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Acute thrombocytopenia after liver transplant: Role of platelet activation, thrombopoietin deficiency and response to high dose intravenous IgG treatment☆

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***Background/Aims*: Thrombocytopenia is common after liver transplantation due to platelet sequestration secondary to hypersplenism. The aim of this study was to further investigate the causes of this condition, as well as the response of thrombocytopenia to high dose intravenous immunoglobulins.**

***Methods*: We retrospectively studied 73 patients who underwent liver transplantation. Out of these 73 patients, 27 had severe thrombocytopenia and were treated with high dose intravenous immunoglobulin. Additionally, we retrospectively studied 8 patients undergoing liver transplantation.**

***Results*: Our data suggest that splenomegaly is not the only factor responsible for thrombocytopenia after liver transplantion and two additional phenomena, namely, reduced platelet production due to reduced thrombopoietin level and sustained platelets activation take part in the pathogenesis of this condition. The infusion of high dose immunoglobulins induced a safe, prompt, complete and persistent resolution of severe thrombocytopenia in more than 70% of patients.**

***Conclusions*: Based on these findings, treatment with high dose intravenous immunoglobulins should be considered in the management of severe thrombocytopenia after liver transplant, although additional randomized trials are warranted.**

***Keywords*: Platelet; Liver transplant; Thrombocytopenia**

1. Introduction

Thrombocytopenia is a common complication among liver transplant recipients [1]. Early after orthotopic liver transplantation (OLT), thrombocytopenia affects ~90% of patients and it results in an average 60% reduction in platelet count [1–4]. Thrombocytopenia peaks 4–5 days following surgery and the platelet count returns to preoperative levels 2–3 weeks after transplant [5]. Nearly 8% of OLT patients suffer from severe thrombocytopenia with platelet count lower than $20 \times 10^3/\mu L$. Moderate and severe thrombocytopenia in the post-OLT setting limits diagnostic assess-

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ments and therapeutic options and, most importantly, constitutes an important risk factor for major bleeding episodes.

Although thrombocytopenia is a frequent complication in OLT patients, little is known about its pathogenesis [6–11]. The aim of this study was to evaluate the relative contribution of reduced bone marrow production and peripheral consumption to thrombocytopenia in OLT patients. Moreover, we wanted to assess the impact of high dose intravenous immunoglobulin (HD-IVIG) treatment on acute thrombocytopenia.

Herein we retrospectively analyzed 73 OLT recipients. This group included 27 patients affected by severe thrombocytopenia ($<15\times10^3/\mu L$) and treated with HD-IVIG (Group A), and the remainders 46 patients with platelet counts above $20\times10^3/\mu L$, which were left untreated (Group B).

Through an extensive serial biochemical profiling in an additional group of 8 consecutive patients we conclude that this particular type of thrombocytopenia is the combined result of persistent reduced systemic thrombopoietin levels in the presence of chronic platelet consumption exacerbated by the surgical procedure.

2. Patients and methods

2.1. Patients and OLT

The retrospective analysis was conducted on a cohort of 73 patients with end-stage liver disease. Patients underwent liver transplant between January 1996 and December 1997 at the Centro Trapianti di Fegato, Niguarda Hospital, Ca'Granda Milan, Italy. OLT was performed according to standard surgical and anesthesiologic procedures with veno-venous bypass in 70% of cases. Graft preservation was attained using University of Wisconsin solution. Immunosuppressive therapy included anti-thymocyte immunoglobulins (Thymoglobulin Mérieux Pasteur, 1.5 mg/kg/day from post-operative day (POD) 1 to POD5, methylprednisone at tapering dose (initial dose 1 mg/kg), azathioprin (1.5 mg/kg per day for 30 days), Cyclosporine A (Sandimmun Neoral, Novartis) aiming at C_0 level within 200 and 350 ng/mL (6 mg/kg/day in two doses). In 27 patients with severe thrombocytopenia (<15,000 platelets/μL), high dose intravenous immunoglobulins (HD-IVIG, Sandoglobulin, Norvartis), 0.4 g/kg/day, were also administered for 5 consecutive days starting at POD3.

