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Heparin-induced anaphylactic and anaphylactoid reactions: two distinct but overlapping syndromes

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Background: Heparin-induced anaphylactic and anaphylactoid reactions are of increasing clinical and scientific interest, particularly given the recent identification of a syndrome of heparin-induced anaphylaxis due to oversulfated chondroitin sulfate (OSCS), a contaminant in certain heparin preparations. However, heparin-induced anaphylactoid reactions also have been reported to be a consequence of immune-mediated heparin-induced thrombocytopenia (HIT). *Objective*: To summarize the clinical features and pathophysiology of two distinct disorders, HIT-associated anaphylactoid reactions as well as anaphylaxis resulting from OSCS-contaminated heparin. *Methods*: We review literature describing these two types of heparin-induced anaphylactic and anaphylactoid reactions, and seek potential pathophysiologic links between them. *Results*: Intravenous bolus heparin administered to patients with circulating 'HIT antibodies', usually as a result of recent heparin therapy, can produce anaphylactoid reactions, probably as a consequence of *in vivo* activation of platelets and, possibly, leukocytes. Affected patients often evince fever/chills, hypertension and/or acute respiratory compromise ('pseudo-pulmonary embolism'). In contrast, heparin-induced anaphylaxis is caused by activation of the contact system, with formation of vasoactive kinins (bradykinin, des-arg^9-bradykinin). This latter syndrome has been linked in an epidemic form to administration of OSCS-contaminated heparin; these reactions feature prominent hypotension and laryngeal edema. Hemodialysis patients are at increased risk for both syndromes. There is evidence that OSCS-contaminated heparin itself increases the risk of HIT compared with non-contaminated heparin. *Conclusion*: Two distinct syndromes of heparin-induced anaphylaxis and anaphylactoid reactions exist. These seem to share certain epidemiologic features, given that OSCS-contaminated heparin can produce anaphylaxis through contact system activation but also could increase risk of HIT and HIT-associated anaphylactoid reactions.

Keywords: anaphylactic(oid) reactions, bradykinin, contact system, contaminant, hemodialysis, heparin-induced thrombocytopenia, oversulfated chondroitin sulfate

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1. Introduction

Heparin-induced anaphylactic and anaphylactoid reactions have been described for > 50 years [1-3]. During the 1970s and 1980s, the time that a new syndrome

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Table 1. HIT is a clinicopathologic syndrome.

Clinical features	Pathologic features
Thrombocytopenia (~ 85 – 95%)*	Anti-PF4/heparin IgG antibodies that activate platelets through the platelet Fcγ receptors, detectable either by:
Thrombosis (50 – 75%)	
Venous	Platelet activation assays or
Arterial	PF4/polyanion immunoassays
Microthrombosis/overt disseminated intravascular coagulation	
Temporal link to immunizing heparin exposure	
Miscellaneous features	
Heparin-induced skin lesions (5 – 10%)‡	
Anaphylactoid reactions (~ 5%)§	

*Thrombocytopenia defined as a 50% or greater decline in the platelet count, or as a decrease to < 100×10^9/l.

‡Estimated frequency among patients who develop HIT while receiving subcutaneous unfractionated heparin thromboprophylaxis post-orthopedic surgery; the frequency probably is considerably lower in other patient populations.

§Most often occur 5 – 30 min following unfractionated heparin bolus administration to a patient who has received heparin in the past 7 – 30 days (and occasionally up to 100 days).

HIT: Heparin-induced thrombocytopenia.

known as immune heparin-induced thrombocytopenia (HIT) began to be more widely recognized, some clinicians observed heparin-induced anaphylactoid reactions in the context of possible HIT [4,5]. These life-threatening reactions typically occurred in patients who had received heparin in the recent past, and who were then administered intravenous bolus unfractionated heparin (UFH). Affected patients presented with life-threatening anaphylactoid reactions – or even fatal cardiopulmonary arrest – occurring several minutes post-bolus. Over time, the link between HIT and such heparin-induced anaphylactoid reactions became established.

Beginning in 2007, a new epidemic of anaphylactic reactions associated with UFH occurred, featuring prominent hypotension, and resulting in some fatalities. These reactions were linked to contact system activation induced by certain batches of UFH manufactured in China. The explanation for these reactions was a contaminant – perhaps better classified as an adulterant – in UFH known as oversulfated chondroitin sulfate (OSCS). This surprising development recalled an intriguing finding in the early history of HIT in which a hypersulfated chondroitin sulfate (essentially identical to OSCS), formerly used to treat degenerative arthritis, was also identified to cause a disorder identical to HIT [6]. Thus, heparin can cause anaphylactic/toid reactions not only as a manifestation of the HIT syndrome, but also through the contact system-activating effects of contaminating OSCS.

The primary aim of our review is to summarize the clinical and laboratory features of these two syndromes of heparin-induced anaphylactic and anaphylactoid reactions. We will describe how these two syndromes – although pathophysiologically distinct – share certain overlapping clinical features. We will also argue that OSCS-contaminated heparin could increase the risk of HIT, compared with UFH products that do not contain this contaminant.

2. Heparin-induced anaphylactoid reactions associated with HIT

2.1 Overview of HIT

HIT is a humoral immune reaction triggered by heparin that results from the formation of platelet-activating IgG antibodies, which recognize multimolecular complexes comprised of the cationic chemokine, platelet factor 4 (PF4) and the anionic sulfated polysaccharide, heparin. HIT is classified as a 'clinicopathologic disorder' as the diagnosis rests on two pillars: one or more clinical features (e.g., thrombocytopenia, thrombosis) as well as detection of pathologic 'HIT antibodies' (**Table 1**) [7]. The most common clinical manifestation of HIT is thrombocytopenia, with at least 90% of patients evincing 50% or greater declines in the platelet count, usually to platelet count nadirs between 20 and 150×10^9/l [8].

The target antigens in HIT are formed when the positive charge of PF4 is neutralized by negatively-charged polyanions. Owing to its wide use, heparin is by far the most common agent that induces HIT antigens, but the resulting anti-PF4/polyanion immune response does not depend exclusively on the presence of pharmacologic heparin [9]. As discussed later, several other polyanions can substitute for heparin in triggering this immune response. Binding of heparin to PF4 results in the formation of 100 – 150 nm linear ridge-like structures with repetitive binding sites [10]. Binding of the antibodies to these multimolecular PF4/heparin complexes [11] results in immune complexes that form *in situ* on platelet surfaces [12]. When the antibodies are of IgG class, the Fc moieties bind to the platelet Fcγ receptors, leading to Fcγ receptor crosslinking, with resulting 'strong' platelet activation that includes the formation of procoagulant platelet-derived microparticles [13]. Concomitant activation of endothelium [14] and monocytes [15] creates a pancellular procoagulant response.

Table 2. Clinical features of anaphylactoid reactions following intravenous bolus heparin.

