Lentiviral gene therapy corrects platelet phenotype and function in patients with Wiskott-Aldrich syndrome

Lucia Sereni, PhD,[a] Maria Carmina Castiello, PhD,[a] Dario Di Silvestre, PhD,[b] Patrizia Della Valle, MS,[c] Chiara Brombin, PhD,[d] Francesca Ferrua, MD,[a,e,f] Maria Pia Cicalese, MD, PhD,[a,f] Loris Pozzi, MS,[c] Maddalena Migliavacca, MD, PhD,[a,f] Maria Ester Bernardo, MD, PhD,[a,f] Claudio Pignata, MD, PhD,[g] Roula Farah, MD,[h] Lucia Dora Notarangelo, MD,[i] Nufar Marcus, MD,[j,k,l] Lorella Cattaneo, MD,[m] Marco Spinelli, MD,[n] Stefania Giannelli, PhD,[a] Marita Bosticardo, PhD,[a]* Koen van Rossem, MD, PhD,[o] Armando D'Angelo, MD,[c] Alessandro Aiuti, MD, PhD,[a,e,f] Pierluigi Mauri, PhD,[b] and Anna Villa, MD[a,p] *Milan, Segrate, Naples, Brescia, Alessandria, and Monza, Italy; Beirut, Lebanon; Petach Tikva and Tel Aviv, Israel; and Brentford, United Kingdom*

Background: Thrombocytopenia is a serious issue for all patients with classical Wiskott-Aldrich syndrome (WAS) and X-linked thrombocytopenia (XLT) because it causes severe and life-threatening bleeding. Lentiviral gene therapy (GT) for WAS has shown promising results in terms of immune reconstitution. However, despite the reduced severity and frequency of bleeding events, platelet counts remain low in GT-treated patients.
Objective: We carefully investigated platelet defects in terms of phenotype and function in untreated patients with WAS and assessed the effect of GT treatment on platelet dysfunction.
Methods: We analyzed a cohort of 20 patients with WAS/XLT, 15 of them receiving GT. Platelet phenotype and function were analyzed by using electron microscopy, flow cytometry, and an aggregation assay. Platelet protein composition was assessed before and after GT by means of proteomic profile analysis.
Results: We show that platelets from untreated patients with WAS have reduced size, abnormal ultrastructure, and a hyperactivated phenotype at steady state, whereas activation and aggregation responses to agonists are decreased. GT restores platelet size and function early after treatment and reduces the hyperactivated phenotype proportionally to WAS protein expression and length of follow-up.
Conclusions: Our study highlights the coexistence of morphologic and multiple functional defects in platelets lacking WAS protein and demonstrates that GT normalizes the platelet proteomic profile with consequent restoration of platelet ultrastructure and phenotype, which might explain the observed reduction of bleeding episodes after GT. These results are instrumental also from the perspective of a future clinical trial in patients with XLT only presenting with microthrombocytopenia. (J Allergy Clin Immunol 2019;■■■:■■■-■■■.)

Key words: *Wiskott-Aldrich syndrome, X-linked thrombocytopenia, gene therapy, platelets*

Wiskott-Aldrich syndrome (WAS; OMIM 301000) is a severe X-linked primary immunodeficiency that affects 4 per million male births, with a life expectancy of less than 15 years in severe cases.[1,2] This syndrome is caused by mutations in the *WAS* gene encoding Wiskott-Aldrich Syndrome protein (WASp), a hematopoietic specific actin regulator.[3,4] Patients with the classical phenotype are characterized by microthrombocytopenia, immunodeficiency, eczema, and increased susceptibility to autoimmunity and malignancies. Patients presenting with only microthrombocytopenia without other clinical manifestations are referred to as having X-linked thrombocytopenia (XLT).[5,6]

Since its identification, WAS has been described as "familial and innate thrombopathia,"[7,8] and low platelet count with