The prospective study included 8 patients who underwent OLT from November 2000 to March 2001 with the procedure described. Tests were performed prior to admission up to POD15 and included cell blood count with differential, coagulation panel (PT, PTT, TT, TR, AT III, FDP, DD, bleeding time), anti-platelet antibodies (at baseline prior to OLT, POD3, and POD8), von Willebrand factor (every other day), functional platelet tests (daily), three-color cytofluorimetric analysis of platelet function and activation with ADP (PAC-1 FITC, CD 62PE, CD61 PerCP), and flow cytometric assessment of reticulate platelet count (daily). Data collected from the surgical patients were then compared with those of two control groups, cirrhotic patients waiting to be transplanted and normal healthy volunteers. The cirrhotic group included 18 patients with HCV-related liver failure, Class Child C, with platelets <100,000/μL in the absence of any known primary or systemic neoplastic disease. The second control group was composed of 7 healthy subjects, who had not been subjected to blood transfusion or collection in the previous month and did not assume NSAID in the previous 2 weeks.

Informed consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in an *a priori* approval by the Niguarda Ca' Granda Hospital Human Research Committee.

2.2. Reticulated platelet count

Reticulated platelet identification and count were performed in peripheral blood. Plasma platelets enriched (PRP) was obtained by spontaneous sedimentation (30 min at room temperature). Cell pellets previously fixed in 1% paraformaldehyde were incubated with 1 mL Trizol (ReticCount®, Becton–Dickinson, Milan) then analyzed (FACSCalibur, Becton–Dickinson) [12]. The total number of reticulated platelets was measured and their percentage with respect to total platelets was computed.

2.3. Platelet activation studies

Platelet activation test was performed in blood samples no later than one hour from sampling. Platelets were stained and analyzed in basal conditions and after ADP exposure for anti-CD61 as specific surface marker, CD62P as activation-dependent surface marker of platelets and PAC1 ligand-mimetic monoclonal antibody that binds only to the activated isoform of GPIIb–IIIa in a manner similar to the physiological ligand (Becton–Dickinson, Milan).

2.4. Anti-platelet antibodies

The presence of anti-platelet antibodies (Allo-antibodies, Auto-antibodies, and Antibodies anti-Glycoprotein) was established in samples of plasma collected at pre-OLT, POD3, and POD8. The immunoenzymatic assay PAK PLUS (GTI Opera, Milan) was employed for antibody detection according to manufacturer's instruction and results interpreted according to current guidelines [12]

2.5. Thrombopoietin (TPO) levels

TPO levels were measured at Pre-OLT, Post-OLT, POD3, POD6, POD8, and POD15 using ELISA in plasma samples collected in the presence of EDTA and stored at −20 °C (Quantikine® R&D, Minneapolis, USA).

2.6. Statistical analysis

Data relative to the retrospective study were expressed as means ± standard deviation (SD) ANOVA, test χ^2 and regression analysis were employed for the statistical analysis. Data relative to the prospective study were expressed as means ± standard deviation (SD), ANOVA and T-test, Paired T-test, Fisher Exact Test have been utilized for the statistical analysis.

3. Results

3.1. Retrospective analysis

In 73 patients undergoing OLT, mean platelet count before surgery was $46\pm25\times10^3/\mu L$. In 4% of these patients, thrombocytopenia pre-OLT was severe (platelet count <15,000/μL) platelet count reduction was moderate (platelet count between 20,000 and 50,000/μL) in 51%, and mild (platelet count between 50,000 and 100,000/μL) in 37% of the patients. At this time, only 8% of the patients had a platelet count greater than 100,000/μL and 83% of the patients had an enlarged

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spleen at physical examination. When compared to pre-OLT values, mean platelet count was reduced by 40% at POD1 ($25 \pm 13 \times 10^3$/μL, $P < 0.05$) and at POD3 ($24 \pm 13.5 \times 10^3$/μL, $P < 0.05$). At POD3, 37% of the patients ($n = 27$) had severe thrombocytopenia (Group A in our study, with an average platelet count of $16 \pm 6 \times 10^3$/μL), in spite of multiple platelet transfusions (8 platelet units/day on average). This group of patients received HD-IVIG at a mean daily dose of 0.4 g/kg/die for five consecutive days from POD3 to POD8. The remaining 63% of the patients ($n = 46$, Group B in our study) had a platelet count >20,000/μL and were left untreated.

The age of the patients and the etiology of liver failure were similar in the two groups (data not shown). At all time points, platelet levels were persistently lower in Group A when compared to the values in Group B. Importantly, in Group A the number of platelets at POD3 correlated significantly with platelet levels pre-OLT ($r = 0.50$, $P < 0.02$). Of relevance, the number of platelet units transfused in the peri-operative period did not differ in the 2 groups.