Timing: onset 5 – 30 min after intravenous heparin bolus (less commonly, following intravenous or subcutaneous low-molecular-weight heparin administration)
Clinical context: recent use of heparin (past 7 – 100 days)
Laboratory features: abrupt, potentially reversible fall in the platelet count
Signs and symptoms:
Inflammatory: chills, rigors, fever and flushing
Cardiorespiratory: tachycardia, hypertension, tachypnea, dyspnea, bronchospasm, chest pain or tightness, and cardiopulmonary arrest
Gastrointestinal: nausea, vomiting and large-volume diarrhea
Neurological: pounding headache, transient global amnesia, transient ischemic attack or stroke

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The consequences of HIT-induced activation of hemostasis are protean, and range from clinically-evident venous and/or arterial thrombosis to overt disseminated intravascular coagulation. At least 50% of all patients affected by HIT develop clinically-evident thrombosis [8,13,16]. Some patients develop necrotizing skin lesions at the sites of heparin injection [17,18]. Approximately 5% of patients identified with HIT develop abrupt onset of various symptoms and signs beginning a few minutes after heparin injection. These have been variably termed as 'anaphylactic', 'anaphylactoid', 'acute systemic reactions' and 'acute cardiovascular collapse' [18-22].

2.2 Clinical features of HIT-associated anaphylactoid reactions

Relatively old medical literature drew attention to occasional acute anaphylactoid reactions following intravenous bolus heparin therapy [1-3]. It is now apparent that some of these reactions were almost certainly due to HIT. For instance, Turcotte *et al.* [3] reported four patients who developed acute pulmonary reactions (with prominent 'bronchospasm') after intravenous bolus UFH. As three of the four patients required one or more above-the-knee limb amputations (a characteristic complication of HIT), it seems very likely that HIT was the explanation for these reactions.

More recent studies, utilizing laboratory tests to detect HIT antibodies, have established a clear link between HIT and heparin-induced anaphylactoid reactions [18-22]. **Table 2** summarizes the clinical features of these acute HIT-associated reactions [18]. Most often, HIT-associated anaphylactoid reactions begin 5 – 30 min following an intravenous bolus injection of UFH. However, in some patients, the reactions occur after intravenous bolus infusion [23] or subcutaneous [24,25] injection of low-molecular-weight heparin (LMWH). Large magnitude declines in the platelet count accompany these reactions, provided that the repeat platelet count is taken during or soon after occurrence. **Figure 1** summarizes the clinical course of a representative patient who developed an acute anaphylactoid reaction following intravenous heparin bolus administration in which HIT was implicated.

Patients can develop any of a large number of signs or symptoms, which can be classified as cardiopulmonary, inflammatory, neurological and gastrointestinal (**Table 2**) [18]. Patients can develop several symptoms and signs simultaneously [19]. Some authors have noted that the presence of skin lesions at heparin injection sites may represent a risk factor for intravenous bolus UFH-induced anaphylactoid reactions [17]. Typically, patients with heparin-induced necrotizing skin lesions have high titers of platelet-activating anti-PF4/heparin IgG antibodies.

Several interesting clinical features are worth highlighting. In contrast to classic anaphylaxis, hypotension is usually not a feature of HIT-associated anaphylactoid reactions, in our experience [18,20,26]. Indeed, affected patients typically exhibit *hyper*tension: 12 of 15 consecutive patients identified as having HIT-associated anaphylactoid reaction had an increase in blood pressure (by at least 30 and/or 15 mm Hg systolic or diastolic blood pressure, respectively (unpublished observations)). Thus, these reactions resemble those linked to transfusion of platelets, particularly in the pre-leukodepletion era, in which leukocyte-derived chemical mediators generated during platelet storage have been implicated [27]. The lack of hypotension is a key feature that distinguishes HIT-associated anaphylactoid reactions from those linked to OSCS-contaminated heparin.

The pulmonary reactions can be prominent, including severe dyspnea and even respiratory arrest [28], thereby mimicking severe pulmonary embolism ('pseudo-pulmonary embolism') [23,29]. Associated severe bradycardia or cardiac arrest can prove fatal or severely disabling (**Figure 1**).

An interesting manifestation is the neurologic syndrome of transient global amnesia (TGA), which has been observed in some patients with HIT-associated anaphylactoid reactions [30]. Affected patients remain alert and communicative with no loss of personal identity; however, they experience striking loss of memory for recent events (retrograde amnesia) and an impaired ability to retain new information (anterograde amnesia) [31]. One theory is that TGA is triggered by cerebral venous congestion that leads to impairment of the mesial temporal areas (amygdala and hippocampus). Episodes usually last from 2 to 12 h, with very low risk of future recurrence.

HIT-associated anaphylactoid reactions have been especially reported in the setting of hemodialysis [22,23,32,33]. We discuss this phenomenon later in section 4.4.

2.3 Pathogenesis of HIT-associated anaphylactoid reactions

The pathogenesis of HIT-associated anaphylactoid reactions is poorly understood. Given the accompanying acute platelet count fall, it seems likely that these reactions result at least in part from acute *in vivo* platelet activation. Concomitant

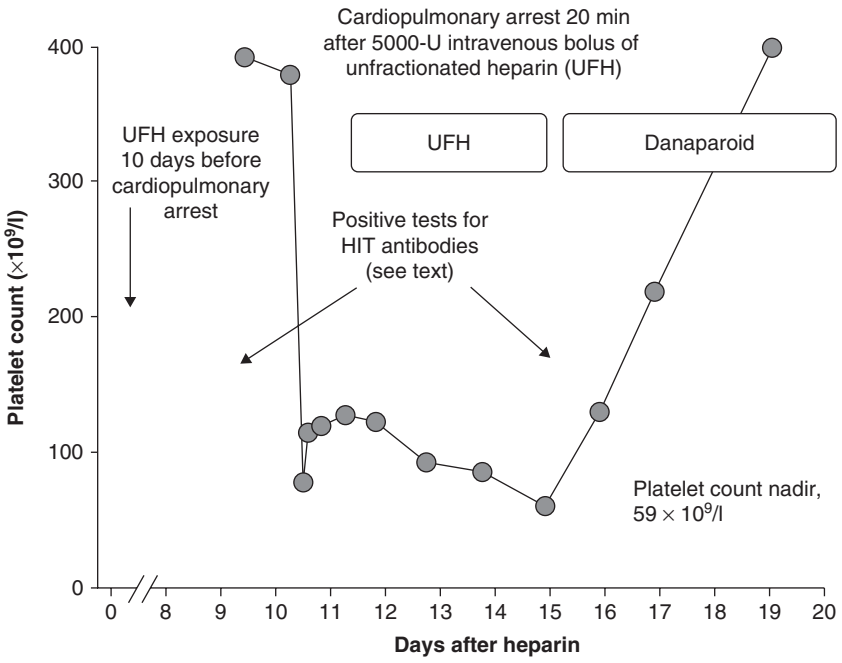

Figure 1. A 53-year-old woman with cardiopulmonary arrest post-intravenous bolus UFH. The patient had received UFH 10 days earlier at another hospital and was subsequently transferred for cardiac surgery. She had severe mitral stenosis and a large dense intra-atrial thrombus. While awaiting surgery, she, therefore, received a 5000-unit bolus of UFH; within 20 min post-bolus, the patient was found to have a 'pulseless/electrical activity arrest'. Following cardiopulmonary resuscitation and administration of epinephrine and atropine, the circulation was restored. CT brain examination showed several cerebral infarcts consistent with a combination of anoxic and ischemic injury. The surgery was cancelled and intravenous heparin by infusion was commenced. The platelet count fell from 127 to 59×10^9/l over the next 4 days and the diagnosis of HIT was suspected and platelet-activating anti-PF4/heparin antibodies were confirmed by laboratory tests. In retrospect, the cardiac arrest post-bolus UFH was suspected to represent an 'anaphylactoid' reaction secondary to acute HIT, based on the abrupt 78% platelet count fall (from 376 to 81×10^9/l). This diagnosis was further supported by positive tests for HIT antibodies in a blood sample obtained one day before the cardiopulmonary arrest. Thus, it seemed that the UFH administered 10 days earlier at the referring hospital triggered the presence of HIT antibodies that accounted for the acute anaphylactoid reaction triggered by bolus UFH administration.

