INCREASED RISK OF THROMBOSIS IN FcγRIIA 131RR PATIENTS WITH HIT DUE TO DEFECTIVE CONTROL OF PLATELET ACTIVATION BY PLASMA IgG2

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Key points

- Normal IgG, and especially IgG2, differentially inhibit platelet activation induced by HIT antibodies according to the FcγRIIA H131R polymorphism.
- This variable effect of IgG probably explains the higher risk of thrombosis in patients homozygous for the FcγRIIA 131R allele.
ABSTRACT

Thrombosis results in heparin-induced thrombocytopenia (HIT) from cellular activation involving Fc receptors. In this study, the FcγRIIA 131RR genotype was found to increase the risk of thrombosis in HIT patients (OR = 5.9; 95% CI 1.7-20). When platelet aggregation tests (PAT) were performed with platelet-rich plasma (PRP), a shorter lag time was measured in 131RR donors than in HR and HH individuals in response to HIT plasma or 5B9, a recently developed humanized monoclonal antibody to PF4/heparin. Importantly, this difference was no longer detectable when PAT were performed with washed platelets, or IgG-depleted PRP. Moreover, polyclonal IgG or monoclonal IgG1 added to IgG-depleted PRP increased the lag time in response to 5B9. HH platelets were also sensitive to IgG2, which in contrast failed to inhibit the response to 5B9 of 131RR platelets. Finally, higher tissue factor mRNA levels were measured in the whole blood of 131RR donors after activation by HIT antibodies, with increased phospholipid procoagulant activity. These results demonstrate that HIT patients homozygous for the FcγRIIA 131R allele have a higher risk of thrombosis, probably due to increased cell activation by antibodies to PF4/H, with a lower inhibitory effect of endogenous IgG, especially from the IgG2 subclass.
INTRODUCTION

Heparin-induced thrombocytopenia (HIT) is an atypical immune complication of heparin treatment, due to antibodies specific to platelet factor 4/heparin (PF4/H) complexes \(^1\). Despite having thrombocytopenia, affected patients usually develop thrombosis, and this particular clinical presentation is explained by the central role of Fcγ receptors in the pathophysiology of HIT, particularly of FcγRIIA that mediates platelet activation induced by IgG/PF4/H immune complexes (IC). In addition, platelets from healthy donors exhibit wide variability in their response to HIT antibodies \(^2-4\), and many patients who synthesize significant levels of antibodies to PF4/H while being treated with heparin do not develop HIT \(^5-7\).

The FCGR2A gene, which encodes FcγRIIA, displays a functional allelic dimorphism generating two codominantly expressed allotypes with either a histidine (H) or an arginine (R) at the amino acid position 131 in the second Ig-like extracellular domain. It has been reported that neither of them binds monomeric IgG \(^8\), but they differ substantially in their ability to bind IgG-containing immune complexes (IC): the 131H allotype binds efficiently human IgG1 and IgG2, whereas the 131R allotype binds human IgG1 but poorly IgG2 \(^9\). The consequences of this differential binding may be important. For instance, individuals homozygous for the 131R allele are at higher risk of serious infection with encapsulated organisms, which are cleared via a predominant IgG2 response \(^10\).

An influence of platelet FcγRIIA H131R polymorphism on the risk of HIT has also been proposed, but with discordant results \(^11-16\) and no association was confirmed \(^17\). However, one study showed an increased frequency of the 131R variant in HIT in patients with thrombosis \(^16\), and we had previously found that platelet counts were lower in FcγRIIA RR patients with antibodies to PF4/H after cardiac surgery \(^6\). Carlson et al assumed that PF4/heparin antibody IC were less efficiently removed from the circulation in FcγRIIA RR
patients with subsequent prolonged IC–dependent activation of platelets. This hypothesis is however unlikely since HIT antibodies to PF4/H are predominantly IgG1\textsuperscript{14}, and the two Fc\(\gamma\)RIIA allotypes have identical affinity for IgG1 IC.