From [a]San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), Division of Regenerative Medicine, Stem Cells and Gene Therapy, [c]the Coagulation Service & Thrombosis Research Unit, and [f]the Pediatric Immunohematology Unit, IRCCS San Raffaele Scientific Institute, Milan; [b]the Proteomic and Metabolomic Laboratory, Institute of Biomedical Technologies, National Research Council (ITB-CNR), Segrate; [d]the University Centre for Statistics in the Biomedical Sciences (CUSSB), Vita-Salute San Raffaele University, Milan; [e]Vita-Salute San Raffaele University, Milan; [g]the Pediatric Section, Department of Translational Medical Sciences, University of Naples Federico II, Naples; [h]the Department of Pediatrics, Division of Hematology–Oncology, Saint George Hospital University Medical Centre, Beirut; [i]the Pediatric Onco-Haematology and BMT Unit, Children's Hospital, ASST Spedali Civili of Brescia; [j]the Department of Pediatrics and [k]the Kipper Institute of Immunology, Schneider Children's Medical Center of Israel, Petach Tikva; [l]the Sackler Faculty of Medicine, Tel Aviv University; [m]SC Pediatria, Ospedale Infantile C. Arrigo, Alessandria; [n]the Pediatric Clinic, MBBM Foundation, Maria Letizia Verga Center, Monza; [o]the Rare Diseases Unit, GlaxoSmithKline, Brentford; and [p]the Milan Unit, Istituto di Ricerca Genetica e Biomedica, Consiglio Nazionale delle Ricerche, Milan.

*Marita Bosticardo, PhD, is currently affiliated with the Laboratory of Clinical Immunology and Microbiology, Immune Deficiency Genetics Section (IDGS), Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland.

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Corresponding author: Anna Villa, MD, San Raffaele Telethon Institute for Gene Therapy (SR-Tiget), Division of Regenerative Medicine, Stem Cells and Gene Therapy, IRCCS San Raffaele Scientific Institute, Milan, Italy. E-mail: villa.anna@hsr.it.

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Abbreviations used
ADP: Adenosine diphosphate
BAFF: B cell–activating factor
CD62P: P-selectin
CT: Closure time
FERMT3: Fermitin family homolog 3
FU: Follow-up
δ-g: Electron-dense granule
GPX1: Glutathione peroxidase 1
GT: Gene therapy
HD: Healthy donor
HMGB1: High-mobility group box 1
HSCT: Hematopoietic stem cell transplantation
LV: Lentivirus
MFI: Mean fluorescence intensity
OCS: Open canalicular system
PRP: Platelet-rich plasma
ROS: Reactive oxygen species
sCD40L: Soluble CD40 ligand
sCD62P: Soluble P-selectin
STAT3: Signal transducer and activator of transcription 3
TEM: Transmission electron microscopy
vWF: von Willebrand factor
WAS: Wiskott-Aldrich syndrome
WASp: Wiskott-Aldrich syndrome protein
XLT: X-linked thrombocytopenia

increased risk of bleeding still remains one of the most significant challenges. Platelet counts in patients range from less than 3,000 up to 70,000 platelets/μL, with a mean platelet volume half of that of healthy donors (HDs).[2,9] Bleeding events range from mild skin petechiae to life-threatening manifestations, such as gastrointestinal and intracranial hemorrhages, which account for about 23% of deaths.[10,11]

Severe thrombocytopenia often requires supportive treatment, including platelet transfusions. Splenectomy can be an effective treatment, especially for patients with XLT without autoimmunity[12]; however, this treatment carries a significant long-term risk of sepsis and is therefore not indicated for all patients, especially if a more definitive treatment is foreseen in the future.[9,13] Recently, new thrombopoietin agonists have been reported to be efficacious in preventing life-threatening hemorrhagic episodes.[14-16]

The current curative treatment for WAS is hematopoietic stem cell transplantation (HSCT) from an HLA-identical donor, which is available only for a minority of patients. Despite an overall 5-year survival of 89.1%, significant morbidity and mortality are still an issue with this procedure, particularly in patients older than 5 years.[17,18] Gene therapy (GT) clinical trials, in which patients receive autologous $CD34^+$ cells transduced with a lentiviral (LV) vector encoding human WASp, are ongoing. Preliminary analyses showed safety and efficacy with improved but not normalized platelet counts and reduced bleeding episodes.[19,20] Several studies have been conducted to elucidate the pathogenesis of thrombocytopenia in patients with WAS. Defective platelet production by megakaryocytes and accelerated peripheral elimination have been proposed as the underlying cause, highlighting an unsolved dichotomy.[21] Megakaryocytes have been described to fail both in the maturation step and in proplatelet production.[21] Conversely, several articles showed that after normal thrombopoiesis, platelets in the periphery are the target of both innate and adaptive immunity.[21] Recently, using a conditional *WAS* murine model lacking WASp only in platelets and megakaryocytes, we dissected intrinsic versus extrinsic platelet defects, demonstrating how mutant platelets induce a specific autoimmune response mediated by macrophages and B cells, leading to thrombocytopenia.[22]

