immunoassay that differentiates between HIT-like and VITT-like anti-PF4 antibodies.[69] Further, having two complementary anti-PF4 immunoassays might be useful for investigating patients with atypical anti-PF4 disorders such as spontaneous HIT (i.e., anti-PF4 disorders associated with non-heparin triggers or in the presence of certain monoclonal antibodies).[83]

For one assay, the LIA, the biological basis for discrepant HIT/VITT antibody reactivity is clear, as a positive test result requires that the patient's anti-PF4 antibodies inhibit binding of a HIT-like monoclonal antibody (KKO) to PF4/PVS complexes,[82] a target site distinct from the epitopes recognized by VITT; potentially, substitution of KKO by the monoclonal antibody, 1E12, which recognizes the binding site of VITT antibodies on PF4, might make this assay suitable for recognizing VITT antibodies.[84]

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DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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