In blood smears collected from patients in both groups, schistocytes were not significantly increased, coagulation parameters were similar, and indirect bilirubin increase was not significantly altered, ruling out systemic microangiopathy or disseminated intravascular coagulation resulted in platelet consumption. Overall survival at six months was 86% (63/73) with early graft dysfunction [7] slightly more represented in Group A than Group B (55% versus 32%, $P = 0.09$).

Incidence of early infections (Group A: 66%; Group B: 54%) and acute rejection episodes (Group A: 22% ; Group B: 13%) were not significantly different in the two groups, ruling out infections and graft rejection as putative mechanisms responsible for severe thrombocytopenia in Group A.

Bone marrow aspirate was performed in 4 patients of Group A after OLT documented megakaryocytic hyperplasia.

The response to treatment with HD-IVIG varied in the patients of Group A; 74% of patients showed a complete response to therapy as documented by the platelet count that was greater than 100×10^3/μL at the time of hospital discharge with only one death at POD180 among these responders while 3 other patients, with partial response to therapy, died between POD 10 and 70 due to multiple organ dysfunction syndrome, spontaneous intracranial hemorrhage in presence of *S. aureus* sepsis and autoptic diagnosed linfoma, respectively.

3.2. Prospective analysis: clinical data and platelet turnover

The systematic evaluation of the hemostatic profile was conducted in eight liver transplant recipients with thrombocytopenia. Two control groups were employed: cirrhotic patients that did not receive liver transplant and healthy subjects. At the time of the admission in the intensive care unit mean platelet count was $58 \pm 19 \times 10^3$ cells/μL. Changes in platelet count over time are shown in Figs. 1 and 2a. One patient developed severe thrombocytopenia at POD7, 10×10^3 cells/μL, and, following 5-day treatment with HD-IVIG, platelet count increased to 100×10^3 cells/μL. In all cases, patients received platelet transfusions of 3–49 units in the intra-operative and post-surgical periods. In the cirrhotic control patients (no-OLT), mean platelet count was $84 \pm 36 \times 10^3$ cells/μL, and, in normal subjects, $290 \pm 15 \times 10^3$ cells/μL.

Platelet production was determined by flow cytometry and data are shown in Figs. 1 and 2. In cirrhotic control patients (no-OLT), the total reticulated platelet (RP) count was $1.3 \pm 1.2 \times 10^3$ RP/μL and the fraction of RPs accounted for $1.6 \pm 1.1\%$. Corresponding values in normal subjects were $5.8 \pm 1.5 \times 10^3$ RP/μL and <2% [10]. In transplant candidates, RP count was $1.03 \pm 0.7 \times 10^3$ RP/μL, which represented $2 \pm 1\%$ of total platelets. After liver transplant, the changes in both RP number and percentage followed a bimodal pattern of increases, with an early significant increase ($2.6 \pm 1.5 \times 10^3$ cells/μL, $6.3 \pm 4\%$) at post-OLT, and a late increase ($4.9 \pm 2.8 \times 10^3$ cells/μL, $6.1 \pm 2.9\%$) at POD8. Nadir ($1 \pm 0.6 \times 10^3$ cells/μL; $3 \pm 1.6\%$) was reached at POD2 and values returned to normal at POD15 ($4.0 \pm 3.0 \times 10^3$ cells/μL; $1.8 \pm 1.5\%$; $P = 0.235$ with respect to healthy subjects).

3.3. Thrombopoietin (TPO) levels

Megakaryocyte mass was determined indirectly by thrombopoietin levels. TPO concentration was mea-

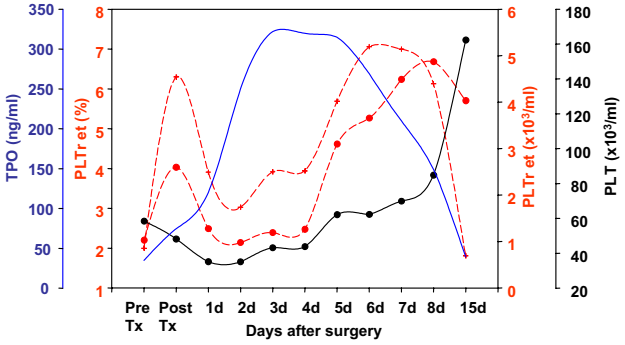

Fig. 1. Platelet counts, reticulated platelet counts and thrombopoietin levels. Platelets (PLT, black circles), absolute number of reticulated platelets (PLT ret, red circles), percentage of reticulated platelets (red crosses) were measured in peripheral blood from 8 patients 1 h before the beginning of the surgical procedures, (PreTx) at the admission in the ICU (PostTx) and at the indicated time points after surgery. Results are expressed as mean values. Thrombopoietin levels (ng/mL, blue lines) were measured in the serum of the same patients at the same time points. [This figure appears in colour on the web.]