Results of HIT antibody tests (tests performed using patient serum):

*Day 9 = 94% serotonin release at 0.3 IU/ml heparin (normal, < 20% release); < 10% release at 0 and 100 IU/ml heparin; commercial enzyme immunoassay from GTI, Inc. (Waukesha, WI, USA) = 0.85 units (normal, < 0.40 units).

‡Day 15 = 93% serotonin release at 0.3 IU/ml heparin (normal, < 20% release); < 10% release at 0 and 100 IU/ml heparin. For all assays, the positive and negative controls reacted as expected.

HIT: Heparin-induced thrombocytopenia; UFH: Unfractionated heparin.

leukocyte activation, with formation of leukocyte-derived mediators of inflammation, is plausible. Given that HIT is caused by platelet-activating IgG, it is likely that the HIT-associated anaphylactoid reactions are also caused by the effects of this class of antibodies. In keeping with this view, Hewitt *et al.* [21] identified only IgG (and, particularly, no IgE) anti-PF4/heparin antibodies in a patient with HIT who developed an anaphylactoid reaction post-UFH bolus.

Thus, it seems that anaphylactoid reactions to heparin share similarities to IgG-mediated anaphylactoid reactions to another anticoagulant, recombinant hirudin [34]. In one hemodialysis patient who manifested recurrent hirudin-induced anaphylactoid reactions, high-titer IgG anti-hirudin antibodies were found, without any evidence for presence of anti-hirudin IgE antibodies, or antibodies against *Candida* species or *Saccharomyces cerevisiae* (yeast is used to produce recombinant hirudin) [35]. Also, in a volunteer who repeatedly received the recombinant hirudin, desirudin, in a clinical study, and then developed cutaneous 'prickling' sensations, generalized urticaria and slight respiratory depression after a subcutaneous dose of hirudin, prick tests and intradermal skin tests gave equivocal results for desirudin, but high-titer IgG (without IgE) anti-hirudin antibodies were identified [36]. It is also known that IgG antibodies can explain allergic reactions to protamine [37], thiamine [38] and dextran [39].

2.4 Epidemiology of HIT

The frequency of HIT varies widely, from a relatively high rate (up to 5% of patients who receive UFH for 2 weeks

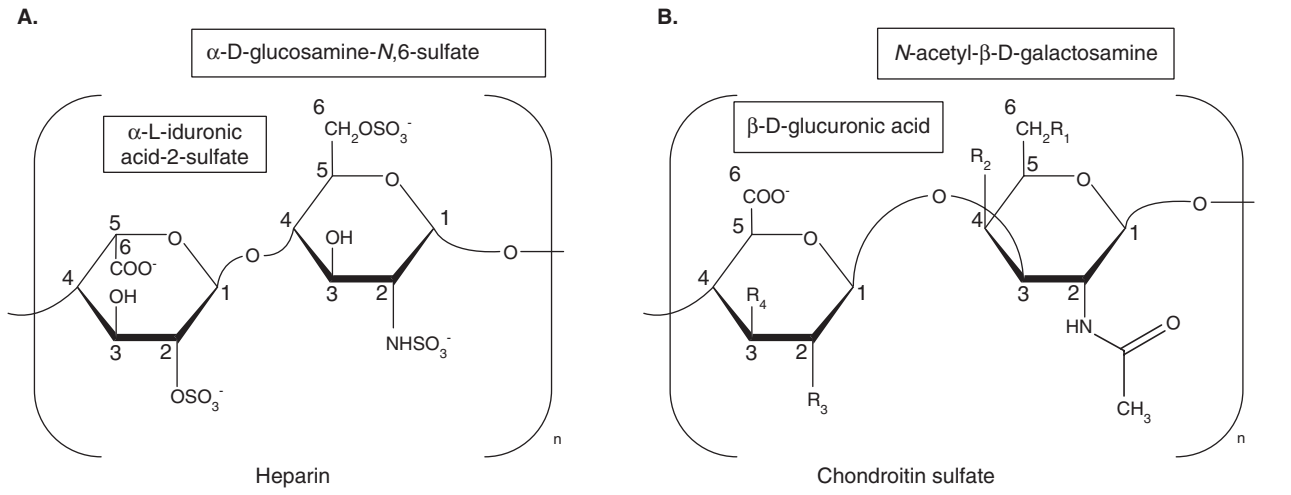

Figure 2. Disaccharide structure of heparin (A) and chondroitin sulfate/OSCS (B). A. The major disaccharide units of heparin consist of α-1,4-linked iduronic acid (usually sulfated at the 2-position, i.e., iduronic acid-2-sulfate) and glucosamine (usually both *N*-sulfonated and 6-*O*-sulfonated (see panel), but sometimes additionally 3-*O*-sulfonated, a substitution crucial for antithrombin-binding activity of the functional 'pentasaccharide' moiety in heparin). A minority of disaccharides within heparin contain glucuronic acid, rather than its epimer, iduronic acid. **B.** The major repeat units of chondroitin sulfate consist of glucuronic acid and *N*-acetylgalactosamine. R_1 – R_4 can be either SO_4^{2-} or OH. Chondroitin sulfate A and C refer to disaccharides that are sulfated at position 4 (R_2) and 6 (R_1) sites, respectively, of the *N*-acetylgalactosamine unit. In contrast, OSCS consists of chondroitin sulfate in which R_1, R_2, R_3 and R_4 all represent sulfate groups, that is, per-*O*-sulfo chondroitin sulfate.
OSCS: Oversulfated chondroitin sulfate.

following orthopedic surgery) to negligible rates (no reported cases among women who receive LMWH during pregnancy) [40]. This variable frequency of HIT is influenced by at least four factors, including type of heparin (UFH > LMWH), type of patient (post-surgery > medical > obstetrical), sex (female > male) and duration of heparin therapy (exposure to heparin for 10 – 14 days compared with exposure for < 5 days) [40,41]. Moreover, there is indirect evidence that in postoperative patients with lower heparin drug levels, immunization rates may be greater than in patients with higher heparin concentrations; this phenomenon could reflect a greater risk of forming stoichiometrically-optimal PF4/heparin complexes at lower (prophylactic-dose) compared with higher (therapeutic-dose) drug levels [42].