The aims of the present study were therefore to elucidate the mechanisms explaining that homozygous Fc\(\gamma\)RIIA 131RR HIT patients have a higher risk of thrombosis, and especially to investigate whether IgG subclasses differentially modulate the platelet response to HIT antibodies according to the Fc\(\gamma\)RIIA polymorphism.
PATIENTS AND METHODS

Patients

The HIT patients and control groups have previously been described by Rollin et al\textsuperscript{18}. The patient group (HIT) comprised 89 individuals including 35 with thrombotic complications and the two control groups Ab\textsuperscript{pos} and Ab\textsuperscript{neg} were composed of 160 and 174 non-HIT patients, having or not developed significant levels of PF4-specific antibodies after cardiac surgery.

Two groups of patients, who had presented either ischemic stroke (n =113) or venous thromboembolism (VTE, n =97) unrelated to HIT, were investigated. In addition, a previously analyzed\textsuperscript{19} control population including 206 healthy subjects was also studied.

All patients and controls were Caucasian and blood samples were collected after obtaining informed consent according to the Helsinki Declaration principles. Moreover, both our institutional Ethics Committee and the Ministry of Research had previously approved collection of DNA from patients with HIT or thrombosis and from healthy controls for genetic studies (Agreement N\textsuperscript{o}s: 2011-N7, 2002-13, DC2008-308, respectively).

Materials

5B9 is a chimeric humanized anti-PF4/H monoclonal IgG1 antibody that we recently developed and that fully mimics human HIT antibodies (Figure S1).

Monoclonal anti-CD32 antibody (clone IV.3) was purchased from Stemcell Technologies. Polyclonal IgG (Privigen) were obtained from CSL Behring. Cetuximab, a chimeric mouse-human IgG1 anti-EGFR (Epidermal Growth Factor Receptor), and Panitumumab, a human IgG2 anti-EGFR, were obtained from Merck-Serono and Amgen SAS, respectively. Both IgG1 and IgG2 antibodies are specific for EGFR, which is not expressed on platelets, and were showed to be monomeric (>98%) with size exclusion
chromatography analysis. Horm type I collagen from equine tendon was obtained from Nycomed and unfractioned heparin (Heparin Choay) from Sanofi.

**Genotyping**

Genomic DNA was extracted from citrated whole blood using the Flexigen DNA kit (Qiagen) according to the manufacturer’s instructions. Genotypes of FcγRIIA H131R polymorphism (rs1801274) were defined by a PCR-RFLP method as previously described.

**Assays of TF mRNA levels after whole blood stimulation by HIT antibodies**

Whole blood collected from healthy donors on 0.129 M sodium citrate was incubated with HIT plasma (1/5 dilution) or 5B9 (10µg/mL) in the presence or absence of heparin (0.5 IU/mL). After 60 minutes of incubation at 37°C, total RNA was isolated using the Qiamp RNA blood Mini kit (Qiagen). PCR were performed using Platinum qPCR supermix (Invitrogen) and a Taqman Probe (Hs00175225_m1, Applied Biosystems). In addition, the expression level of the CD14 gene was evaluated using Platinum qPCR SYBRgreen supermix (Invitrogen) and 3 pmol of forward (5’-GGTTCCCTGCTCAGCTACTGG-3’) and reverse (5’-TAGGTCCCTCGAGCGTCAGTT-3’) specific primers. The relative increase in TF mRNA levels was quantified after incubation of HIT antibodies and heparin, comparatively to the condition without heparin, and using the 2−ΔΔCt method.

**Assay of plasma procoagulant activity after whole blood stimulation by 5B9**

In selected experiments, platelet-depleted plasma was obtained after two centrifugations (2250g, 15 min) following incubation of whole blood with 5B9 (10µg/mL) with or without heparin (0.5 IU/ml). The Procoag-PPL assay (Diagnostica Stago) evaluating the procoagulant activity of phospholipid microparticles was then performed. Briefly, 25 µL
of test plasma were mixed with 25 μL of phospholipid-depleted human plasma and prewarmed for 2 minutes at 37°C. Coagulation was then triggered by adding 100 μL of factor Xa/calcium reagent (containing 0.01 U/mL bovine factor Xa in a buffered calcium solution), followed by the measurement of clotting time using a KC4 instrument (Stago). The relative decrease in clotting time was calculated for each donor after stimulation with 5B9 and heparin, compared to the experimental condition without heparin.

