Chapter

Update on Mechanisms, Pathogenicity, Heterogeneity of Presentation, and Laboratory Diagnosis of Heparin-Induced Thrombocytopenia

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Abstract

Heparin-induced thrombocytopenia (HIT) is the most life-threatening adverse effect of heparin therapy and is provoked by the development of drug-dependent antibodies. It occurs more frequently in patients with cardiac or orthopedic surgery or severe circulatory diseases, and the risk depends on the patient pathological status. As heparin is an anticoagulant used for treating thrombotic events or their risk, this iatrogenic complication has a paradoxal effect as it can induce thromboembolic diseases, frequently associated to severe morbidity or fatal outcomes. Diagnosis involves clinical evaluation of disease probability and laboratory tools for testing the presence of heparin-dependent antibodies with immunoassays or their capability to activate platelets with functional assays. Antibodies developed when stoichiometric complexes of platelet factor 4 (PF4) with heparin are formed during therapy. In few cases non-platelet factor 4 antigens can be involved. Antibodies can remain asymptomatic, but pathogenicity occurs in the presence of high concentrations of IgG isotype antibodies, with high avidity: they target and activate platelets or endothelial cells exposing heparin-PF4 (HPF4) complexes and produce thrombocytopenia and sometimes thrombosis. Risk factors which favor the development of antibodies and their pathological effect are discussed. The present understanding of mechanisms underlying disease development and diagnostic strategies of this heparin adverse effect is presented.

Keywords: platelet factor 4, heparin, antibodies, pathogenicity, thrombosis, thrombocytopenia, diagnosis

1. Introduction

The major adverse effect of heparin therapy concerns probably the development of thrombocytopenia and thromboembolic complications, which are directly caused by the drug itself [1–6]. This heparin paradox is associated with a characteristic platelet fall and thrombosis in some heparin-treated patients, especially when unfractionated heparin (UFH) is used, but it can also occur with low molecular weight heparin (LMWH) therapy. The patient's clinical context can favor the

development of this iatrogenic complication, called heparin-induced thrombocytopenia (HIT) without or with associated thrombosis (HITT). When this complication occurs, it requires an immediate management with the withdrawal of heparin and use of an alternative anticoagulant [7–11]. If incorrectly managed, it can rapidly cause severe burden and become life-threatening. This complication is reported to occur in about 1–5% of patients treated with UFH and 0.2–0.5% of those treated with LMWH, but the incidence highly depends on the clinical context [1, 3, 5, 6]. Cardiology or orthopedic surgery, trauma, circulatory diseases, and the presence of tumors are increased risk factors for that disease. A recent meta-analysis in the USA reported a different HIT/HITT incidence and clinical association than that usually accepted but shows that it remains a critical clinical issue in hospitals [5]. The first alert signal for HIT is a platelet count drop by more than 30–50% between two successive measures, occurring between 5 and 15 days following the onset of heparin therapy, in the absence of any other thrombocytopenia cause (**Table 1**). However, platelet fall can develop earlier if patients have been exposed to heparin during the 3 months preceding the treatment. The mechanisms producing HIT involve the generation of a heparin-dependent antibody, usually of the IgG isotype (but IgA and IgM isotypes can also be present). This antibody has been demonstrated to be targeted to complexes of heparin and platelet factor 4 (PF4) in most of the cases [12, 13], but non-PF4 antigens can be present in some atypical patients [14–17]. Frequently, heparin-dependent antibodies, including IgG isotypes, are asymptomatic [18, 19]. They are symptomatic and harmful only in a few subsets of affected patients. What renders the antibodies pathogenic in those patients is not totally understood, but some evidence becomes available. IgG isotypes present at high concentration, and with high avidity, provoke frequently the development of disease [19, 20]. Clinical diagnosis and laboratory diagnosis are of high importance to rapidly identify the patients with an active disease and treat them [6, 8, 21–24]. It includes a multiple strategy approach:

1. early detection of patients with thrombocytopenia

2. evaluation of their HIT clinical score

3. testing for the presence of heparin-dependent antibodies with immunoassays

4. checking the capability of these antibodies to activate platelets in a heparin-dependent mode [25].

It is of essence to duly characterize patients with HIT: if this complication is excluded, it is not necessary to deprive them from heparin, as it is the most effective anticoagulant in many acute conditions. Conversely, if HIT/HITT is confirmed, it is

- Clinical context with blood activation
- Cardiac/orthopedic surgery
- Malignancy
- Autoimmune disorders
- UFH > LMWH
- Heparin therapy duration (≥5 days)
- Re-exposure to heparin <100 days

Table 1.
Risk factors for HIT development during heparin therapy.

mandatory to not reintroduce any heparin treatment and to switch to an alternative anticoagulation. In this book chapter, we will present and discuss the following: (a) the present understandings of conditions which can favor development of heparin-dependent antibodies in heparin-treated patients; (b) why antibodies generate HIT or HITT only in a few subset of patients; (c) the mechanisms of action of these antibodies, as they are presently understood; (d) the available laboratory tools and their indications; (e) the diagnostic strategy for rapidly characterizing patients at risk; (f) the occurrence of atypical presentations of HIT in patients with pre-existing antibodies to PF4 or to interleukin 8 (IL8) or with antibodies to protamine sulfate (PrS); and (g) cross-reactivity of the various polysaccharide anticoagulants in immunoassays and functional methods. This chapter mainly focuses on antibodies generated to heparin-PF4 (HPF4) complexes, which concern most of the patients with HIT/HITT, but other non-PF4 antigens can be involved in few cases and will be rapidly discussed.

2. Development of heparin-induced thrombocytopenia

Heparin-dependent antibodies develop in many patients treated with UFH or LMWH. Their incidence is higher in patients undergoing extracorporeal circulation (ECC) for cardiopulmonary bypass (CPB) or extracorporeal membrane oxygenation (ECMO) [5, 26, 27]. Antibodies' development is not rare during heparin therapy, but they are often of the IgM isotype with a rapid reversal without any clinical incidence [18, 19]. They can also be of the IgA or IgG isotypes, and the three isotypes are associated in many patients and are frequently asymptomatic. IgGs have been demonstrated to be those which can become pathogenic, especially when present at high concentration, with high affinity for their target heparin-dependent antigen [19, 25]. A subset of the IgG isotype heparin-dependent antibodies can then activate platelets [20], which induce thrombocytopenia, platelet aggregation, and sometimes thrombosis. HIT was first characterized for the white thrombus formed in arteries (platelets and white blood cells), when patients with this complication were first identified, but there is evidence now that arterial (about 30% of cases) or venous (about 70% of cases) thrombosis can occur [28]. Skin necrosis at the injection site or elsewhere, or thrombosis, frequently at limb extremities, is often observed, but thrombosis can occur at many different sites.