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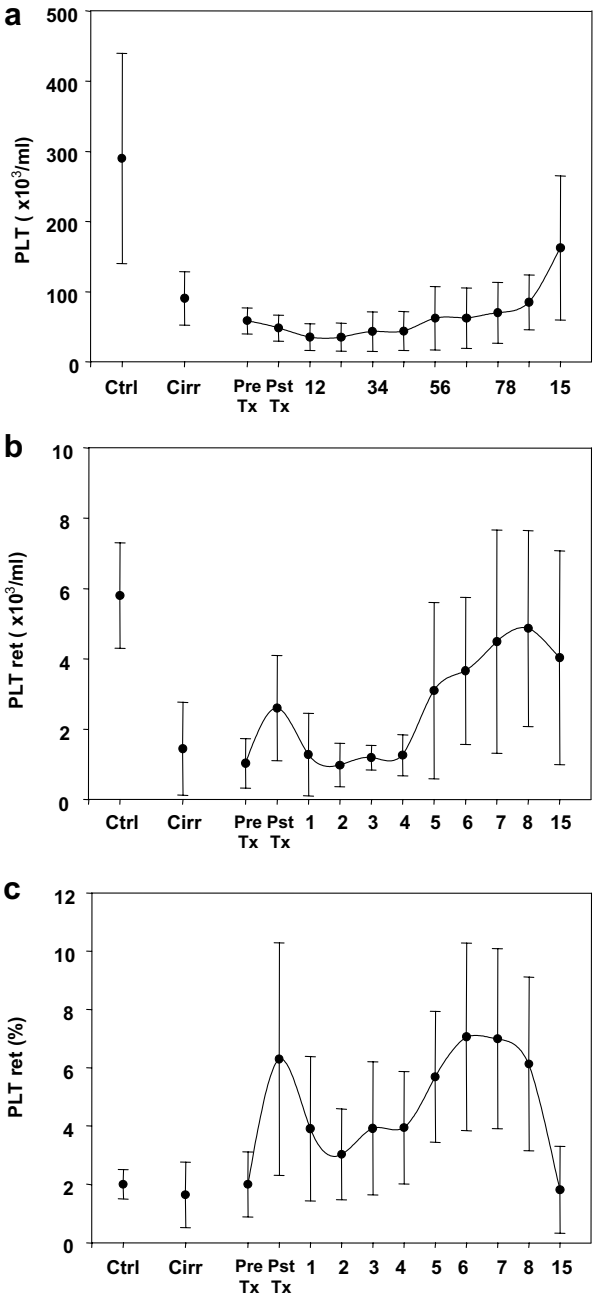

Fig. 2. Platelets, reticulated platelet count in normal controls, cirrhotic controls and time course evaluation in transplanted patients. Platelets (PLT, a), absolute reticulated platelets (PLT ret, b), percentage of reticulated platelets (PLT ret, c) were measured in peripheral blood in healthy controls (see Section 2), cirrhotic patients and transplanted patients, at the indicated time points after surgery. Results are expressed as mean values ± SD.

sured in the peripheral blood of eight surgical patients (Fig. 1). At pre-OLT, median TPO levels were reduced (34.5 ± 14.5 pg/mL). TPO level rose more than 2-fold immediately after surgery (post-OLT) with respect to pre-OLT, but the difference was not statistically significant ($P = 0.154$). TPO level peaked on POD3, when a