**Platelet aggregation tests and serotonin release assays**

Whole blood from 70 healthy aspirin-free donors (30 males and 40 females) was collected according to the recommendations of the ISTH in Acid-Citrate-Dextrose supplemented with PGE1 (0.1mM). Platelets were washed and suspended at a final count adjusted to 350x10⁶/mL. In addition, whole blood from healthy donors was collected on 0.129 M sodium citrate and platelet rich plasma (PRP) was isolated (mean platelet count = 352 ± 82x10⁶/mL). All platelet aggregation tests (PAT) were performed in an APACT 4 aggregometer (ELITech Group). PAT were performed with 15µL of HIT plasma, or with 18 and 36 µg/mL of 5B9, both in the presence of 0.5 IU/mL of heparin. In addition, a PAT was also performed with 0.5 µg/mL of collagen.

In selected experiments, citrated plasma of a healthy donor was depleted of endogenous IgG using a Hitrap protein G column (GE healthcare, Life Sciences). Washed platelets were then suspended in autologous IgG-depleted plasma at a final count adjusted to 350x10⁶/mL to obtain IgG-depleted PRP. PAT were performed using 5B9 (18µg/mL) and heparin (0.5 IU/mL) with IgG-depleted PRP in the absence or presence of different concentrations of polyclonal IgG (i.e. 1, 2, 3, 4, 5 and 10 mg/mL), or monoclonal IgG1 and IgG2 (1, 2 or 4 mg/mL).

Serotonin release assays (SRA) were performed as previously described.
Flow cytometry assay of monomeric IgG binding to platelet FcγRIIA

MoAb IV.3 is a mouse IgG2b that blocks the binding of IgG to FcγRIIA\textsuperscript{23}. We therefore evaluated the ability of monomeric IgG to inhibit the binding of FITC-conjugated moAb IV.3 to FcγRIIA of platelets from HH or RR donors, using a procedure similar to an assay we had developed to evaluate IgG/FcγIIIA interactions\textsuperscript{24}. Briefly, platelets (5×10\textsuperscript{4} in 10µL) were incubated with varying concentrations of polyclonal IgG, IgG1 or IgG2 (30 min). Platelets were then incubated with FITC-conjugated IV.3 (1 µg/mL, 30 min) and analyzed by flow cytometry after adding 300 µL of PBS containing 1% BSA. The results were expressed as the percentage of inhibition of IV.3 binding: (% of IV.3 positive platelets in the absence of IgG - % of IV.3-positive platelets in the presence of IgG)×100/(% of IV.3-positive platelets in the absence of IgG).

Statistical analysis

Chi squared analysis was used to compare the genotypes and allele frequencies in HIT patients and controls. The Kruskall Wallis, Mann Whitney U and t tests were performed to analyze the aggregation parameters and the data obtained after whole blood stimulation in relation to the FcγRIIA H131R polymorphism. P values below 0.05 were considered significant.
RESULTS

The frequency of the FcγRIIA 131R isoform is higher in HIT patients with thrombosis

Genotypes and allele frequencies were similar in HIT patients and the two groups of controls (Ab\textsuperscript{neg} and Ab\textsuperscript{pos}) (Figure 1A). However, the 131R allele was more frequent in HIT patients with thrombotic complications compared to those without thrombosis (59% vs. 38%, respectively, p=0.012). Moreover, homozygous RR subjects were more numerous in HIT patients with thrombosis than in those without (34.5% vs. 8.5%, respectively, p=0.008). The FcγRIIA 131RR genotype therefore appeared to significantly increase the risk of thromboembolic complications in HIT (OR = 5.9; 95% CI 1.7-20).

On the other hand, FcγRIIA genotypes and allele frequencies were similar in patients with VTE or ischemic stroke and in healthy subjects (Figure 1B), supporting the hypothesis that the effect of the FcγRIIA H131R polymorphism on the risk of thrombosis in HIT was specific to HIT.