Here we performed a detailed analysis of platelet phenotype and function in patients with WAS before and after LV-GT. Our data show that platelets isolated from untreated patients with WAS have abnormalities in ultrastructure and function, as well as a perturbed proteomic profile and metabolic activity. These defects significantly improve after GT in correlation with the restoration of WASp expression in platelets. Overall, this study provides new evidence of the pathogenic dysregulation of cellular mechanisms in platelets from patients with WAS, providing further clarification of the effect of WASp on the structure and function of platelets compared with data reported thus far in the literature.[21] It is the first study that demonstrates the extent to which LV-GT is able to correct morphologic and functional platelet abnormalities in patients with WAS, restoring platelet morphology, activation, and function and preventing the occurrence of bleeding manifestations starting at very early time points after treatment. These findings will be instrumental also for a perspective clinical trial targeting patients with XLT in whom the correction of platelet phenotype in the absence of other concomitant clinical manifestations is of primary importance also to prevent life-treating complications that can arise in these patients with their progression to a more severe phenotype.[23]

METHODS

Experimental design

Data reported in this article were obtained from blood samples from patients with WAS and HDs. Diagnosis of WAS was based on clinical findings and confirmation using genetic analysis. Table E1 in this article's Online Repository at www.jacionline.org describes the clinical features and analyses performed for each patient. Details about the experimental design and GT procedure are described in the Methods section in this article's Online Repository at www.jacionline.org and extensively reported elsewhere.[19]

Sample collection

Complete peripheral blood count analysis was performed at Laboraf (Ospedale San Raffaele, Milano, Italy). Platelet-rich plasma (PRP) was obtained by centrifuging peripheral blood (Citrate .105M/3.2%, BD Vacutainer; BD, Franklin Lakes, NJ) for 10 minutes at 700 rpm, as previously described.[22] Plasma was collected by centrifuging blood (K2EDTA 7.2 mg, BD Vacutainer) for 10 minutes at 2000 rpm.

Cytofluorimetric staining

Cell suspensions were stained with the following anti-human antibodies: anti-PAC1 (PAC-1) from BD PharMingen (San Diego, Calif); anti-CD61 (VI-PL2) from BioLegend (San Diego, Calif); and anti–P-selection (CD62P; Psel.KO2.3) from Invitrogen (Carlsbad, Calif). Intracellular WASp staining was performed as previously described[24] or by using a purified mouse anti-human WASp antibody (Clone 5A5) from BD PharMingen. All the flow cytometric samples were acquired with a FACSCanto II system (BD) and analyzed with FlowJo software (TreeStar, Ashland, Ore).

Electron microscopy

Transmission electron microscopy (TEM) was performed, as previously described.[22] Briefly, PRP was centrifuged for 7 minutes at 3200 $\times g$, and the pellet was fixed, washed, dehydrated, and embedded in Epon (Sigma, St Louis,