Given these considerations, it can be expected that most cases of HIT-associated anaphylactoid reactions would occur in patients who recently received postoperative thromboprophylaxis with UFH (i.e., the most common setting for HIT) and who then received intravenous bolus UFH because of (initially unrecognized) HIT-associated thrombosis. Given recent trends towards greater use of safer heparins (e.g., LMWH, fondaparinux) and more frequent suspicion of HIT when symptomatic thrombosis complicates UFH or LMWH thromboprophylaxis, it is not surprising that the frequency of HIT-associated anaphylactoid reactions has decreased in recent years (unpublished experience of the authors).

2.4.1 HIT-associated immunization is associated with heparin chain length and degree of sulfation

The mechanism and structural requirements for complex formation between sulfated carbohydrates, especially heparin, and proteins, such as PF4, are well characterized, and certain structure–function relationships have been established.

Heparin is a polydisperse mixture of glycosaminoglycans (GAGs) with molecular mass (MM) ranging from 5 to 30 kDa (average MM, 13 kDa) [43]. It is composed of alternating D-glucosamine residues 1→4 linked to uronic acid, either L-iduronic acid or D-glucuronic acid. The principal repeating unit in heparin is the trisulfated disaccharide [→4)-*O*-α-L-iduronic acid-2-sulfate (1→4)-α-D-glucosamine-*N*, 6-sulfate (1→] (**Figure 2A**), which represents about 60% of the heparin chain. With a degree of sulfation (DS) ratio (i.e., the mean number of sulfate groups per disaccharide) of 2.0 – 2.5, heparin has the highest charge density among naturally-occurring animal GAGs. By binding to domains bearing positively-charged amino acids (especially arginine and lysine), heparin interacts with many proteins, resulting in manifold biological activities.

Heparin binds to PF4 through electrostatic interactions with the positively-charged residues on the surface of PF4, which occur independent of any anticoagulant capacity of the heparin. Binding of a single heparin molecule of 16 – 18 monosaccharides spans about 50% of the PF4 tetramer. At clinically relevant concentrations of heparin and high concentrations of PF4, several PF4 tetramers compete

for heparin binding. This permits binding of a single heparin chain to more than one PF4 tetramer. Particularly if a heparin molecule is longer than 16 monosaccharides, it is able to bind to, and thereby bridge, two PF4 tetramers. Thus, at certain concentrations of UFH and PF4, large, linear, ridge-like, multimolecular complexes are formed [10,44]. The molar ratios required for complex formation with PF4 increase in the order: UFH < LMWH < heparan sulfate < dermatan sulfate < chondroitin-6-sulfate (chondroitin sulfate C) < chondroitin-4-sulfate (chondroitin sulfate A) [45] (**Table 3**). Heparin molecules consisting of < 10 disaccharides complex with PF4 less efficiently [46]. Different relative sizes, amounts and stability of the complexes may be responsible for the observed differences in immunogenicity (UFH > LMWH, fondaparinux) and clinically relevant crossreactivity (UFH, LMWH > fondaparinux) [47,48].

With a panel of different synthetic and structurally well-defined sulfated polysaccharides (β-1,3-glucan sulfates), which varied in their DS, MM, sulfation pattern and chemically introduced glycosidic side chains, we further characterized the structural characteristics of polysaccharides required to induce changes in PF4 resulting in HIT antibody binding [46]. Uronic acids, amino groups, or the α-1,4- or β-1,4-glycosidic linkages found in heparin are not essential for these biological properties. Rather, the most important factor influencing formation of HIT antibody-binding sites on PF4 is the DS of the polyanion [46]. The MM is the second most important structural parameter for the immunogenicity of a sulfated polysaccharide, with UFH (13 kDa) representing an optimal chain length for forming immunogenic complexes with PF4. The third most important feature is chain branching. Compared with linear glucan sulfates, glycosidically branched compounds with similar DS and MM values show increased interaction with PF4. Branching changes the 3D structure of the polysaccharide chains, resulting in enhanced flexibility, and improves interaction with proteins. In addition, as side chains are more accessible to sulfation, they provide clusters of negative charges [49], further facilitating binding to PF4 and resulting antigenicity.

2.4.2 OSCS (Arteparon) as a cause of HIT

Chondroitin sulfate is comprised of alternating β-D-glucuronic acid and sulfated *N*-acetyl-β-D-galactosamine units (**Figure 2B**). Depending on the location of the sulfate groups on the *N*-acetylgalactosamine residues, various types of chondroitin sulfate are distinguished (e.g., 4- and 6-sulfation denote chondroitin sulfate A and C, respectively) (**Table 3**). In OSCS, obtained by chemical sulfation of chondroitin sulfate, all 4 hydroxyl groups labeled 'R' in **Figure 2B** are replaced by sulfate groups. OSCS structurally corresponds to the pharmacologic agent, Arteparon® (Luitpoldwerke, Munich, Germany) [50], which is a chemically-modified, high-sulfated GAG derived from bovine cartilage. Arteparon can cause HIT (including HIT-associated anaphylactoid reactions). In Section 3, we discuss the very recent discovery that this tetrasulfated disaccharide repeat form of OSCS is the principal contaminant responsible for the recent epidemic of heparin-induced anaphylactic reactions [50-52].

Arteparon administered by intramuscular or intraarticular route was used from 1970 until ~ 1994 for treatment of degenerative joint disease [53]. In the 1980s, the first evidence emerged suggesting that Arteparon could induce antibodies resembling heparin-induced antibodies [54,55]. In 1991, we observed a patient who developed a clinical syndrome mimicking the symptoms and signs of HIT when receiving Arteparon, and who then developed acute HIT when heparin was given shortly afterwards [6]. We here provide the original case description (with comments) to highlight the clear association between HIT antibody-associated anaphylactoid reactions triggered by Arteparon: "A 53-year-old male patient suffering from 'knee pain' was treated with Arteparon® (Luitpoldwerke, Munich, Germany) by weekly intraarticular injections into the right knee on six occasions from December 1990 until the end of January 1991. After the fifth injection headache, dyspnoea and fever occurred in the patient. Upon the sixth injection 1 week later the patient experienced a shaking chill, fever of 38.5°C [*comment*: *features consistent with HIT-related anaphylactoid reactions*] and acute pain in the left leg [*comment*: *DVT is a typical feature of HIT*]. Ten days later the patient was admitted to the hospital with clinical signs of pulmonary embolism [*comment*: *typical feature of HIT*]. Perfusion scintigraphy of the lungs revealed multiple occlusions of the vessels …. Phlebography showed an approximately 10-day-old deep venous thrombosis of the left calf. At that time, the platelet count was 180×10^9/l. Unfractionated heparin was instituted by intravenous infusion at a dose of 500 IU/h. The next day the platelet count dropped to 50×10^9/l [*comment*: *illustrates the in vivo cross-reactivity of OSCS-induced antibodies with heparin*]. …. [W]hen low platelet counts persisted … without obvious reasons, further laboratory investigations confirmed the diagnosis of [heparin-induced thrombocytopenia]. All heparin preparations were discontinued…. Coumarin was started…. The platelet counts normalized within 3 d." During laboratory workup, crossreactivity of typical HIT antibodies with Arteparon was shown, with an interesting bell-shaped curve of reactivity (minimal reactivity at very low or very high concentrations of OSCS), essentially identical to the reaction pattern induced by heparin in the presence of antibodies induced by heparin [6].