The potency of HIT antibodies to stimulate TF synthesis and induce procoagulant activity is higher in individuals homozygous for the FcγRIIA 131R isoform

A significant increase in TF mRNA levels was found for all donors tested after HIT plasma samples had been added to whole blood with heparin (0.5 IU/mL), compared to the experimental conditions without heparin (median increase in TF mRNA level = 4.2-fold). Interestingly, this relative increase in TF mRNA level was greater after stimulation of whole blood from homozygous FcγRIIA RR donors (median value = 5.6-fold vs. 3.1-fold for HH donors, p=0.02, Figure 2A). This result was also confirmed after addition to whole blood of 5B9 and heparin with a 7.5-fold increase in TF mRNA levels in RR individuals compared to 4.5 and 2.9-fold in the HR and HH groups, respectively (p=0.003, Figure 2B). Noticeably, TF
gene expression was also strongly inhibited when HIT plasma was co-incubated with IV.3 (Figure S2).

5B9 with heparin also induced a procoagulant activity dependent on phospholipid microparticles that was variable according to the FcγRIIA polymorphism. Therefore, the relative shortening of plasma clotting time measured was greater in RR subjects than in the other genotypes (median decrease in clotting time = 46.2% vs. 31.7% and 28.1% in RR, HR and HH groups, respectively, p=0.007, Figure 2C).

The FcγRIIA H131R polymorphism influences platelet activation induced by HIT antibodies only in the presence of plasma

To investigate the influence of FcγRIIA H131R polymorphism on the platelet response to HIT antibodies, we first performed SRA, and the release measured was identical when washed platelets from healthy donors homozygous for the 131R and 131H FcγRIIA isoform were incubated with HIT plasma and heparin (Figure 3A). PAT were then performed with 70 healthy donors and similar results were also obtained according to the FcγRIIA H131R polymorphism after activation of washed platelets by HIT antibodies (Figure 3B). In contrast, when PAT were performed with PRP, the lag time measured was shorter in healthy donors homozygous for the FcγRIIA 131R isoform compared to that of HH and HR individuals (median = 201 vs. 287 and 310 sec respectively, p= 0.025).

A wide variability in platelet response to 5B9 and heparin was also evidenced in PRP, particularly when the lower concentration of antibody (18µg/mL) was tested, with an absence of aggregation in 23 of the 70 donors studied. Importantly, 22 of non-responders to 5B9 were carriers of the FcγRIIA H allele and only one was an RR homozygote (Figure 3C). In addition, the lag-time measured with 5B9 was shorter when the PRP from RR subjects was tested (median = 471 vs. 750 sec in carriers of the H allele, p = 0.02). However, this apparent
influence of the FcγRIIA H131R polymorphism in PRP was no longer visible when a higher concentration of 5B9 (36µg/mL) was used (data not shown). On the other hand, the lag-times measured in response to 18µg/mL of 5B9 were similar when PAT were performed with washed platelets whatever the FcγRIIA H131R genotype (Figure 3C). Finally, and as expected, no differences were also found when PAT were performed with collagen (0.5 µg/mL), both with PRP and washed platelets (Figure 3D).

These results together strongly supported the hypothesis that the FcγRIIA H131R polymorphism influences the platelet response to HIT antibodies, but this effect required the presence of plasma components, and an influence of endogenous IgG was therefore hypothesized.

*Endogenous immunoglobulin subclasses differentially modulate platelet activation induced by HIT antibodies according to the FcγRIIA H131R polymorphism*

No differences were found regarding total IgG and IgG subclass levels in any subgroup compared to others, both in healthy donors and in HIT patients (Figure S3). In particular, levels of IgG2 that corresponded to about 35% of total IgG (versus approximately 48% of IgG1) were similar in homozygous donors for the H and R alleles.

When the platelet response to 5B9 of IgG-depleted PRP from FcγRIIA HH and RR donors was evaluated, the lag times obtained in both groups were shorter than with native PRP and identical to those measured with washed platelets (Figure 4A and B). As expected, no effect of IgG depletion on the platelet response to collagen in PRP was observed (Figure S4A). The addition of polyclonal immunoglobulins to IgG-depleted PRP before performing PAT resulted in a dose-dependent increase in the lag time, and the platelet response to 5B9 was similar to those obtained with PRP when 5 mg/ml or 10 mg/ml of IgG were present (Figure 4C and D). In addition, this effect of polyclonal IgG, that we also confirmed with PAT performed using human HIT plasma (Figure S5), was more obvious with HH platelets
than in RR donors. Indeed, the lag times were significantly longer with HH platelets than with RR cells as soon as 3 mg/ml of polyclonal IgG were added to IgG-depleted PRP (mean value = 996 vs. 412 sec, p= 0.015) (Figure 4E). Moreover, the platelet response to 5B9 was fully abolished with 4 or 5 mg/ml of polyclonal IgG only in HH donors.