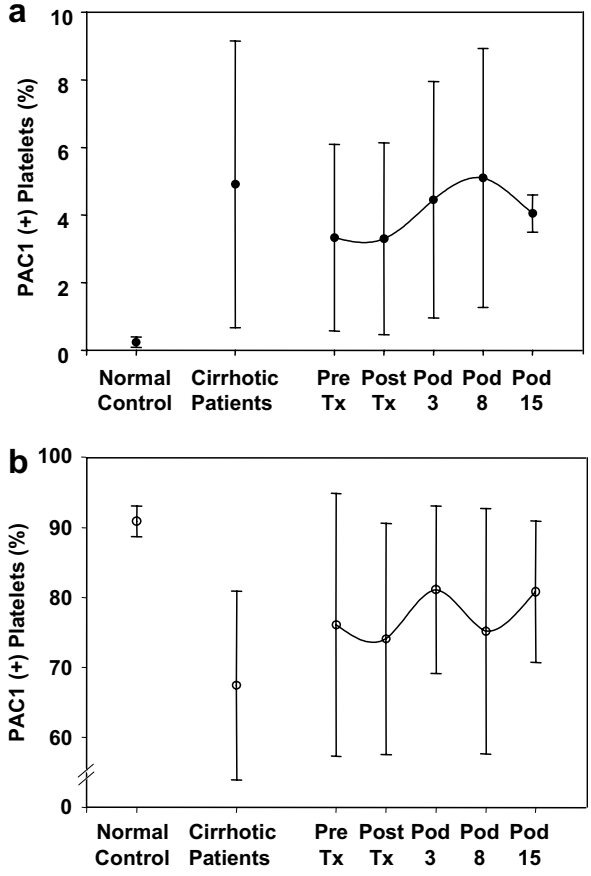

Fig. 3. Platelet activation ex vivo or upon in vitro ADP stimulation. Platelet activation in the same group of patients as described in the legend to Fig. 2 was monitored by measuring the percentage of platelets expressing PAC1 (see Section 2) ex vivo (a) or upon in vitro ADP stimulation (b). Results are expressed as mean values ± SD.

nearly 10-fold increase with respect to pre-operative levels was found ($P = 0.004$), and returned to normal values by POD15.

3.4. Platelet activation state

Blood samples were analyzed by flow cytometry using monoclonal antibodies for CD62 and PAC1. In normal subjects, baseline platelet activation was extremely low; PAC1 binding was observed in $0.24 \pm 0.15\%$ of the platelets present in the blood sample (Fig. 3a) with a mean expression of PAC1 equal to 2.8 ± 0.5 Linear Units (LU). Following exposure to ADP, the fraction of activated platelets increased to $91 \pm 2\%$ (Fig. 3b) and mean expression PAC1 reached 106 ± 15 LU. Non-transplanted cirrhotic patients showed dim PAC1 binding in $5 \pm 4\%$ of platelets (Fig. 3a) and the fluorescence intensity was 3.4 ± 0.7 LU. In the presence of ADP, the fraction of platelets capable of binding PAC1 increased to $67 \pm 14\%$ (Fig. 3b) with a mean fluorescence intensity equal to 56 ± 13 LU.

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In transplanted patients before surgery, basal PAC1 binding involved $3.3 \pm 2.76\%$ platelets (Fig. 3a) with a mean fluorescence intensity of 3.1 ± 0.7 LU. After ADP exposure, $76 \pm 19\%$ platelets were PAC1 binding (Fig. 3b) with a fluorescence intensity of 73 ± 28 LU. After organ transplant, basal platelet activation was sustained and changed as a function of time (range 3.3–5.1%; Fig. 3a), as well as the reduced response to ADP exposure (range 74–81%, range 59.9–72.0; Fig. 3a and b).

Although the fraction of basal activated platelets was much higher in cirrhotic patients with respect to healthy controls ($P = 0.027$), the degree of activation measured as fluorescence intensity of PAC1 did not differ in the two groups ($P = 0.06$). However, platelet reactivity to ADP was much lower in cirrhotic patients than in healthy individuals. In this regard, the fraction of ADP-responsive platelets decreased by 26% in cirrhotic patients with respect to normal controls ($P = 0.002$) and the fluorescence intensity of PAC1 binding by 42% ($P = 0.001$). The attenuated response to ADP challenge points to the reduced functional competence of platelets in cirrhotic patients. Conversely, transplanted patients showed an 8-fold increase in basal activated platelets with respect to healthy subjects ($P = 0.027$). This difference was not associated with changes in the level of expression of PAC1 that was similar in the two groups. In transplanted patients, the expression of PAC1 in response to ADP was significantly lower than in controls (-31%, $P = 0.032$).

4. Discussion

Our study included the largest number of patients in which post-liver transplant thrombocytopenia was treated with HD-IVIG.

Although this trial requires further confirmation because of its non-randomized nature, this study strongly indicates that the infusion of high dose immunoglobulins induced a prompt, complete and persistent resolution of severe thrombocytopenia in more than 70% of patients. Moreover, the safety of this treatment is evident; in this regard, the type and prevalence of early post-surgical complications were comparable to those found in control patients.