In addition to platelet activation, heparin-dependent antibodies can induce activation of endothelial cells (ECs) and of monocytes, and they can release tissue factor (TF) from these cells, which contribute to thrombosis [29–31]. Platelet-leukocyte aggregates are also formed and contribute to thrombogenicity. When this multiple blood activation process is initiated, it is enhanced at pathological sites where platelet and white blood cells can be chemo-attracted and accumulate with a high density. If blood activation and prothrombotic process are strong enough to overwhelm the antithrombotic body's defenses, thrombus formation occurs. The first clinical warning for HIT is the occurrence of thrombocytopenia, with a characteristic time kinetics from the onset of therapy, when other causes of decreased platelet counts are excluded [6, 25, 28]. Platelet fall typically occurs between 5 and 15 days following the initiation of treatment, as shown in **Figure 1**, except if patients already received heparin within the 100 preceding days or in the rare cases with pre-existing anti-PF4 antibodies. Thrombocytopenia can then develop earlier and possibly just at the onset of heparin therapy. In HIT/HITT thrombocytopenia is usually moderate, between 20 and 100 giga platelets per liter (G/L), and it is rarely very severe (<10 G/L). When it develops, the clinical probability for HIT/HITT

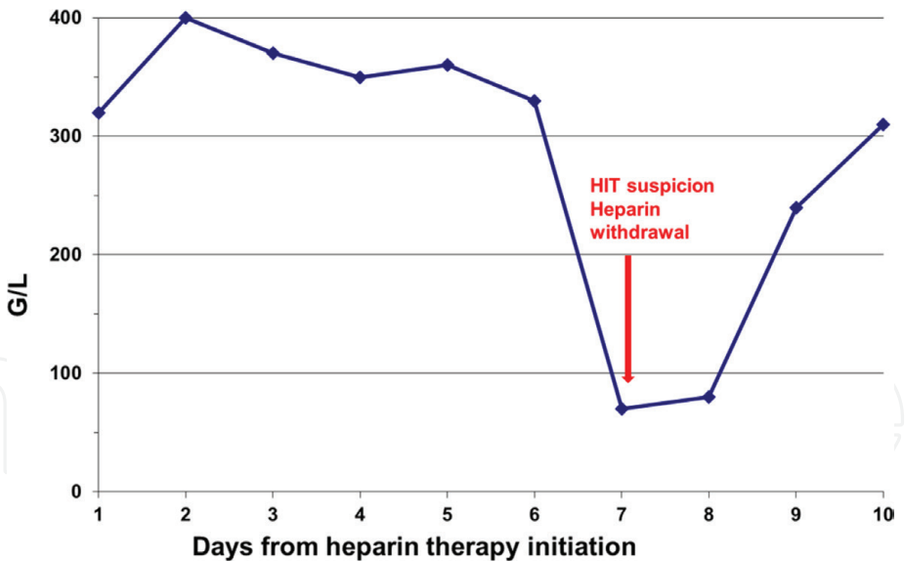

Figure 1.
Typical platelet count kinetics in heparin-treated patients who develop heparin-dependent antibodies responsible for heparin-induced thrombocytopenia.

Quote	Points (0, 1, or 2 for each of 4 categories: maximum possible score = 8)		
Score	2	1	0
Thrombocytopenia	>50% fall from baseline or platelet nadir 20 to 100 x 10^9/L	30 to 50% fall from baseline or platelet nadir 10 to 19 x 10^9/L	Fall <30% from baseline or platelet nadir <10x10^9/L
Timing of platelet count fall or other sequelae	Clear onset between days 5 and 10, or less than 1 day (if heparin exposure within past 100 days).	Consistent with immunization but not clear (e.g., missing platelet counts) or onset of thrombocytopenia after day 10	Platelet count falls too early (without recent heparin exposure)
Thrombosis or other sequelae (e.g., skin lesions)	New thrombosis; skin necrosis; post heparin bolus acute systemic reaction	Progressive or recurrent thrombosis; erythematous skin lesions; suspected thrombosis not yet proven	None
oTher cause for thrombocytopenia	No other cause for platelet count fall is evident	Possible other cause is evident	Definite other cause is evident
Pretest probability score: 6 to 8 = High; 4 to 5 = Intermediate; 0-3 = Low			
Adapted from Warkentin TE. Br J Haematol. 2003;121-535-55.			

Table 2.
The pretest probability for HIT based on the 4Ts score.

must be evaluated. It is an important criterion for estimating the risk to develop this complication in heparin-treated patients, and various pretest methods for estimating disease risk are available. The most frequently used is the 4Ts score [28], which considers four major criteria: the presence of thrombocytopenia, the timing of platelet count fall, the occurrence of new thrombosis or sequelae, and the investigation of other causes of thrombocytopenia. For each criterion, a score from 0 to 2 is given, as shown in **Table 2**. It allows to classify patients from 0 to 8 (risk is low for 0–3, intermediate for 4–5, and high for ≥6, indicating an elevated disease probability). In cardiology patients with ECC, thrombocytopenia is frequently observed, and HIT can be identified when a biphasic platelet count kinetics is present: in the absence of HIT, thrombocytopenia is progressively corrected, but, if present, platelet count starts to increase and falls again when symptomatic antibodies develop [27].

Other HIT clinical evaluation approaches have been proposed (such as the expert score), but the 4Ts score remains the most widely used. When HIT is suspected, heparin treatment must be stopped and replaced by another anticoagulant. The possible drugs which can be used include argatroban, direct oral anticoagulants (DOACs), danaparoid sodium, fondaparinux, and bivalirudin [7, 9–11, 32, 33]. Nevertheless, if HIT is excluded, heparin can be reintroduced, as it can be of full benefit for the patient, especially in cardiac surgery and circulatory diseases. Establishing rapidly a safe and reliable diagnosis of HIT is then of essence for the right management of patients [6, 25, 28].