On the other hand, the addition of monoclonal IgG1 to IgG-depleted PRP at concentrations similar to those usually present in normal plasma resulted in a similar prolongation in the lag time of HH and RR platelets in response to 5B9 (Figure 4F). In contrast, IgG2 strongly inhibited the response to 5B9 only of platelets expressing the H isoform, with a dose-dependent increase in lag time, whereas no effect was observed on RR platelets (Figure 4F).

As expected, neither polyclonal IgG nor monoclonal IgG1 and IgG2 modified the platelet response to collagen according to the FcγRIIA H131R polymorphism (Figure S4B).

Finally, competitive flow cytometry assays were performed to evaluate the ability of monomeric IgG1 and IgG2 to bind FcγRIIA receptors. As shown in Fig 4G and 4H, a dramatic inhibition of mAb IV.3 binding to both HH and RR platelets (# 90% and 70%, respectively) was achieved by 2.5 mg/mL of either polyclonal human IgG or monoclonal IgG1. By contrast, when monoclonal IgG2 was added at similar concentration, a significant inhibition of IV.3 binding to platelets was obtained only with HH platelets (# 60%) but not with RR platelets (<15%, Figure 4H). These findings indicated that HH platelets bind both monomeric IgG1 and IgG2 when present at physiologic concentrations, while RR platelets exclusively bind monomeric IgG1.
DISCUSSION

Given the central role of FcγRIIA in the pathogenesis of HIT, several studies have investigated the influence of the H131R polymorphism on the risk of HIT but no association was demonstrated\textsuperscript{17}. The present study confirmed this conclusion, but also showed that the frequencies of the R allele and of the homozygous RR genotype were significantly higher in HIT patients with thromboembolic complications than in those with thrombocytopenia alone. Only three studies had previously investigated the influence of the FcγRIIA H131R polymorphism on the risk of thromboembolic complications in HIT. In the first, 36 patients (including 23 with thrombosis) were studied and no association was found\textsuperscript{14}. Conversely, Carlsson et al. found in a larger study that the frequency of the 131RR genotype was over-represented in patients with thrombotic events\textsuperscript{16} (with a rate of 37%, similar to the 34.5% found in the present study). Scarparo et al. had also reported an increased frequency of the FcγRIIA R/R genotype in HIT patients with thrombosis, but this association was significant only when combined with HPA-1a/b or PECAM1-V/V125 gene polymorphisms\textsuperscript{25}.

The H131R substitution is located in the extracellular domain of FcγRIIA, which interacts with the Fc fragment of IgG and does not influence the expression level of this receptor on the platelet surface\textsuperscript{12}. On the other hand, FcγRIIA appears to be physiologically important for α\textsubscript{IIb}β\textsubscript{3}-mediated outside-in signaling and thrombus formation\textsuperscript{26}. However, our results failed to suggest any prothrombotic effect of the R allele in homozygous patients with a stroke or venous thromboembolism, and therefore the impact of the FcγRIIA H131R polymorphism on the risk of thrombosis seems to be very specific to HIT.

HIT is a particular clinical prothrombotic syndrome, and Carlson et al. hypothesized that IgG/PF4/heparin IC might be less efficiently removed from the circulation of patients with the FcγRIIA 131RR genotype, thereby prolonging cell activation and increasing the risk of thrombosis\textsuperscript{16}. However, HIT antibodies to PF4/H are predominantly IgG1\textsuperscript{14}, and only
marginal differences in the affinity of human IgG1 IC to the H and R allotypes of FcγRIIA have previously been reported. Moreover, no significant difference in circulating anti-PF4/H antibody levels in relation to the FcγRIIA polymorphism was evidenced in our cohort (data not shown). We therefore postulated that the greater risk of thrombosis in HIT patients with the RR genotype was related to more efficient cellular activation by HIT antibodies, independently of the clearance of IC.