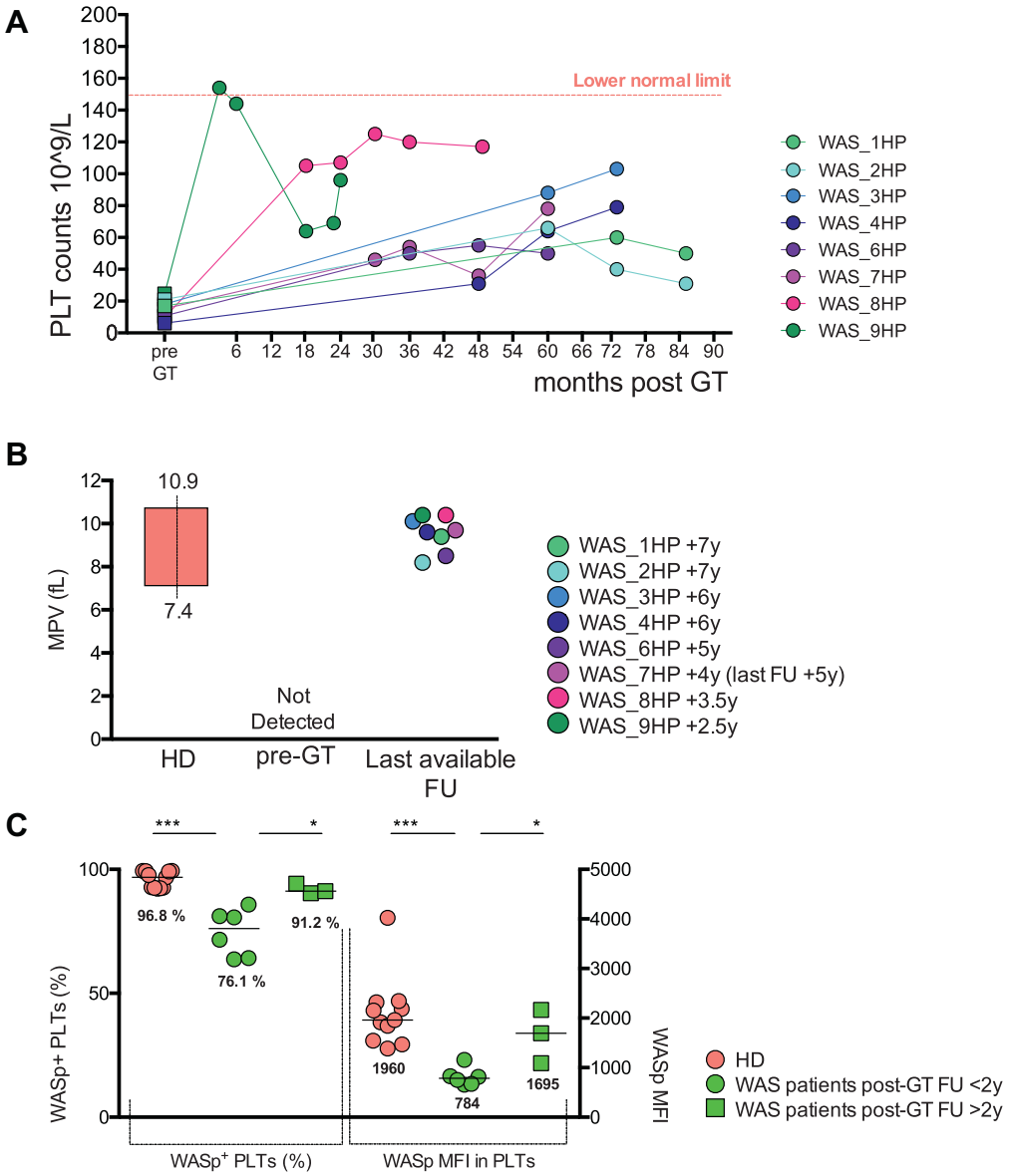


FIG 1. Platelet *(PLT)* counts and WASp expression in HDs and post-GT patients. **A,** Platelet counts are reported for the first 8 patients enrolled in the TIGET-WAS trial (WAS_1HP to WAS_9HP); measurements were taken at the defined time points indicated in Table E1. The lower normal limit for HDs (150×10^9/L) is reported. **B,** mean platelet volume *(MPV)* is reported for HDs and patients (WAS_1HP to WAS_9HP) at the last FU indicated in Table E1. HD values are reported as reference. For patient WAS_7HP, MPV analysis is available at 4 years after GT instead of 5 years after GT. **C,** WASp expression is reported in HDs and post-GT patients in terms of percentages of $WASp^+$ platelets or MFIs on the whole platelet population. Analysis was performed for research purposes and not for the official case report form. Median values are reported below the groups analyzed. Statistical analysis in Fig 1, *C,* was performed by using the Mann-Whitney test. A *P* value of less than .05 was considered statistically significant. $*P < .05$ and $***P < .001$.

Mo). Sections were examined on an LEO 912AB transmission electron microscope (Zeiss, Oberkochen, Germany).