3. Heparin-dependent antibodies in clinical settings

HIT/HITT occurs in some of the patients who develop heparin-dependent antibodies, a major risk factor for the disease occurrence. In most of the cases, they are targeted to stoichiometric complexes of heparin and PF4 (HPF4) and are of the IgG isotype but are the only ones present in few patients with atypical HIT/HITT antibodies to IL8 or to PrS [12, 14, 15, 17]. In rare cases, the antibody specificity remains non-identified, although patients present the suggestive clinical complication of heparin therapy. What causes the heparin-dependent antibodies' generation is not yet fully understood, but drug immunogenicity tends to develop when heparin forms complexes with its high-affinity binding blood protein, PF4, a chemokine from the CXC family [34–36], and eventually IL8 [37, 38]. In healthy individuals, PF4 is normally present at very low concentrations in blood circulation (<10 ng/ml). It is released from platelets' α-granules upon activation or aggregation, as a complex of eight PF4 tetramers with a platelet proteoglycan dimer, with a molecular weight (MW) of about 350 kDa. This complex is rapidly cleared from circulation as PF4 is captured by endothelial cells' glycosaminoglycans (GAGs) and

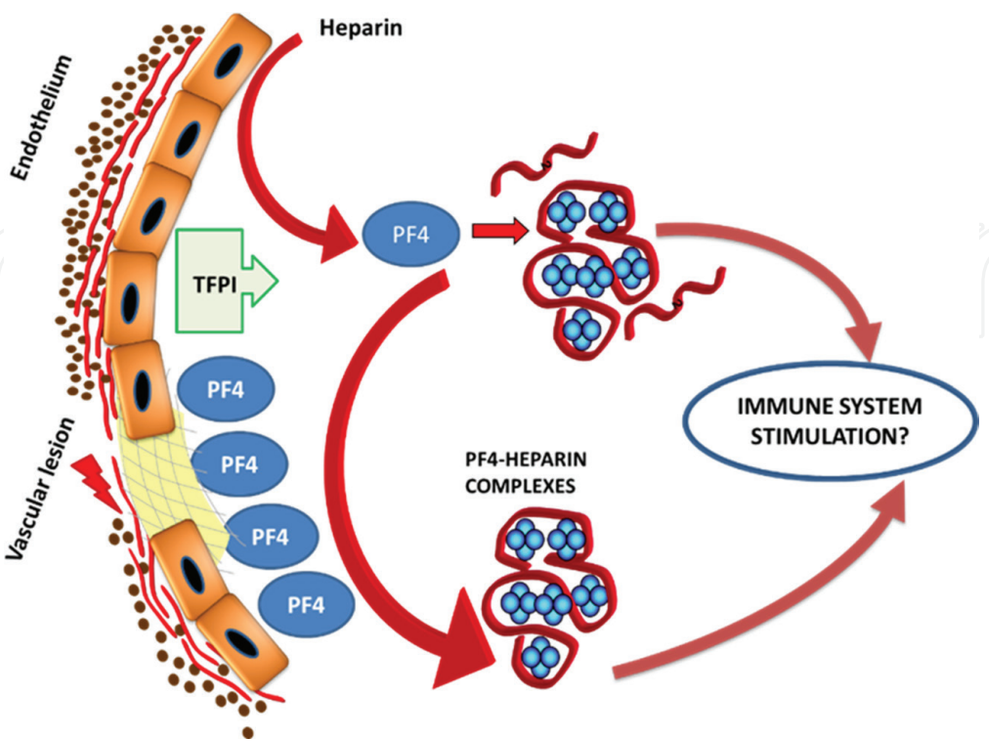

Figure 2.
At the onset of heparin therapy, TFPI and PF4 "storage pool" are displaced from endothelial cells and released into blood circulation. Heparin complexes with PF4, and this can stimulate the immune system (especially if heparin and PF4 stoichiometric concentrations are met), and antibodies to these complexes are generated.

remains in this endothelial storage pool. In patients with inflammation or blood activation, PF4 concentrations can be much higher, either in blood circulation or on the endothelial storage pool. In addition, at pathological sites, platelets and white blood cells can be chemo-attracted and stimulated. Much higher PF4 concentrations can be present at these sites. At the onset of heparin therapy, PF4, which has a higher affinity for this drug than for GAGs or physiological proteoglycans, forms complexes with it, as presented in **Figure 2**. In some circumstances these complexes can activate the immune system and induce the generation of antibodies. The immune response can be innate, mediated via the toll-like receptors, and adaptive with a T cell-mediated response, followed by the generation of antibodies. The three isotypes (IgG, IgA, or IgM) can be present [13, 19], but IgGs are formed very rapidly, which is unusual in the early stage of the immune response, and IgGs can become rapidly pathogenic [19]. In rare cases, only IgA (especially in patients with cancer) or IgM isotypes are identified [39]. Following heparin treatment cessation, antibodies disappear from blood circulation within about 3 months. The respective concentrations of PF4 and heparin in blood circulation or at pathological sites are key factors for inducing immunogenicity [40, 41]. The clinical context is then a risk factor for heparin-dependent antibodies' development. Another initial cause which can favor generation of antibodies has been described and concerns a previous exposure of patients to bacterial infections [42]. PF4 can complex with bacterial polysaccharides and then becomes immunogenic. The immune response induces generation of antibodies to this chemokine. When patients with this former stimulation receive heparin, PF4 released from endothelium forms HPF4 complexes which reactivate the immune system (**Figure 1**), and IgG isotypes are rapidly generated. In addition to PF4, heparin treatment (and more especially LMWH) can also release tissue factor pathway inhibitor (TFPI) bound to ECs into blood circulation. No immune reaction to TFPI has been observed until now, but its increased concentration contributes to elevate the anticoagulant activity of heparin at the beginning of treatment.

4. Heparin-dependent antigens in HIT

The major heparin-dependent antigen involved in HIT/HITT is PF4, a CXC chemokine present in platelet α-granules and released upon platelet activation and aggregation. PF4 is a 70 amino acid (AA) protein with a MW of 7800 kDa, released in blood circulation as a tetramer with a MW of about 30 kDa [34–36, 43]. This chemokine has a structure involving one α-helix and three β-sheets organized in an antiparallel manner; it is highly electropositive, with many lysine and arginine residues, and has two disulfide bridges per monomer. The tetramer is organized in such a way that it exposes an external ring of positive charges, as shown in **Figure 3**. The formation of HPF4 complexes depends on the respective concentrations of heparin and PF4 [13, 24, 44]. Stoichiometric complexes are formed at a concentration of about 150 μg of heparin (i.e., about 27 IU UFH) per mg of PF4 (**Figure 4**). High- and low-affinity heparin molecules have the same reactivity with PF4, as well as LMWH, and the sulfation grade is of essence for these interactions. Patients who develop antibodies are those with the highest extracellular concentrations of PF4 in blood circulation, or at pathological sites, and with heparin concentrations permitting the formation of stoichiometric HPF4 complexes.