2.4.3 Other polyanions reported to cause HIT-mimicking syndromes

Beside OSCS, certain other polyanions can induce antibodies indistinguishable from those that cause HIT. This includes PI-88, a phosphosulfomannan mixture [56] evaluated as an anticancer agent. During Phase I studies, the

Table 3. Main disaccharide units of mammalian glycosaminoglycans, arranged by increasing DS.

Glycosaminoglycan	Main components of disaccharide unit	DS
Hyaluronic acid	Glucuronic acid; *N*-acetylglucosamine	0
Keratan sulfate	Galactose; *N*-acetylglucosamine	~ 0.6
Chondroitin sulfate A	Glucuronic acid; *N*-acetylgalactosamine-4-sulfate	~ 0.8
Chondroitin sulfate C	Glucuronic acid; *N*-acetylgalactosamine-6-sulfate	~ 0.8
Dermatan sulfate	Iduronic acid; *N*-acetylgalactosamine-4-sulfate	~ 1.4
Heparan sulfate	Glucuronic acid; *N*-acetylglucosamine	0.8 – 1.8
LMWH	Depolymerized forms of UFH (see below)	2.0 – 2.5
UFH	Iduronic acid-2-sulfate (or glucuronic acid); glucosamine-*N*,6-sulfate	2.0 – 2.5
OSCS	Glucuronic acid-2,3-sulfate; *N*-acetylgalactosamine-4,6-sulfate	4.0

Higher values of DS and chain length increase binding to PF4. DS denotes the mean number of sulfate (SO_4^{2-}) groups per disaccharide unit. The distinction between heparan sulfate and heparin is not precise; heparin is the preferred term if the content of *N*-sulfate groups largely exceeds that of *N*-acetyl groups and the content of *O*-sulfo groups exceeds that of *N*-sulfo groups. UFH of bovine lung origin also has higher DS than UFH of porcine mucosal origin (~ 2.5 versus 2.1, respectively).
Information to prepare this table obtained from published sources [43].
DS: Degree of sulfation; LMWH: Low-molecular-weight heparin; OSCS: Oversulfated chondroitin sulfate; UFH: Unfractionated heparin.

dose-limiting toxicity of PI-88 was immunologically-mediated thrombocytopenia similar to HIT [57]. Another implicated polyanion is pentosan polysulfate, which can induce formation of HIT antibodies together with thrombocytopenia and thrombosis [58]. This agent is available as an oral formulation (Elmiron®, Baker Norton Pharmaceuticals, Inc., Miami, FL, USA) in the US for treatment of chronic bladder inflammation (interstitial cystitis). In Germany, it is formulated for subcutaneous application as prophylaxis of venous thrombosis (Fibrezym®, bene-Arzneimittel GmbH, Munich, Germany) as well as for oral administration for treatment of peripheral arterial disease (Pentosanpolysulfat SP54®, bene-Arzneimittel). In contrast, dextran, dermatan sulfate, *N*-desulfated heparin or sulfated glucosamine [59] have not been reported to interact with PF4 in a way to induce the HIT antigens.

3. Heparin-induced anaphylaxis associated with OSCS-contaminated heparin

3.1 Epidemic of heparin-induced anaphylactoid reactions

Beginning in 2007, hundreds of patients in the US and Europe were recognized who developed serious adverse effects within minutes of receiving intravenous UFH, with features of hypotension, angioedema and swelling of the larynx [60-64]. The Centers for Disease Control and Prevention were first notified on 7 January 2008 of allergic-type reactions that began on 19 November 2007, at a pediatric hospital, with eight episodes observed among four pediatric patients undergoing hemodialysis over a 2-month period. Case-finding efforts by the Centers for Disease Control and Prevention resulted in a dialysis supply company indicating that they had received reports of 50 such reactions among adult hemodialysis patients in six states. A common factor (identified in > 90% of cases) was use of heparin (1000 U/ml) from 30 or 10 ml vials manufactured by Baxter. On 17 January 2008, based on these reports, Baxter announced a voluntary recall of nine heparin lots that had been produced at a single plant in Changzhou, China.

The mean time to a reaction after exposure to heparin was 5 min among patients undergoing hemodialysis [52]. Hypotension and nausea were the most prominent features (~ 50% each), with dyspnea, vomiting, tingling, angioedema and flushing also representing common symptoms and signs (each observed in ~ 20 – 30% of cases confirmed to have received OSCS-contaminated heparin) [52]. Pruritus, urticaria and chills were infrequent (≤ 3%). As of 13 April 2008, there were 81 reports of death linked to anaphylaxis associated with heparin (since 1 January 2007) [51].

Most cases were identified in patients receiving UFH for hemodialysis, although one cardiac care facility also observed severe allergic-type reactions among cardiac patients who had received heparin from lots that were subsequently recalled [60]. The conclusion followed that "the temporal and geographic distribution of these reactions in a discrete population of patients suggests common exposure to a health-care product with wide distribution in the United States" [60]. A subsequent epidemiologic study using case-control methodology identified heparin manufactured by Baxter Healthcare as the factor most strongly associated with these reactions (present in 100% of case facilities versus 4.3% of control facilities; $p < 0.001$) [52].

3.2 Identification of OSCS as the heparin contaminant

Given the immediately preceding role of intravenous UFH administration, and the spike in such cases of UFH-associated anaphylaxis, the possibility of a contaminant or impurity in heparin was investigated. In a landmark paper, Guerrini *et al.* [50] identified the contaminant as OSCS. An initial comparison of six UFH lots that were associated with adverse clinical symptoms against four control lots using 1D

NMR revealed an unusual series of *N*-acetyl signals. Moreover, the specific profile was indicative of the presence of *O*-substituted *N*-acetylgalactosamine residues (not found in heparin). Subsequent investigations utilizing a variety of analytic techniques (e.g., total correlation spectroscopy, rotating-frame nuclear Overhauser effect spectroscopy) revealed the contaminant to consist of a polymeric repeat of *N*-acetylgalactosamine linked to glucuronic acid through β-linkages. These workers successfully isolated this contaminant from heparin through addition of an organic solvent (ethanol) and by degrading the heparin using nitrous acid.

Further detailed structural analyses of the isolated contaminant revealed it to contain a disaccharide repeat unit of glycuronic acid linked β1→3 to a β-*N*-acetylgalactosamine. Of note, this disaccharide had an unusual sulfation pattern, with sulfate groups at the 2-*O* and 3-*O* positions of the glucuronic acid and at the 4-*O* and 6-*O* positions of the galactosamine (**Figure 2B**). Essentially, this tetrasulfated repeating disaccharide represented complete sulfate substitution of the four hydroxyl groups of chondroitin sulfate (two each on the uronic acid and galactosamine moieties). Thus, OSCS consists of per-*O*-sulfated chondroitin sulfate and has a DS that approaches 4.0.

Guerrini *et al.* remarked that such a contaminant was "highly unusual", as it has never been isolated from animal tissues, and, therefore, was unlikely to have been produced naturally. They also noted that the contaminant is structurally identical to Arteparon. Further, because chemically-synthesized OSCS has anti-thrombin (anti-IIa) activity [65], OSCS-contaminated UFH would 'pass' coagulation screening assays. The only logical conclusion: OCSC is not an accidentally-introduced impurity (such as dermatan sulfate in some UFH preparations) but rather an intentionally-added adulterant [66].