Thrombotic complications in HIT result from multicellular activation that involves platelets, monocytes, neutrophils and endothelial cells, with an important role of tissue factor and procoagulant microparticles in triggering and amplifying the coagulation cascade. Accordingly, a significant increase in TF gene expression was measured when plasma samples from HIT patients were incubated in whole blood from healthy donors with heparin, and this effect was stronger with 5B9, a chimeric IgG1 antibody to PF4/H complexes that mimics human HIT antibodies. More importantly, 5B9 and human HIT antibodies stimulated TF gene activity more effectively when added to the whole blood of donors homozygous for the FcγRIIA R allele. In addition, the phospholipid procoagulant activity (PPA) evaluated by measuring the plasma clotting time of these individuals was greater after activation by 5B9 and heparin, this effect probably related to the microparticles produced by activated platelets and monocytes. The monocyte activation by PF4/H/IgG IC can be direct or indirect, involving platelet-monocyte interactions, but Kasthury et al. demonstrated that direct activation of monocytes by HIT antibodies mainly depended on FcγRI receptors, without a significant role of FcγRIIA. However, our results showing that TF gene expression after stimulation in whole blood was inhibited by IV.3, strongly supported a major contribution of FcγRIIA receptors in cell activation induced by HIT antibodies. It has been recently shown that monocytes preferentially bind HIT IC because of the higher affinity of monocyte surface glycosaminoglycans for PF4 compared to platelet chondroitin sulfate molecules. However,
monocytes express only 5% of the total amount of FcγRIIA receptors in whole blood\textsuperscript{30}, while 80% are present in circulating platelets\textsuperscript{31}, and about 15% in neutrophils\textsuperscript{32}. The activation of monocytes in HIT might therefore also depend \textit{in vivo} on platelet activation induced by IgG/PF4/H complexes. In this regard, PAT clearly demonstrated that the FcγRIIA H131R polymorphism influenced the platelet response to HIT antibodies. However, significant differences with shorter lag times were visible only when PAT were performed with PRP, and not with washed platelets. Moreover, this variation in lag time, likely reflecting a variable capability of HIT IC to crosslink FcγRIIA receptors and activate platelets, disappeared when the PRP tested was IgG-depleted, or when PAT were performed with a higher concentration of 5B9 (i.e. 36 μg/ml). These results therefore suggested that normal plasma IgG were competing with 5B9, with an influence of the FcγRIIA dimorphism that was no longer present when the amount of HIT antibodies was too high. Furthermore, the lag time obtained with IgG-depleted PRP was always similar to those measured with washed platelets from the same donor after activation by 5B9. Finally, the addition of polyclonal IgG to IgG-depleted samples partly inhibited the platelet response to 5B9 and HIT human antibodies, but this effect was more pronounced with platelets expressing the 131H isoform. These findings are also in agreement with previous studies showing that addition of high concentrations of polyclonal IgG to washed platelets inhibited platelet activation and aggregation induced by HIT plasma\textsuperscript{33,34}, and with the fact that few HIT patients have been successfully treated by IV infusion of immunoglobulins\textsuperscript{35,36}. The plasma IgG fraction therefore appeared to be critical in contributing to the variations in platelet response to HIT antibodies according to the FcγRIIA polymorphism. Moreover, this effect was probably independent of the plasma levels of total IgG and IgG subclasses, which were similar in the healthy donors tested whatever their FcγRIIA genotype.
The H and R allotypes of FcγRIIA bind differentially IgG2, which represent about 35 to 40% of total IgG present in polyclonal IgG and plasma samples used in our study, but they interact similarly with IgG1 and IgG3 IC8. We therefore assumed that the difference in platelet response in PRP according to the FcγRIIA polymorphism was mainly related to the plasma IgG2 subclass. However this hypothesis presupposed that monomeric IgG present in the plasma can bind efficiently to FcγRIIA, although this interaction has been considered to be minimal8. Our results obtained with aggregation tests and flow cytometry analysis unambiguously demonstrated that monomeric IgG2 bound efficiently to the H allotype at physiological concentrations and decreased the platelet activation induced by HIT antibodies. On the other hand, monomeric IgG1 bound efficiently to both H and R allotypes, with similar inhibition of HIT antibody-mediated platelet activation.