If heparin treatment is given through continuous infusion, heparin concentration remains constant in blood circulation, and the risk to form stoichiometric HPF4 reactive complexes is reduced. When heparin is given through the subcutaneous route, blood concentrations present high variations, from <0.1 IU/ml at trough to >0.7 IU/ml at peak. For current curative UFH treatments (2–3 injections/day),

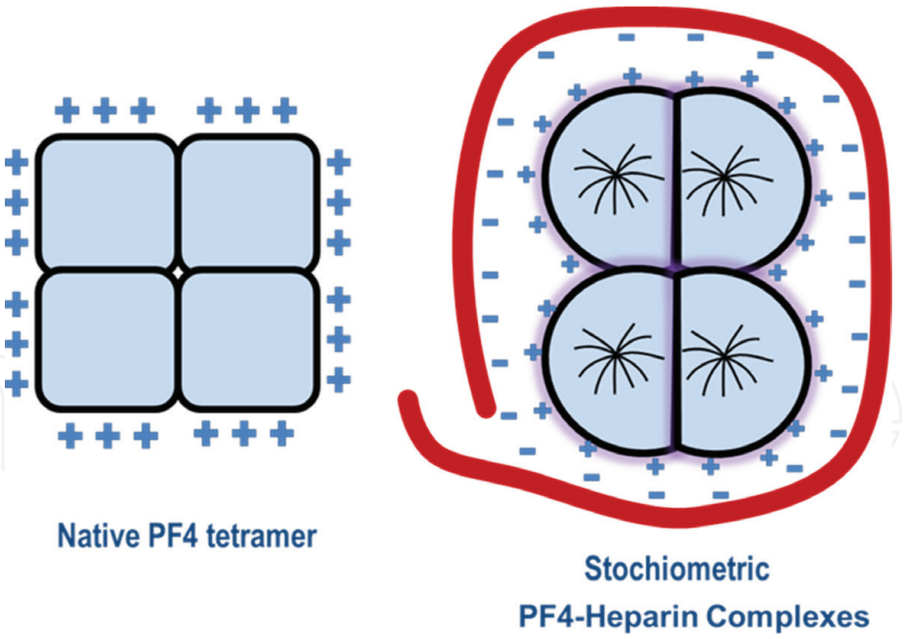

Figure 3.
Reaction of heparin with PF4 tetramers at stoichiometric concentrations. There is an intimate interaction between the ring of positive charges on the PF4 tetramer and the negative charges of the sulfated polysaccharide, heparin. This strong interaction induces an alteration of PF4 structure, rendering it immunogenic. Heparin (UFH or LMWH) molecules with at least 12 monosaccharides are required for this interaction.

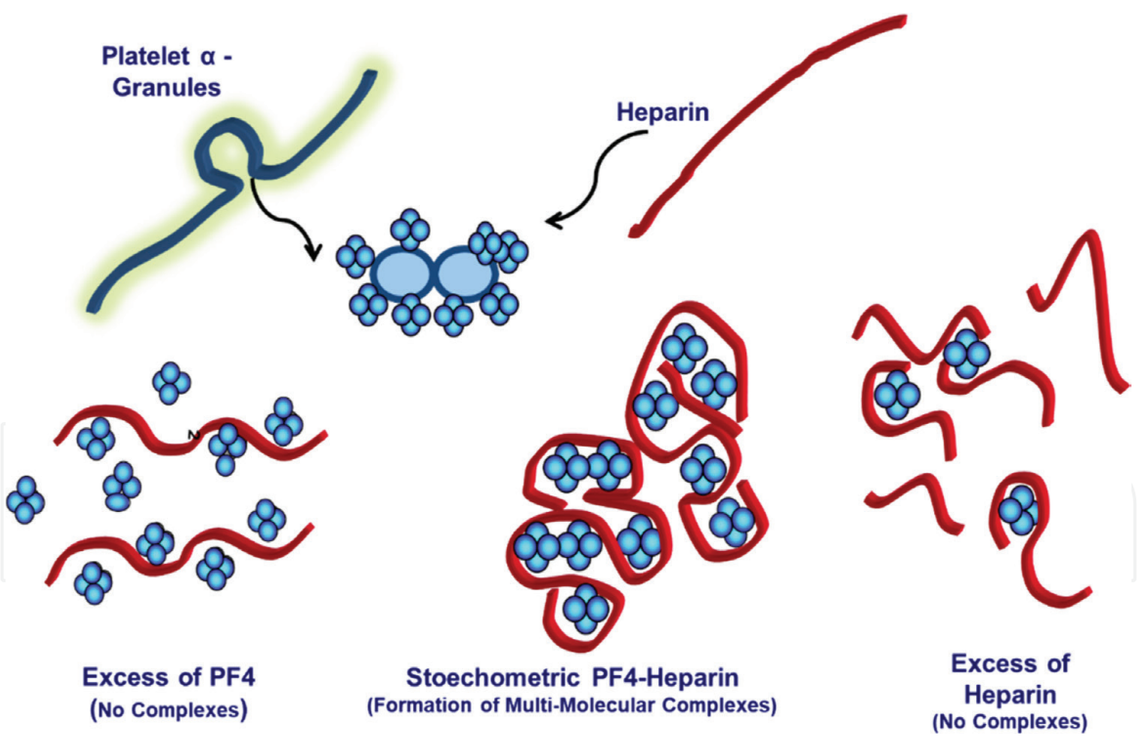

Figure 4.
PF4 is released from platelets as a complex with a proteoglycan dimer and is displaced by heparin for which it has a higher affinity. Complexes of heparin and PF4 depend on their respective ratios. When heparin and PF4 are at a stoichiometric concentration, large multimolecular complexes are formed and can be exposed on platelets or other blood cells. They can bind heparin-dependent antibodies and focus the deleterious immune reaction onto these cells. This can induce HIT or HITT in some patients.

the PF4 concentrations needed for forming stoichiometric complexes must be of about 4 μg/ml for heparin concentrations ≤0.1 IU/ml (trough) or of ≥28 μg/ml for heparin concentrations ≥0.7 IU/ml (peak). Required PF4 concentrations are high comparatively to expected heparin concentrations in blood circulation, even in

disease states, but these high concentrations could be present at pathological sites. In ECC, blood heparin concentrations are high (about 4–5 IU/ml) and constant: formation of stoichiometric complexes can only occur with 25–30 μg/ml of PF4, which is unlikely. For information, the total amount of PF4 releasable from platelets, when they are totally activated and aggregated, is of about 5 μg per ml of blood (depending on platelet count and PF4 content; it is of ±12.5 ng/10^6 platelets).