3.3 OSCS contamination of LMWH

LMWH preparations are derived from UFH, using a variety of enzymic or chemical methods; for example, controlled depolymerization using nitrous acid (dalteparin), heparin lyase (tinzaparin), periodate-oxidation (centaxarin), alkaline treatment (enoxaparin) and hydrogen peroxide (ardeparin). Zhang *et al.* [67] studied the effect of these various techniques used to manufacture the aforementioned LMWH preparations on degrading OSCS alone or mixed with UFH. These studies showed that the methods used to manufacture dalteparin, tinzaparin and centaxarin would not depolymerize OSCS. Thus, these three LMWH preparations, if derived from a contaminated UFH source, would contain intact, resistant high-molecular-weight OSCS contaminant that could, however, be easily identified by NMR spectroscopy. On the other hand, enoxaparin and ardeparin prepared from OSCS-contaminated UFH would contain partially degraded OSCS that would be more difficult to detect using analytical methods. LMWH preparations obtained from an OSCS-contaminant source heparin could, in theory, cause anaphylaxis, although to our knowledge such adverse reactions have not been reported.

3.4 Role of OSCS in causing anaphylaxis

Using *in vitro* assays for measuring activation of the contact system, Kishimoto *et al.* [51] showed how OSCS-contaminated UFH can produce anaphylaxis. These workers found that the OSCS-contaminated heparin lots, as well as a synthetic OSCS standard, directly activated the kinin-kallikrein pathway in human plasma, leading to bradykinin (BK) generation. Further, two complement-derived anaphylatoxins, C3a and C5a, were produced. Complete concordance between use of OSCS-contaminated plasma lots and generation of kallikrein activity was observed. Interestingly, these reactions showed a bell-shaped curve, that is, kallikrein activity was produced by OSCS-contaminated UFH at concentrations of 2.5 and 25 μg/ml, but not at 0.25 and 250 μg/ml. With purified or synthetic OSCS, the reactions occurred at 10-fold lower concentrations, that is, at 0.25 and 2.5 μg/ml. These findings were consistent with the OSCS contaminant representing ~ 20% of the mixture. The bell-shaped reaction profile is characteristic of GAG-induced responses [68] (see also section 2.4.2), perhaps reflecting inhibition of factor XIIa at very high GAG concentrations, as kallikrein activity was not generated when factor XII-depleted plasma was used, or by complex formation (as shown for complexes of UFH and PF4 in HIT [9]).

Figure 3 illustrates the consequences of activation of the contact system by OSCS and certain other polyanions. Although heparin is able to activate factor XII in purified systems, only polyanions with DS values that approach 4.0 sulfates per disaccharide (such as OSCS and dextran sulfate) are able to activate factor XII in plasma (perhaps, anti-thrombin found in plasma prevents heparin-induced activation of factor XII). The marked hypotension characteristic of OSCS-induced anaphylaxis is believed to be owing to the vasodilatory effects of BK, as well as C3a and C5a. The usual lack of pruritus and urticaria argue against a major role for mast cell or basophil activation, which would lead to histamine release.

To further corroborate the role of OSCS in inducing hypotension, pigs were treated with a single intravenous dose (5 mg/kg) of OSCS-contaminated heparin and synthetic OSCS [51]. Compared with controls (heparin, chondroitin sulfate), some of the swine treated with OSCS developed marked hypotension, although all pigs uniformly showed marked increase in plasma kallikrein activity in association with injection of OSCS-contaminated heparin or synthetic OSCS (but not control UFH or chondroitin sulfate). The finding that most pigs did not develop hypotension parallels the fact that most human recipients of OSCS also do not develop any obvious adverse effect.

3.5 Polyanion-induced activation of contact system

The role of OSCS-contaminated heparin in triggering contact system activation has features reminiscent of certain hypotensive reactions linked to exposure of blood to large areas of artificial surfaces. These include hemodialysis membranes