These results therefore strongly support the concept that, in addition to IgG1, endogenous IgG2 (these two classes corresponding to >90% of total plasma IgG) may reduce the avidity of HIT immune complexes on the surface of platelets (and maybe of monocytes) and subsequent cell activation in patients expressing the 131H isoform, thereby protecting them from thrombosis. However, since IgG2 is unable to bind to the FcγRIIA R allotype, the modulation of platelet activation by HIT antibodies in RR patients can only depend on IgG1 (corresponding to about 55% of total plasma IgG), therefore contributing to greater pathogenicity of PF4-specific antibodies, with an increased risk of thrombosis (Figure 5). It can be postulated that, in addition to the variable intrinsic sensitivity of platelets to HIT IgG, other parameters such as the specificity and the concentration of anti-PF4/H antibodies or PF4 levels on the platelet surface, the plasma concentrations of IgG1 and IgG2 and FcγRIIA H131R polymorphism may be critical in modifying the risk of thrombosis in HIT patients. It would therefore be of interest to study these parameters in a large population of HIT patients but also in other medical situations associated with immune-mediated thrombocytopenia and
thrombosis such as primary APS, in which the FcγRIIA H131R polymorphism may also influence the pathogenic effects of IgG2 antibodies.

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AUTHORSHIP

JR performed and designed the research, analyzed the data and wrote the paper. CP, GT and YG, designed the research, analyzed the data, and wrote the paper. VGG performed research, analyzed the data, and wrote the paper. HCS, DL, and AS performed research and analyzed the data.

CONFLICTS OF INTERESTS

The authors declare there are no competing financial interests.

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LEGENDS TO FIGURES

Figure 1: Genotype and allele frequencies of FcγRIIA H131R polymorphism.

Panel A: Analysis of the three groups of patients (Ab\textsuperscript{neg}, Ab\textsuperscript{pos} and HIT). HIT patients were also stratified according to the presence (HITT) or absence (HIT) of thrombotic complications. No information was available about thrombotic events in 5 HIT patients.

Panel B: Analysis of H131R polymorphism in patients with venous thromboembolism (VTE) or stroke and in healthy subjects.

p value was calculated using the Chi-squared test. MAF = minor allele frequency.

Figure 2: Influence of FcγRIIA H131R polymorphism on tissue factor gene expression and procoagulant activity induced by HIT antibodies in whole blood.

Panel A: Relative increase in tissue factor (TF) levels measured by qPCR (see methods) after addition of HIT plasma and heparin (0.5 IU/mL) to the whole blood from 24 donors (HH n=12 and RR n=12), compared to the condition without heparin. Plasma samples from 5 HIT patients with (A\textsubscript{405nm} values between 1.6 and 3 in PF4-specific ELISA and positive SRA) were tested. p values were calculated using the Mann Whitney U-test.

Panels B and C: Relative increase in TF mRNA levels (B) and shortening of plasma clotting time (C) measured after addition to the whole blood of 20 donors (HH n=8; HR n=6; and RR n=6) of 5B9 (10 µg/mL) with heparin (0.5 IU/mL), compared to the condition without heparin. p values were calculated using the Mann Whitney U-test.

Figure 3: Influence of FcγRIIA H131R polymorphism on platelet activation induced by HIT antibodies.

Panel A: Serotonin release assay performed with washed platelets from FcγRIIA 131HH (n = 5) and RR donors (n = 5) incubated with HIT plasma sample that contained high titer of
activating PF4-specific antibodies ($A_{405nm} = 2.7$ in ELISA), and different concentrations of unfractionated heparin (UFH).

**Panels B, C and D:** Lag times measured with platelet rich plasma (PRP) and washed platelets (WP) from 70 healthy donors according to to FcγRIIA H131R genotypes (i.e. RR $n=15$; HR $n=32$; HH $n=23$). The median values are indicated as solid lines. Aggregation tests were performed with unfractionated heparin (0.5 IU/mL) and the HIT plasma tested in SRA as described above (panel B) or 5B9 (18 µg/mL) (panel C) or with collagen (0.5 µg/mL) (panel D).

The Mann Whitney U-test was performed to compare lag times in relation to FcγRIIA polymorphism and p values are displayed.