But PF4 can accumulate and be at higher concentrations at pathological sites. Immunogenic stimulation occurs when body detects a non-self-component, which can be heparin used as anticoagulant. When bound to PF4, it forms large complexes, which can activate the immune response, which is targeted to these complexes and possibly extended to PF4 itself, through epitope spreading. Generated antibodies can be considered as alloantibodies. In few cases, PF4 antibodies can be pre-existing chronically or generated transitory as a side response to an infectious disease [14, 42, 45].
Anti-PF4 autoantibodies can bind to HPF4 complexes formed during heparin therapy and are then targeted to platelets or other blood cells which expose HPF4 complexes, focusing the deleterious immune response [22, 29, 30, 46]. In few cases, non-PF4 antigens can be involved [14, 15, 17, 46, 47]. HIT/HITT presentation and disease kinetics are then frequently atypical, although a moderate or characteristic thrombocytopenia develops during heparin therapy. IL8 has been reported in some patients as another heparin-dependent antigen in HIT/HITT. Anti-IL8 antibodies are pre-existing in many patients with chronic inflammation and are generated as a regulatory response to control this pathological context. Pathogenicity can occur because IL8 can bind heparin, and these complexes are fixed onto platelets and other blood cells through IL8 receptors (IL8-RA and IL8-RB) or through direct heparin binding [37, 38].
Interestingly, heparin binding to platelets increases with their activation grade. Anti-IL8 antibodies then focus the immune response deleterious effects to blood cells exposing heparin IL8 complexes which are then activated or destroyed. Neutrophil-activating peptide 2 (NAP-2), the β-thromboglobulin precursor, is another platelet CXC chemokine reported as a possible heparin-dependent antigen in rare HIT cases [14]. Lastly, in patients undergoing ECC [16, 26, 27], heparin is used as anticoagulant and is neutralized with a defined concentration of PrS at the end of the process. Anti-PrS or anti-heparin-PrS antibodies have been the only ones identified in few patients treated with heparin and presenting with a HIT-/HITT-like syndrome [17], with a possible fatal outcome. These antibodies can activate platelets in the presence of heparin [15, 46, 47]. Recent investigations have shown that anti-PrS antibodies are rather frequent in patients receiving this drug for heparin neutralization, but only very few of them develop severe clinical complications. Recurrent ECC in the same patient, with various exposures to heparin and PrS over time, can be an increased risk for development of antibodies and associated pathogenicity, with a HIT-/HITT-like syndrome.

5. Pathogenicity and mode of action of heparin-dependent antibodies

Heparin-dependent antibodies, and especially those to HPF4 complexes, induce thrombocytopenia and thrombosis in some clinical circumstances [46]. Particularly IgG isotypes can activate platelets, ECs, or other white blood cells such as monocytes, when they bind to their target antigenic structure, present at the surface of these cells [28, 29, 44, 46, 47]. There is now evidence that heparin and HPF4 complexes bind to platelets' surface, and this binding increases with their activation grade. HPF4 complexes fix antibodies and target the immune response, provoking platelet activation, aggregation, and interaction with other blood cells. During the process, IgGs react with platelet CD32, which is the FcγRIIa receptor [44, 46]. This contributes to amplify platelet activation and aggregation. The CD32 surface density is an

important factor for the amplitude of platelet activation induced by antibodies. In patients with platelets presenting a CD32 polymorphism (131 Arg-His), activation is enhanced: the 131-Arg-His heterozygous or 131-His-His homozygous CD32 phenotypes are more reactive than the 131-Arg-Arg one. The patient propensity to develop HIT or HITT can depend on platelet activation grade and density or polymorphism of CD32. Antibodies to HPF4 can activate ECs and monocytes, favoring the release of TF, a potent procoagulant starter [29, 30]. In patients with HIT/HITT, neutrophils are activated and form aggregates with platelets, which can be detected in blood circulation. Therefore, the presence of anti-HPF4 IgG antibodies initiates multiple abnormal activities in blood circulation, which induce platelet activation and destruction and a concomitant prothrombotic risk (**Figure 5**). Blood activation can be out of control from body's antithrombotic defenses, which are overwhelmed, and thrombosis occurs. Interestingly, thrombosis tends to occur at pre-existing pathological sites, where blood activation and inflammation are already activated, and the risk is greatly amplified by anti-HPF4 antibodies, as summarized in **Figure 4**. We have the experience that an additional factor is very important for the initiation and amplification of the pathological process. This concerns the antibody avidity for HPF4 complexes [20]. In three patients with HIT or HITT, we succeeded to separate anti-HPF4 IgGs into two groups: the most important (>90%) one had a low affinity for HPF4 and no or only a weak platelet activation capacity, while the minor one (≤10%) activated highly platelets, as evidenced with the C_{14}-serotonin release assay. In few cases, only IgA isotypes specific for HPF4 complexes were identified in patients with HITT and malignant diseases. Although rare, IgAs can be pathogenic in some autoimmune disorders [49, 50], and this is not unexpected to note their effect in HIT. More rarely, IgM can be present at high concentration in patients with HIT, without IgGs. The mechanisms involved are not totally understood, but recently it was demonstrated that anti-HPF4 IgM antibodies can activate complement and induce platelet destruction [39]. Altogether, the different activities described here above help to understand why HPF4 antibodies, including IgG isotypes, can remain

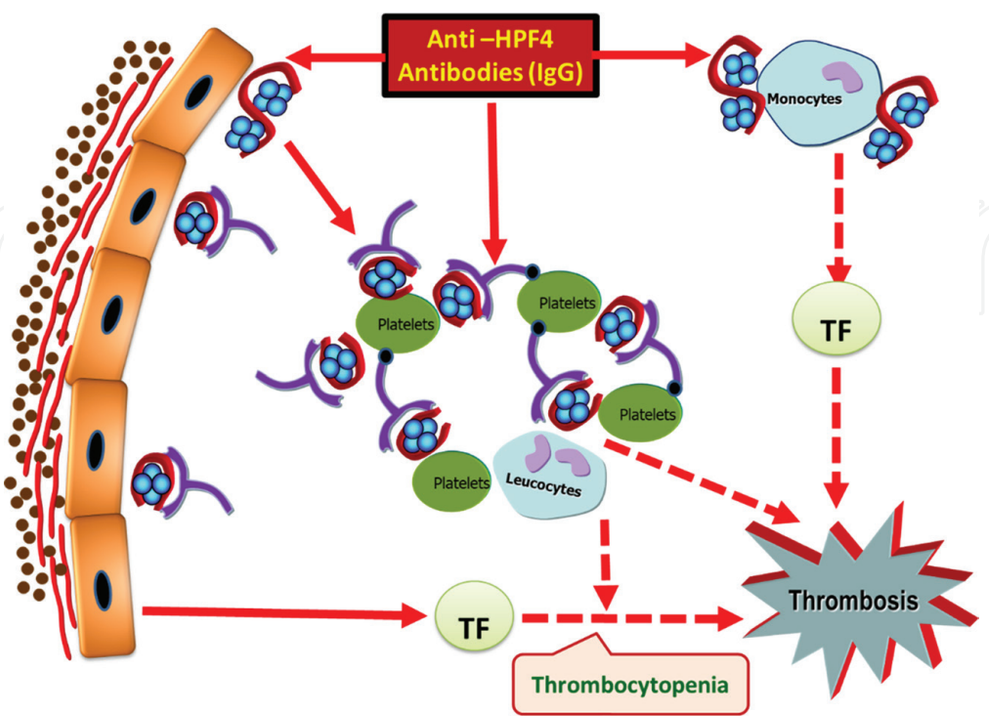

Figure 5.
Scheme showing how heparin-dependent antibodies, targeted to HPF4 complexes, bind to platelets and endothelial cells but also to monocytes and induce platelet and EC activation, monocyte stimulation, release of TF, and formation of aggregates, all contributing to thrombocytopenia and thrombosis.