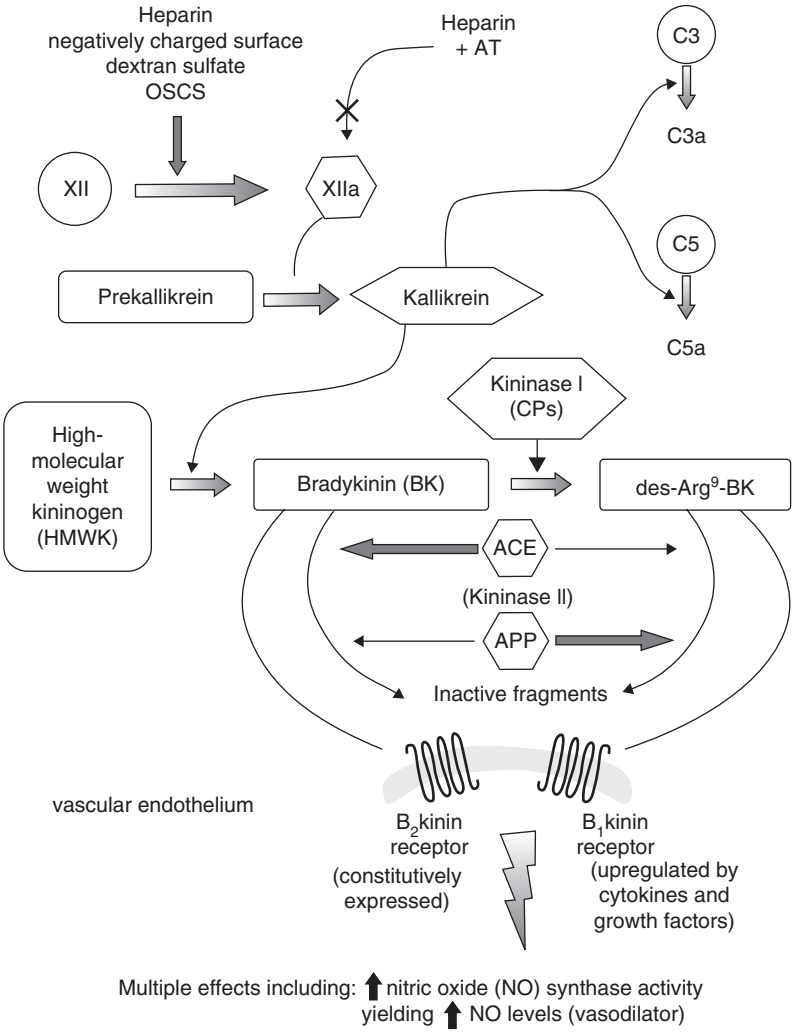

Figure 3. Consequences of activation of the contact system by polyanions including OSCS. Some polyanions, such as dextran sulfate and OSCS, and negatively charged surfaces (e.g., certain hemodialyzers, blood filters) directly facilitate autocatalysis of factor XII to the active enzyme, factor XIIa. Although heparin also facilitates this process, it additionally catalyzes the inhibition (through AT) of XIIa; therefore, pharmacologic heparin – unlike OSCS – usually does not trigger contact system activation. Factor XIIa leads to activation of the intrinsic pathway of coagulation (not shown), and also activates plasma prekallikrein to kallikrein. Kallikrein converts HMWK to BK, as well as the complement factors C3 and C5 to their respective anaphylatoxins, C3a and C5a. BK is converted by 'kininase I' (a term that denotes various carboxypeptidases, for example, carboxypeptidases N, M and U) to its main metabolite, des-arg^9-BK. Both BK and des-arg^9-BK are short-lived vasoactive kinins ($t_{1/2}$, ~ 30 sec and 8 min, respectively), which activate cells through the B_2 and B_1 kinin receptors, respectively. B_1 receptors are upregulated by cytokines and growth factors, whereas the B_2 receptors are constitutively expressed. Activation of the B_2 and B_1 receptors leads to numerous effects, including increased endothelial cell nitric oxide synthase activity, which results in increased levels of nitric oxide, a potent vasodilator. Regulation of the kinin system includes the enzyme, peptidyl-dipeptidase A, also known as ACE and 'kininase II'. Whereas ACE is the major enzyme that degrades BK, another enzyme, APP, is the major enzyme that degrades des-arg^9-BK. Therefore, acquired (e.g., ACE inhibitor therapy) or constitutive defects (e.g., APP deficiency) are risk factors for the development of anaphylactic and other hypersensitivity reactions that result from activation of the contact system.

ACE: angiotensin converting enzyme; APP: Aminopeptidase P; AT: Antithrombin; BK: Bradykinin; CPs: carboxypeptidases; HMWK: High-molecular-weight kininogen; NO: nitric oxide; OSCS: Oversulfated chondroitin sulfate.

(discussed later in this review), apheresis machines [69], staphylococcal protein A columns [70] and use of negatively-charged bedside leukoreduction filters for blood transfusion [71-73]. Although activation of the contact system by these medical devices is usually compensated for by most patients, a subset of patients with other risk factors (e.g., use of ACE inhibitors, prostatectomy-induced release of prostate-specific kallikreins, inherent defects in kinin degradation such as deficiency in aminopeptidase P) can develop clinical symptoms [74]. Indeed, the interaction of several risk factors in contributing to these reactions has been emphasized [75]. Other factors besides negatively-charged surfaces may also cause these reactions. For example, hypotensive reactions related to ionic radiographic contrast materials have been linked to the presence of sulfated polysaccharides in these preparations [76].

4. Hemodialysis-associated anaphylactic/anaphylactoid reactions

4.1 Hemodialysis patients: an at-risk population

Given their dual exposure to heparin and artificial surfaces, hemodialysis patients represent a high risk group for anaphylactic/toid reactions owing to contact system activation, acute HIT or other mechanisms. Several risk factors are evident. First, hemodialysis patients typically receive an intravenous bolus of heparin before starting dialysis. Second, the heparin exposures are repetitive, perhaps favoring immunization, but at the very least will result in a repeat bolus application occurring during a time of acute antibody increase. Third, dialysis systems can expose patients to other immunogenic materials (e.g., latex). Fourth, the large negatively-charged artificial surfaces itself can activate the contact system. These several potentially interacting risk factors infer that clinical breakthrough of anaphylactoid reactions is more likely than in other patient populations.

4.2 Contact system activation in hemodialysis

Contact system activation resulting from hemodialysis using high-flux polyacrylonitrile membranes (e.g., AN69) is one mechanism of anaphylactoid reactions [77], particularly in patients receiving ACE inhibitors (e.g., captopril, enalapril, lisinopril) [78,79] or, to a lesser degree, angiotensin receptor antagonist therapy (e.g., losartan) [80]. Exposure of the plasma to the negatively-charged polyacrylonitrile membranes leads to increase in BK concentrations [81]. BK and des-arg^9-BK increase endothelial cell nitric oxide (NO) synthase activity, and the increase in NO levels produce vasodilatation, as well as angioedema and hypotension (by increasing cGMP) in vascular smooth muscle cells [82]. ACE inhibitors, by impairing BK degradation by ACE, increase the risk for this type of reaction [83]. Although BK generation is enhanced particularly by polyacrylonitrile (e.g., AN69), another hemodialysis membrane, cuprophane (cuprammonium cellulose), seems to increase NO levels independent of BK generation [82]. These latter dialyzers have also been associated with anaphylactoid reactions [84].

4.3 Non-HIT allergic reactions in hemodialysis

The differential diagnosis of hemodialysis-associated anaphylactic/toid reactions is wide, and includes hypersensitivity to, or toxicity of, various components of the dialysis membrane [77]. For example, IgE-mediated hypersensitivity against ethylene oxide (a gas previously used to sterilize hemodialysis membranes) has been reported [85,86]. Other IgE-mediated allergic reactions can result from exposure to formaldehyde [87], natural rubber latex [77] or iron (administered as iron-dextrose) [88]. Also, use of UFH or LMWH has been claimed to be a cause of anaphylaxis during hemodialysis for reasons other than HIT [77,89,90]. Whether these heparin-induced hypersensitivity reactions are linked to the so-called 'delayed-type hypersensitivity' reactions of UFH or LMWH (which manifest as non-necrotizing skin lesions at heparin injection sites in the absence of anti-PF4/heparin antibodies [18,91,92]) is unknown [77]. A further complicating issue is that heparin 'allergy' in a hemodialysis patient can also be caused by preservatives in the heparin preparation such as paraoxybenzoic esters [93].

4.4 HIT-associated anaphylactoid reactions in hemodialysis patients

Several reports have described HIT-associated anaphylactoid reactions in the setting of hemodialysis performed with UFH or LMWH [22,23,32,33]. An intriguing report by Tholl *et al.* [32] reported a man who underwent hemodialysis uneventfully for several years; however, post-parathyroidectomy, he began to develop anaphylactic reactions soon after initiating hemodialysis. Clinical features included abrupt platelet count decreases (consistent with HIT) as well as hypotension (not characteristic of HIT-associated anaphylactoid reactions). As the patient had clear evidence of HIT antibodies, but also a risk factor for hypotension related to contact system activation (ACE inhibitor therapy), perhaps several factors interacted to explain the clinical picture.

In the series describing four patients with acute cardiorespiratory collapse associated with bolus heparin administration, Mims *et al.* [22] noted that one of the patients had a known diagnosis of serologically-confirmed HIT, but received (contrary to physician orders) UFH boluses at the time of three hemodialyses, each one of which was complicated by 'wheezing', 'acute dyspnea' and 'endotracheal intubation.'

Alonso *et al.* [33] reported a 77-year-old woman who developed 'anaphylaxis' within 10 min of starting her fifth hemodialysis session. Similar symptoms – vomiting, tachypnea and wheezing – occurred during five consecutive hemodialysis sections and were accompanied by abrupt decreases in the platelet counts. Subsequent hemodialysis using hirudin as an anticoagulant was uneventful. The presence of anti-PF4/heparin antibodies was confirmed in this patient.