The etiopathogenesis of this form of thrombocytopenia is still controversial. The laboratory and clinical evidence collected in our work excluded that infection, myelosuppression secondary to drugs and autoimmunity played a significant role in the onset of this defect. Our data suggest that the combination of chronic peripheral activation in the presence of a central maturation deficit is the primary condition responsible for the thrombocytopenia post-liver transplant.

Absence of antibodies directed against anti-human platelet antigens (HPA), anti-major histocompatibility complex (MHC) class I and against cardiolipine ruled out autoimmunity and alloimmunization.

Additionally, hepatitis C virus (HCV) has been previously reported as a cause of immune thrombocytopenic purpura (ITP): however this pathogen was equally represented in both groups and anti-platelet antibodies were absent [9].

Four bone marrow aspirates were performed and, in all cases, marked megakaryocytic hyperplasia was detected ruling out drug induced myelosuppression.

Rates of early infection and acute rejection episodes were similar in the two groups, excluding infection as a putative mechanism responsible for this specific condition.

Conversely, the transplanted patients studied here clearly demonstrated that platelet production/maturation was defective together with constitutive platelet activation and consumption.

Peripheral consumption could not be attributed to microangiopathic phenomena for the absence of abnormally high von Willebrand polymers (data not shown), schistocytes and normal hematologic profile in both groups. However, cirrhotic patients from the surgical group and end-stage liver disease control group had abnormally high levels of platelet activation (i.e. consumption) coupled with a reduced functional competence. These two conditions were documented by the increased levels of PAC1 and CD62-P in resting mature platelets and by the reduced levels of the same antigens after ADP-induced activation [13].

Basal platelet activation and impaired platelet reactivity were also present and persisted during the entire hospitalization period [14,15].

The mechanisms that trigger platelet activation in cirrhotic and post-OLT patients are multiple and complex. Several processes result in platelet activation in end-stage liver failure, including vascular shunts, microcirculation alteration, platelet membrane abnormalities dictated by the disarray of lipoprotein metabolism [16]. Recent studies using mouse models of acute viral hepatitis – the most frequent cause of cirrhosis in our cohort of patients – show that platelets become activated in the inflamed liver [17]. Whether similar mechanisms occur in chronically infected patients and contribute to peripheral platelet consumption remains to be determined.

The failure of the bone marrow to efficiently produce and mobilize platelets during this thrombocytopenic status could be related to depressed levels of TPO observed in end-stage liver failure. TPO increase could sustain platelet turnover following liver transplant [10,11,18,19] as demonstrated here by RP absolute number and percentage which showed a parallel pattern of changes with an early peak in the peri-operative time and a second peak at POD6-POD8, followed by a progressive RP decrease. The post-OLT peak in TPO levels

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preceded by two days the appearance of RPs and by four days the normalization of platelet count [20,21].

Interestingly, the early post-operative peak in RP production and release could be associated to the intra-operative massive transfusions of plasma from healthy donors which contained physiologic concentration of TPO and therefore was capable of rapid induction of maturation of hyperplastic megakaryocytic marrows. Based on these findings, erythropoietin [22] or newer TPO agonists, i.e. AMG531 currently in phase 2 clinical trial [23], could have a role in the management of platelet disorders in end-stage liver disease [12,24–29].

The HD-IVIG correction of the thrombocytopenia is remarkable. Several mechanisms have been proposed to account for the action of HD-IVIG [30,31]. Our data point towards competitive blockade of Fcγ receptor on macrophages with subsequent reduced platelet clearance and/or attenuation of complement-mediated damage associated with ischemia/reperfusion of the graft. The molecular mechanism by which activated platelets are cleared from the circulation, however, remains to be determined. Even though less efficient, platelets remain effective [32]. By interfering with platelet clearance, HD-IVIG sustained total platelet count preventing the sudden and persistent thrombocytopenia in liver transplant patients until de novo TPO was synthesized by the newly grafted liver.

In conclusion, these observations provide clinical and experimental evidence for the efficacy of IgG in sustaining platelet count, possibly by interfering with the removal of pre-activated platelets while bone marrow normalizes platelet peripheral levels in response to exogenous transfusion-derived and de novo synthesized TPO.

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Informed consent was obtained from each patient included in the study and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in an *a priori* approval by the Niguarda Ca' Granda Hospital Human Research Committee.

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