asymptomatic in many patients and produce (especially IgG isotypes with high HPF4 affinity) HIT or HITT only in a few of them. The pathogenic process is multifactorial and involves activation and interaction of various blood cells, with the prothrombotic activity of TF. Patients' pathophysiological history and clinical status provide additional risk factors for the occurrence of disease [5, 6].

Nevertheless, there is still a fortuity context for the occurrence of the HIT/HITT complication, which relies on the formation of the immunoreactive HPF4 complexes, requiring defined concentrations of PF4 and heparin, exposed on blood cells [48]. This is a pre-requisite condition for permitting the binding of antibodies and starting the pathogenic process. This explains why this disease develops so rapidly when the critical conditions are met.

6. Diagnosis of heparin-induced thrombocytopenia

Many different assays are available for the diagnosis of heparin-dependent antibodies and for testing their capability to activate platelets. They are classified into two groups: immunoassays [23, 25, 51, 52], developed following the discovery of PF4 as the major target heparin-dependent antigen, and functional assays, performed with a low and a high heparin concentration, which were already used before [53]. A murine monoclonal antibody (KKO) has been developed and mimics HIT-associated antibodies, with platelet activation capability [54]. Here below we discuss the laboratory methods, and their combination, for the diagnosis of HIT/HITT. Diagnosis combines the clinical probability pretest with laboratory investigations [25]. For laboratory testing, the specimen used is plasma or serum for immunoassays and citrated plasma or heat-inactivated serum for functional assays. These techniques provide a laboratory support to establish, confirm, or exclude the diagnosis of HIT/HITT and must always be used in association with the pretest clinical probability. When HIT is suspected with a characteristic thrombocytopenia, heparin must be discontinued and replaced with another anticoagulant.

6.1 Immunoassays for heparin-dependent antibodies

With the discovery of the major target antigen for heparin-dependent antibodies, i.e., HPF4 complexes, immunoassays were developed, optimized, and standardized [23, 24, 52]. The first immunoassay introduced was a two-site enzyme-linked immunosorbent assay (ELISA), for measuring antibodies to HPF4 [12]. The antigen, HPF4, is coated on the plate, which is then saturated and stabilized. A well-defined stoichiometric concentration of PF4 tetramer and heparin (about 150 μg heparin per mg PF4) must be used for presenting epitopes reactive with antibodies. Heparin-dependent antibodies can be caught from the diluted tested plasma or serum (usually a 1:100 dilution is used), during the first incubation step. Following a washing step, the immunoconjugate, specific for human immunoglobulins or their isotypes, is introduced, and a second incubation step is performed. The immunoconjugate is often a rabbit or goat antibody, specific for human whole immunoglobulins (IgGAM) or for only an isotype (IgG, IgA, or IgM), and labeled with peroxidase. In current practice, this tag reagent is an antihuman IgG-peroxidase conjugate. Following a new washing step, the substrate is introduced, and a color develops. Tetramethylbenzidine (TMB) with hydrogen peroxide (H_2O_2) is now the most often used substrate, producing a blue color, which turns yellow when the reaction is stopped with sulfuric acid. Absorbance is measured using a microplate reader at 450 nm. Different variant methods have been introduced. Heparin can be replaced with another sulfated polymer (electronegative) such as polyvinyl sulfonate. However, using heparin matches better

with the context of antibody generation and in vivo pathogenicity. Magnetic latex particles can be used in place of the solid phase capture micro-ELISA. Different tag antibody labels can be used instead of peroxidase, such as alkaline phosphatase (with its appropriate substrate). The "enzyme-substrate" detection system with chemiluminescence or fluorescence can also be used (direct measurement). Combining latex magnetic particles and chemiluminescence or fluorescence allows immunoassay automation. Lastly, performing immunoassays in the presence of an excess of heparin allows confirming antibody specificity [25, 55].

Figure 6 shows the general immunoassay principle for detecting heparin-dependent antibodies. For testing the non-PF4 antigen-dependent antibodies, similar immunoassays can be designed by replacing PF4 with the concerned protein (e.g., IL8 or PrS). We developed an original patented approach, where heparin in excess is coated in the presence of PrS and remains biologically available. The tested patient's sample is then incubated in the presence of a concentrated platelet lysate (containing all the platelet releasable proteins, but not plasma factors). If antibodies are present, a ternary complex is formed between tetrameric PF4 (or eventually another platelet protein), immobilized heparin, and antibodies. Caught antibodies are then detected as previously described [24]. This method offers a kinetic model for testing antibodies and mimics their binding to heparin-protein complexes bound onto platelet or blood cell surfaces. This assay reflects better the mechanisms occurring in pathology and offers improved and optimized sensitivity and specificity.

6.2 Platelet activation methods for disease confirmation

Functional assays rely on testing the capability of heparin-dependent antibodies to activate platelets at a low (0.1–1.0 IU/ml) and a high (10–100 IU/ml) heparin concentration. In HIT/HITT, platelets are only activated at the low heparin