5. Does OSCS-contaminated heparin increase the risk of HIT?

The many similarities between OSCS-induced anaphylaxis and HIT-associated anaphylactic/toid reactions, as well as higher sulfation grade (DS value) representing a key risk factor for polysaccharide-induced immunization, raise the important epidemiologic question as to whether heparin that is contaminated by OSCS has a greater risk of causing HIT compared with uncontaminated heparin. Unlike acute anaphylactic reactions resulting from administration of OSCS-contaminated heparin, the link between HIT and a 'bad batch' of heparin (i.e., one containing OSCS) might not be readily apparent to the clinician. Many patients are exposed in a given hospital stay to more than one preparation of heparin, and physicians who might become suspicious of the direct culpatory role of heparin because a patient developed acute anaphylaxis in a few minutes after receiving the heparin bolus might not be similarly suspicious if a patient developed HIT, for the reason that a certain frequency of HIT is expected in any medical practice in which heparin is commonly administered. Furthermore, even large medical centers see only several patients per year who develop well-documented cases of HIT, and even a relative increase of 100% in HIT frequency caused by OSCS-contaminated heparin might only represent a small increase in absolute numbers, especially if OSCS-contaminated heparin was found in only one of several lots of heparin in clinical use in any given institution.

Recently, we provided evidence suggesting that OSCS-contaminated heparin might have increased the risk of HIT during the recent epidemic [94]. Given that OSCS-contaminated heparin was distributed in Germany, but not in Canada, we hypothesized that there may have been an increase in cases of HIT in the former, but not the latter, country. We, therefore, compared the frequency of laboratory-documented HIT in our two laboratories (McMaster University and Ernst-Moritz-Arndt University in Canada and Germany, respectively) during the approximate 4-month period in which OSCS-contaminated heparin was distributed. We compared the absolute numbers of cases in each of our laboratories against the year-over-year earlier 4-month period. We found that whereas the numbers of cases in the Canadian laboratory remained relatively stable, a doubling of cases was identified in the German laboratory [94] (**Table 4**). Given the incidence of HIT, with approximately one or two dozen cases a year in hospitals having about 1000 beds (and performing cardiac surgery), it is conceivable that the number of patients affected by an increased incidence in HIT owing to OSCS-contaminated heparin might have been substantially larger than the number of patients affected by direct anaphylaxis induced through contact system activation.

The very high DS of OSCS (4.0) contaminating UFH is a key feature supporting a higher immunization rate compared with non-contaminated UFH. Scientific rationale for this concept includes a comparison of the structural features, immunogenicity and HIT-provoking potential between UFH preparations obtained from different animal sources, bovine lung and porcine mucosa. Although the mean MM of these two UFH types is similar, the DS for bovine lung UFH is higher than that for porcine mucosal UFH (~ 2.5 versus ~ 2.1, respectively) [95]. This biochemical difference probably explains the greater immunogenicity of bovine lung UFH [96], as well as its higher risk for causing HIT [40], compared with porcine mucosal UFH.

6. Expert opinion

The clinician who is called to assess a patient who has developed an anaphylactic or anaphylactoid-type reaction soon after heparin administration must consider several diagnostic possibilities with distinct pathophysiologic explanations. First, this clinical picture could be a sign of acute HIT that has resulted from the administration of heparin to a patient who has already-circulating HIT antibodies. This diagnosis would be suggested by the following features: i) the patient does not show features of classic anaphylaxis (e.g., urticaria, angioedema, laryngeal edema); ii) hyper-, rather than hypotension, is evident; iii) certain characteristic features of HIT-associated anaphylactoid reactions are present, including fever and rigors, TGA or cardiopulmonary arrest; and iv) there is a large magnitude decline in the platelet count. Such a patient's blood sample should be investigated for characteristic 'HIT antibodies', including blood samples obtained shortly before the anaphylactoid reaction. In most cases, the history will also reveal that the patient had been exposed to heparin in the recent past, typically about 1 – 4 weeks before the acute administration of heparin that has led to the anaphylactoid reaction. This temporal association relates to the usual minimum time of about 1 week to form high levels of HIT antibodies, which typically peak about 2 weeks after the immunizing heparin exposure, and which usually remain at relatively and clinically-significant levels only for the next few weeks.

The clinical management of a patient with acute HIT falls outside the scope of this review article, but would include such issues as heparin cessation and avoidance, use of an alternative non-heparin anticoagulant and postponing warfarin therapy [97]. In addition, the specific consequences of the anaphylactoid event itself could require urgent intervention, for example, intubation for a patient with respiratory arrest or severe pulmonary compromise.

Current tests for HIT antibodies are highly sensitive for detecting the pathogenic antibodies implicated in HIT-associated anaphylactic reactions [98]. Consequently, a negative test infers that HIT is not the mechanism for the patient's anaphylactic reaction. As discussed earlier, there

Table 4. Patients with positive tests for HIT: comparison of the period of heparin contamination with an earlier period.

Country	OSCS-contaminated UFH distributed	Period before contamination (1 November 2006 to 28 February 2007)	Period of contamination (1 November 2007 to 28 February 2008)	Increase in no. of patients with positive tests
		No. of patients with positive test/total no. tested		
Canada	No*	45/373	51/409	+ 13.3%
Germany	Yes	32/459	64/405	+ 100.0%

Patients were considered to have positive tests for antibodies that cause HIT if both a washed-platelet activation assay and a PF4-dependent enzyme immunoassay were positive. Patients who underwent repeated tests were counted only once. For the McMaster laboratory, 13 and 11 patients who met the criteria for HIT, respectively, during the period before contamination and the period of contamination were local patients (i.e., from Hamilton, Ontario); for the Greifswald laboratory, the corresponding figures for local patients were 2 and 5 patients, respectively.

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*A recall of minimal amounts of OSCS-contaminated products occurred in Canada, without any reports of anaphylactic reactions.

HIT: Heparin-induced thrombocytopenia; OSCS: Oversulfated chondroitin sulfate; UFH: Unfractionated heparin.

also are many other explanations for anaphylaxis in certain clinical settings, such as hemodialysis. Here, heparin may predispose to contact system activation, particularly if certain genetic or other risk factors are present (e.g., use of ACE inhibitors). Plus, non-HIT allergic reactions may also be present. It is to be expected that the identification of the role of OSCS-contaminated heparin in causing serious morbidity and mortality, the development of specific tools to identify such contamination (e.g., NMR spectroscopy), and greater regulatory and industry oversight over heparin manufacturing will make the epidemic of OCSC-contaminated heparin-induced anaphylaxis a historical curiosity that nevertheless has had profound implications for drug manufacturing. (However, the recent raid by federal officials of a manufacturing facility in Cincinnati, OH, USA [99] suggests that continuous pharmacovigilance may be needed to avert continuing contamination in the foreseeable future.) Also, given the development of medical care in many countries of the world, where until recently treatments such as hemodialysis were rarely available, there will most likely be increasing demands for heparin, and the requirement for many countries to produce this anticoagulant using their own limited resources. Thus, the need to apply inexpensive methods [100] to screen for OSCS-contaminated heparin will be a continuing need for any organization involved in manufacturing and/or distribution of this indispensable drug.

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T E Warkentin has received speaking and/or consulting fees from GTI, Inc., GlaxoSmithKline, Organon, Inc. (part of Schering-Plough) and Sanofi-aventis as well as research funding from GlaxoSmithKline.

A Greinacher has received speaking and/or consulting fees from Mitsubishi Pharma and Organon and speaking fees from GlaxoSmithKline and Novartis.

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