To perform whole-mount TEM, a volume of 7 μL of PRP (1×10^6 platelets/μL) was placed on a Formvar/Carbon Coated-Nickel 200 Mesh (Electron Microscopy Sciences, Hatfield, Pa) for 5 minutes, washed, dried for 20 minutes, and, without fixation, imaged directly with the LEO 912AB at 80 kV. Images were analyzed by using ImageJ software (National Institutes of Health, Bethesda, Md).

Platelet staining

Platelet activation was performed, as previously described.[22] Briefly, platelets were stained with CD61, CD62P, and PAC1 and then activated with adenosine diphosphate (ADP; 0.1 μmol/L to 20 mmol/L; Sigma-Aldrich, St Louis, Mi) for 15 minutes. Activation is expressed as percentages of $CD62P^+$ or $PAC1^+$ platelets; CD62P/CD61 or PAC1/CD61 mean fluorescence intensity (MFI) ratios were calculated to normalize activation marker expression for the platelet surface.[15]

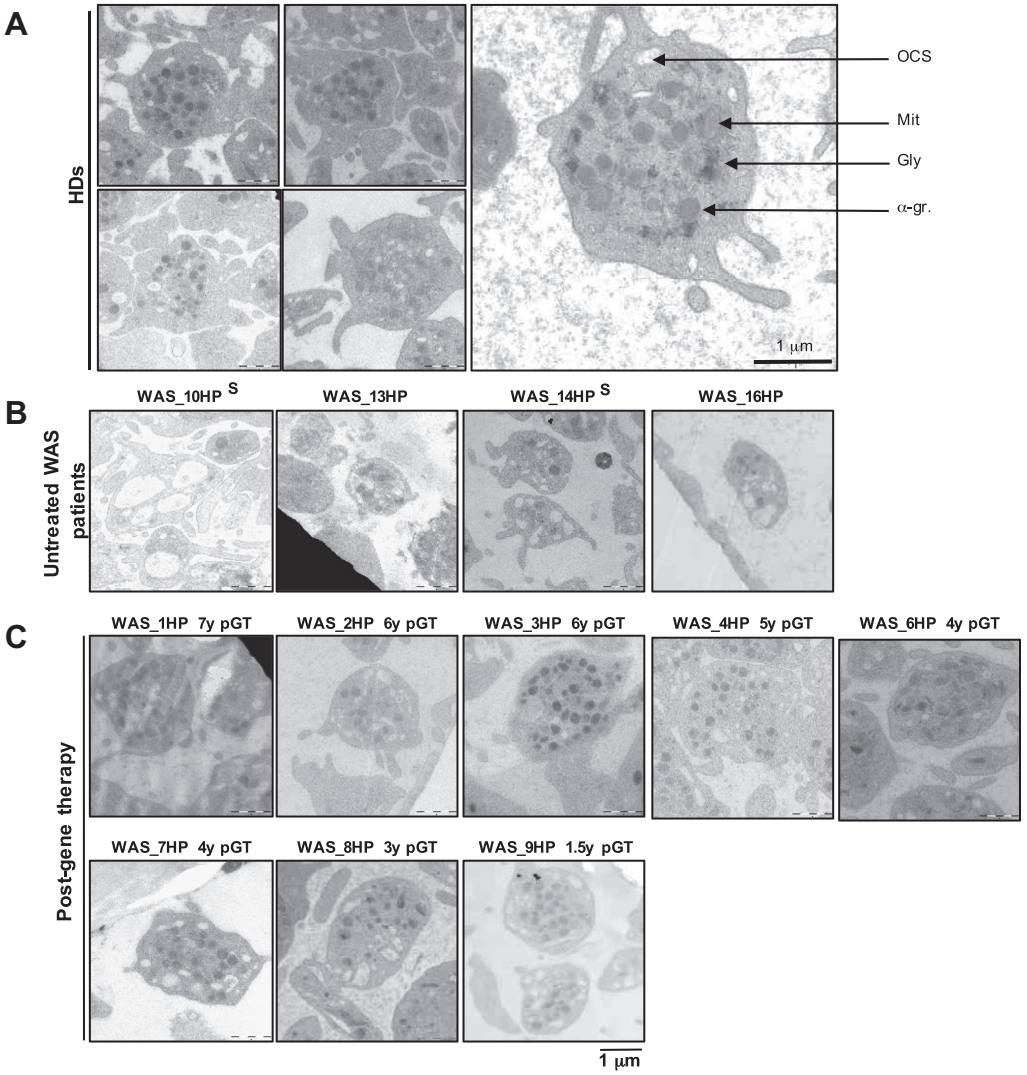


FIG 2. Platelet *(PLT)* ultrastructure in pre-GT and post-GT patients. **A,** TEM images of platelets isolated from HDs. Several internal structures are depicted in the picture: OCS, mitochondria *(Mit)*, glycogen granules *(Gly)*, and α-granules *(α-gr.)*. **B** and **C,** Representative TEM images of platelets isolated from pre-GT (Fig 2, *B*) or post-GT (Fig 2, *C*) patients with WAS. For each post-GT patient, the FU period is indicated as years after GT *(y pGT)* or days after GT *(d pGT)*. Images have been acquired with a 2048 × 2048–pixel camera. *Scale bars* are shown. *S* refers to splenectomized patient.