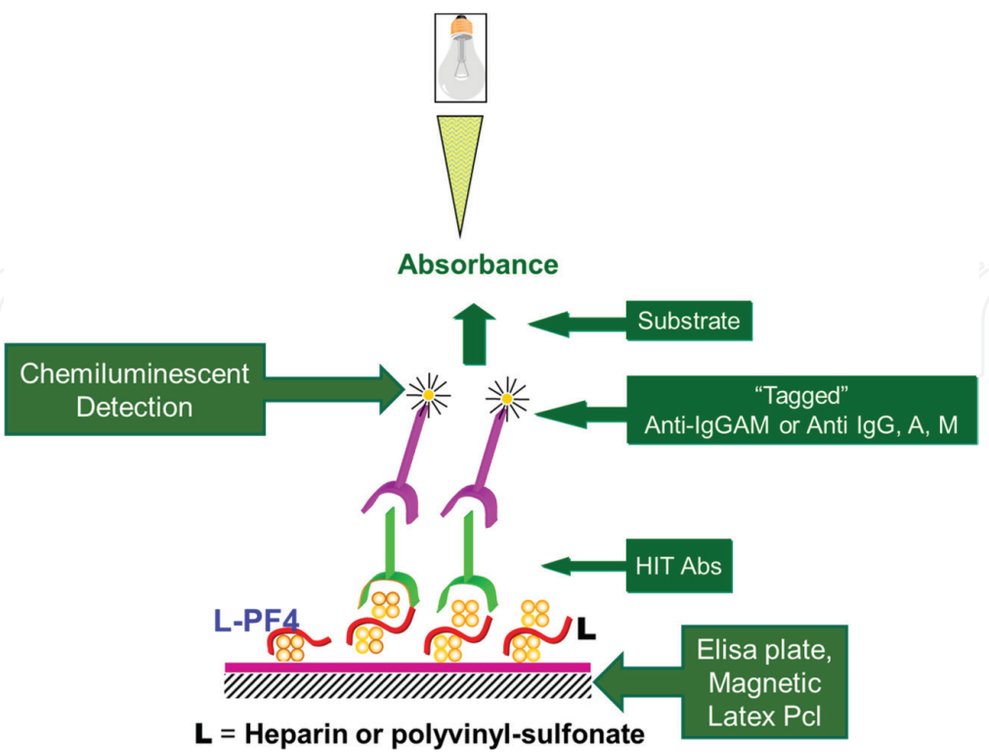

Figure 6.
General principle of immunoassays used for heparin-dependent antibodies, either globally or for specific isotypes. Enzyme tag with substrate is used in ELISA. Chemiluminescent immunoassays, using magnetic latex particles, can be automated on immunological analyzers. Using heparin in excess in sample diluent allows confirming antibody specificity.

concentration. Functional assays need to use normal donor platelets, freshly prepared. They must be duly selected for the right reactivity. This is the constraint which limits the use of this technique. Platelets are used as platelet-rich plasma (PRP) or as washed platelets. What induces donor to donor responsiveness in platelet activation assays used for HIT antibodies is not totally understood. The CD32 platelet density or His polymorphism could favor reactivity. In practice, platelets need to be qualified with a known positive sample for their appropriateness. Frequently, platelets from four normal donors are used, and the assay is positive if at least two out of the four donors give a positive platelet activation test. Other factors can regulate platelet activity, and interestingly washed platelets are usually more reactive than PRP. This can be explained by some platelet activation induced by the washing process, and a higher amount of PF4 is present on platelet surface. Functional assays concern PAT, SRA, heparin-induced platelet activation (HIPA), and flow cytometric assays (FCA); but other assays have been reported and elegantly reviewed in 2017 [53]. PAT is a simple aggregation assay performed with PRP and the tested patient citrated plasma. SRA is performed with washed platelets labeled with C_{14}, incubated with tested patient's plasma, and released C_{14}-serotonin is measured. HIPA is also performed with washed platelets, incubated with the tested sample, and platelet activation/aggregation is visually evaluated. FCA is a technique that requires to mix PRP (washed platelets are possible) with patient's citrated plasma and to measure platelet activation through the expression

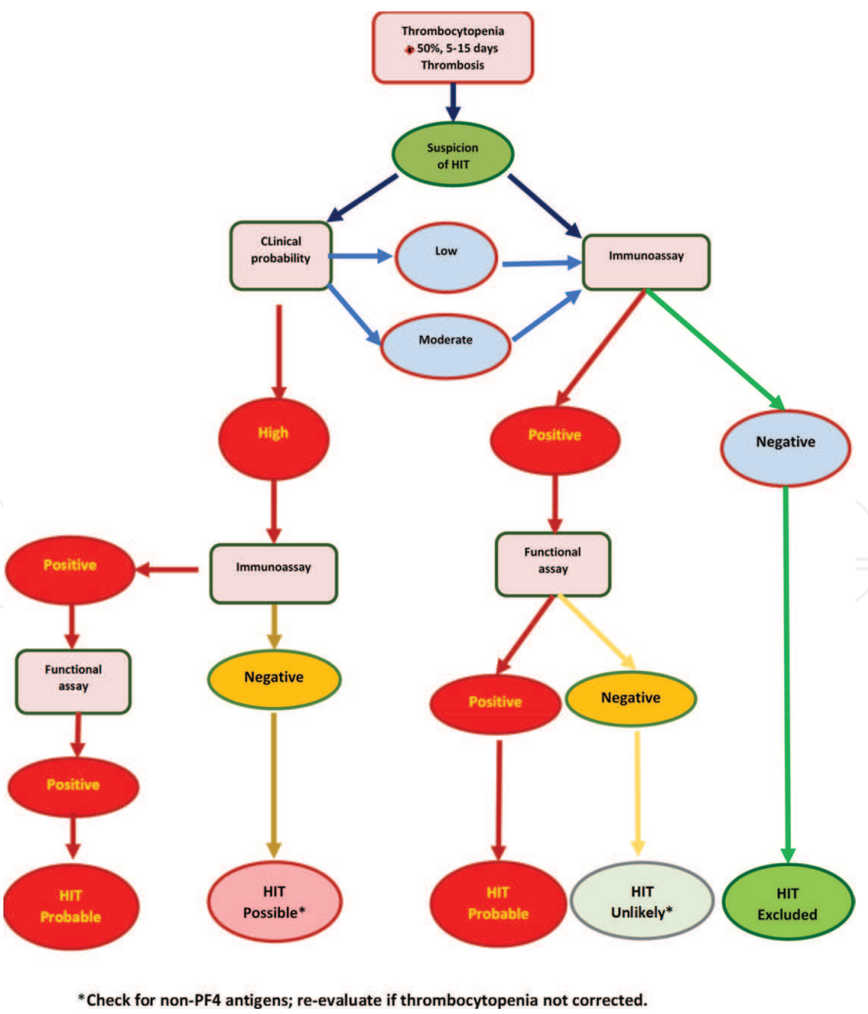

Figure 7.
Scheme showing the algorithm for the diagnosis of HIT/HITT: when suspected (thrombocytopenia and/or thrombosis), the disease diagnosis involves the clinical probability estimation and laboratory testing, first with immunoassay, which allows ruling out disease when not present, and then with a confirmatory functional assay.

of P-selectin [24]. FCA can also be used for the measurement of antibody-induced release of platelet microparticles. SRA is considered as the reference and most sensitive method. PAT has a poor sensitivity. HIPA needs trained laboratory operators and is mainly used in Germany and some neighboring countries. FCA is now a more standardized approach and looks promising but needs to be confirmed through practical experience in clinical laboratories. This method can be available in many centers for testing in emergency, provided a flow cytometer and fresh platelets are available.