**Figure 4: Influence of normal IgG and monoclonal IgG1 or IgG2 on platelet response induced by 5B9 with heparin and relationship with the FcγRIIA H131R polymorphism.**

**Panels A and B:** Representative aggregation profiles obtained after addition of 5B9 (18 µg/mL) and heparin (0.5 IU/mL) to washed platelets (WP), platelet rich plasma (PRP) and IgG-depleted PRP from homozygous 131HH (A) and RR (B) donors. **Panels C, D, E:** Representative aggregation profiles (C, D) and mean lag times ($±$ 1 SEM) (E) obtained after addition of 5B9 (18 µg/mL) and heparin (0.5 IU/mL) with varying concentrations of polyclonal IgG to IgG-depleted PRP from homozygous 131HH (C) and RR (D) donors. The t-test was used to compare the data obtained in the 2 groups of donors. **Panel F:** Mean lag times ($±$ 1 SEM) measured after addition of 5B9 (18 µg/mL) and heparin (0.5 IU/mL) with varying concentrations of human monoclonal IgG1 (cetuximab) or IgG2 (panitumumab) to IgG-depleted PRP from homozygous donors FcγRIIA 131RR and 131HH. The t-test was used to compare the values obtained in the 2 groups of donors. *p<0.05 and ** p<0.01.
Panels G and H: Inhibition of moAb IV.3 binding (expressed in %) on HH and RR platelets by polyclonal IgG (G) and human monoclonal IgG1 or IgG2 (H). The binding of FITC-conjugated IV.3 on platelets from HH and RR donors in the presence of increasing concentrations of polyclonal human IgG or monomeric IgG1 (cetuximab) or IgG2 (panitumumab) was assessed by flow cytometry. The data are representative of flow cytometry analyses performed with 3 different pairs of HH and RR donors.

Figure 5: Proposed schematic representation of the interaction of HIT IgG and normal IgG subclasses with platelets according to the FcγRIIA H131R polymorphism.

Panel A: In FcγRIIA HH immunized patients, IgG2 (in green) and IgG1 (in dark blue) may bind to FcγRIIA receptors (in blue). This interaction thereby reduces the ability of HIT IgG (in red) associated with PF4/H complexes to bind via their Fc fragment either in cis (intraplatelet) or in trans (interplatelet) FcγRIIA receptors at sufficient extent to activate platelets.

Panel B: In FcγRIIA RR immunized patients, IgG2 are not able to bind platelet FcγRIIA receptors that therefore remain available to crosslink with a larger number of HIT IgG/PF4/H complexes. This results in more potent platelet activation with the release of larger amounts of phospholipid procoagulant microparticles. Together with enhanced synthesis of TF, this effect probably explains the higher risk of thrombosis in patients homozygous for the FcγRIIA R allele.
Figure 3

A

Maximum of platelet activation (%)

<table>
<thead>
<tr>
<th>Heparin (IU/mL)</th>
<th>RR (n=7)</th>
<th>HH (n=7)</th>
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<tbody>
<tr>
<td>0</td>
<td></td>
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<tr>
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<tr>
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<tr>
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</tbody>
</table>

B

HIT plasma

Lag time (sec)

<table>
<thead>
<tr>
<th>PRP</th>
<th>RR</th>
<th>HR</th>
<th>HH</th>
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</thead>
<tbody>
<tr>
<td>WP</td>
<td>RR</td>
<td>HR</td>
<td>HH</td>
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</tbody>
</table>

C

5B9

Lag time (sec)

<table>
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<th>HH</th>
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</thead>
<tbody>
<tr>
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<td>RR</td>
<td>HR</td>
<td>HH</td>
</tr>
</tbody>
</table>

D

Collagen

Lag time (sec)

<table>
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<th>HH</th>
</tr>
</thead>
<tbody>
<tr>
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<td>RR</td>
<td>HR</td>
<td>HH</td>
</tr>
</tbody>
</table>
Figure 5

HH: Lower risk of thrombosis

RR: Higher risk of thrombosis

Symbols: IgG1, IgG2, HIT Ab, PF4, Heparin, Microparticles, FcγRIIA
Increased risk of thrombosis in FcγRIIA 131RR patients with HIT due to defective control of platelet activation by plasma IgG2

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