6.3 Diagnostic approach for HIT/HITT

The diagnosis of HIT/HITT must be done accurately and reliably for a safe management of concerned patients [25]. The first alert signal is thrombocytopenia occurring 5–15 days following the onset of heparin therapy or earlier if the patient had a previous exposure to that drug within the 3 preceding months. HIT, or HITT if thrombosis is present, is then suspected and must rapidly be confirmed. If this complication is excluded, patients can continue to receive heparin, the most effective anticoagulant in many critical clinical situations. If the disease is confirmed or cannot be excluded, or if HIT is suspected but the diagnosis cannot be conducted, heparin must be replaced by another anticoagulant, according to the clinical context and practitioners' experience. **Figure 7** shows an algorithm for establishing or excluding the diagnosis of HIT. When HIT is suspected, the pretest clinical probability must be evaluated with the 4Ts method or another one in use in the clinical setting [28]. The 4Ts score is simple and relatively well-standardized. When HIT/HITT is suspected, heparin is immediately stopped, and another anticoagulant is used to avoid any risk of severe complication. Nevertheless, the diagnosis must be established and confirmed, as the patient can need heparin later. The first laboratory investigation involves immunological testing for antibodies. If the test is negative, and the clinical probability is low or moderate, HIT can be excluded. But if clinical probability is high, HIT cannot be excluded and remains possible with non-PF4 heparin-dependent antigens involved. If positive, antibodies are present. HIT develops mainly when IgGs are generated and present at high concentration. Many authors consider that HIT occurs when the optical density (OD) in ELISA is >1.00 (the cutoff value for the positive range being at ≥0.5). When the IgG immunoassay is positive, a functional assay must be performed for confirming the diagnosis, as many heparin-dependent antibodies are asymptomatic. This functional assay must be as sensitive and specific as possible. In any case if clinical probability is high, the possibility of HIT complication remains present, whether the laboratory testing is. Testing must be repeated [56], and other antigens than HPF4 can be investigated.

7. Cross-reactivity of the various heparins, heparin-like compounds, or danaparoid sodium

HIT/HITT is associated with UFH or LMWH therapy, both drugs being sulfated polysaccharides. Other heparin-like anti-FXa anticoagulants, such as fondaparinux or danaparoid sodium, do not generate drug-specific antibodies [7, 10]. However, cross-reactivity of these drugs with antibodies present in patients with characterized HIT/HITT can be observed in laboratory assays [57]. This cross-reactivity has been reported for danaparoid sodium when it is tested in the immunoassay at a high concentration in the presence of PF4 (about 3.00 mg danaparoid sodium per mg of PF4). This can be due to the high-affinity heparan sulfate component present in this drug, which represents about 4% of the total. Cross-reactivity has also

been reported in functional assays. However, there is no evidence that danaparoid sodium can generate drug-induced antibodies, and cross-reactivity is opposed by the other non-affinity components (about 80% low-affinity heparan sulfate, 12% dermatan sulfate, and 4% chondroitin sulfate), present in large excess, which disrupt the possible complexes formed, as do other low-sulfated polysaccharides [9, 58]. Therefore, there is no evidence that danaparoid sodium can provoke HIT/HITT, and the reported results and long-term clinical experience in many countries suggest that cross-reactivity is totally inhibited by the major non-affinity fractions. Furthermore, danaparoid sodium at therapeutic concentrations can inhibit the heparin-induced platelet aggregation. Conversely, pentosan polysulfate was found to be as effective as heparin and to form complexes with PF4 at similar ratios than UFH, for binding all heparin-dependent antibodies [8, 14]. Lastly, fondaparinux is not expected to induce drug-dependent antibodies or to cross-react with existing antibodies [32].

8. Conclusions and perspectives

In this chapter we have reviewed the present understanding of the generation of heparin-dependent antibodies in UFH- or LMWH-treated patients, which are the primary cause for HIT/HITT and a major adverse effect of heparin therapy. This risk is much higher when UFH is used, and disease develops more frequently in some clinical situations including cardiac or orthopedic surgery, traumatology, or malignancy. The occurrence of HIT/HITT tends to decrease thanks to a better control of therapy with UFH, shorter treatment times, and the use of LMWH when possible. When heparin therapy needs to be stopped, a large panel of alternative anticoagulants is available, although in some applications heparin remains the most effective one.

The mechanisms, which can induce generation of heparin-dependent antibodies, and pathogenicity for some of them have been extensively described and discussed in literature [14, 19, 20, 22, 42, 46]. Immunization develops when defined concentrations of heparin and PF4, forming stoichiometric multimolecular complexes, are present. In vivo, immunogenic complexes with PF4 can also be formed with other polyanions such as polyphosphates [59]. Various isotypes can be generated, IgM, IgA, or IgG, but almost all clinical complications of this iatrogenic disease are reported with IgGs present at high concentration and with high affinity. For HIT/HITT pathological development, various patient-associated and fortuity factors are required. Stoichiometric HPF4 complexes must be present for stimulating the immune system and developing antibodies but also for expressing pathogenicity. Heparin-dependent antibodies are harmful only if they bind to target antigenic structures (mainly HPF4 stoichiometric complexes), present on platelets, ECs, or other blood cells, focusing the immunological response. The immune system is then deviated from its protective role and destroys the patient's own cells [60].

HIT/HITT diagnosis is of essence for confirming or excluding this disease, and heparin treatment can be continued if the risk is ruled out. The first step when thrombocytopenia and/or thrombosis occur is to suspect this heparin adverse effect and to evaluate the pretest clinical probability. The 4Ts score is frequently used and allows risk classification from 0 to 8, 6–8 being the highest risk. Concomitantly, performing an immunoassay allows to detect and to measure IgG heparin-dependent antibodies targeted to HPF4. If the assay is negative and if clinical probability is low or moderate (score ≤ 5), HIT can be excluded, but patients need to be monitored closely, especially if thrombocytopenia is not corrected. If positive, IgG heparin-dependent antibodies are present, and HIT probability is higher if ELISA OD ≥ 1.00. Finally, the use of functional assays allows differentiating asymptomatic antibodies from those

which can activate platelets and provoke disease. The presence of HIT or HITT is confirmed when the functional assay is positive [60]. However, even when negative, if clinical probability is high (6–8), HIT/HITT remains possible, and patients must be managed accordingly. Heparin cannot be continued in any patient with a possible or probable HIT diagnosis, and an alternative anticoagulant must be used.

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