Intracellular reactive oxygen species (ROS) production was measured with the ROS-ID Total ROS/Superoxide detection Kit (Enzo Life Sciences, Farmingdale, NY), according to the manufacturer's instructions.

PFA-100 test

Platelet aggregation was tested on peripheral blood (Citrate .105M/3.2%, BD Vacutainer in a PLT Function Analyzer 100 [PFA-100, Siemens, Berlin, Germany]) by using ADP-collagen cartridges, according to the manufacturer's instructions. Hematocrit values of analyzed patients were in the range of normality. A standard curve was generated to account for differences in platelet counts, as described in the Methods section in this article's Online Repository.

ELISA tests

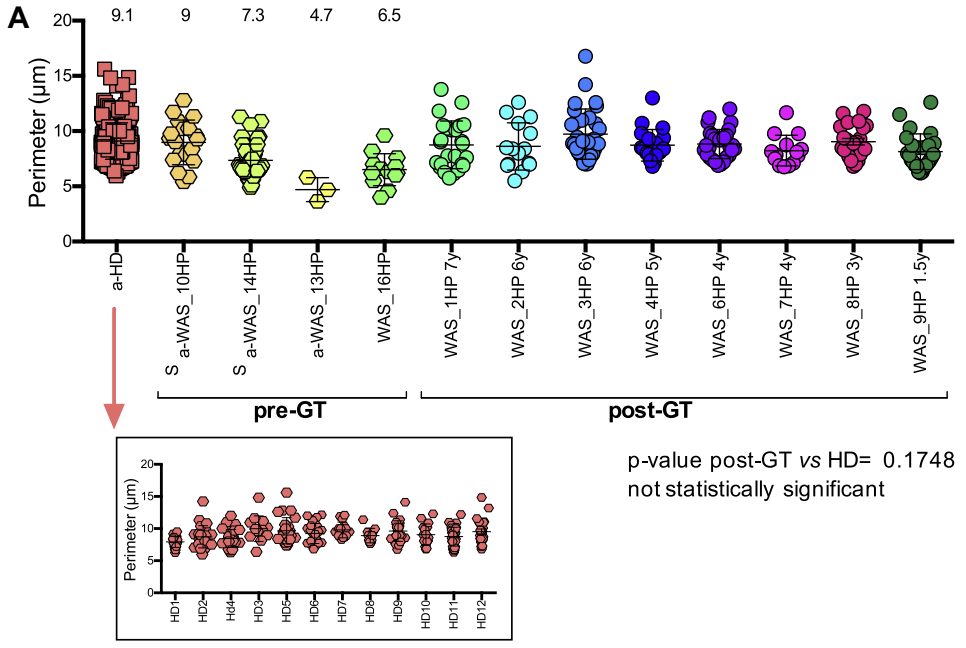
ELISA kits to evaluate concentrations of soluble P-selectin (sCD62P), soluble CD40 ligand (sCD40L), and B cell–activating factor (BAFF) in plasma were purchased from R&D Systems (Minneapolis, Minn) and used according to the manufacturer's instructions; sCD62P and sCD40L levels were normalized for platelet counts and expressed as nanograms per mL per 1000 platelets. Von Willebrand factor (vWF) collagen binding ability was measured by using the "ASSERACHROM VWF:CB" ELISA Kit from Diagnostica STAGO (Asnieres sur Seine, France).

Sample preparation and proteomics analysis

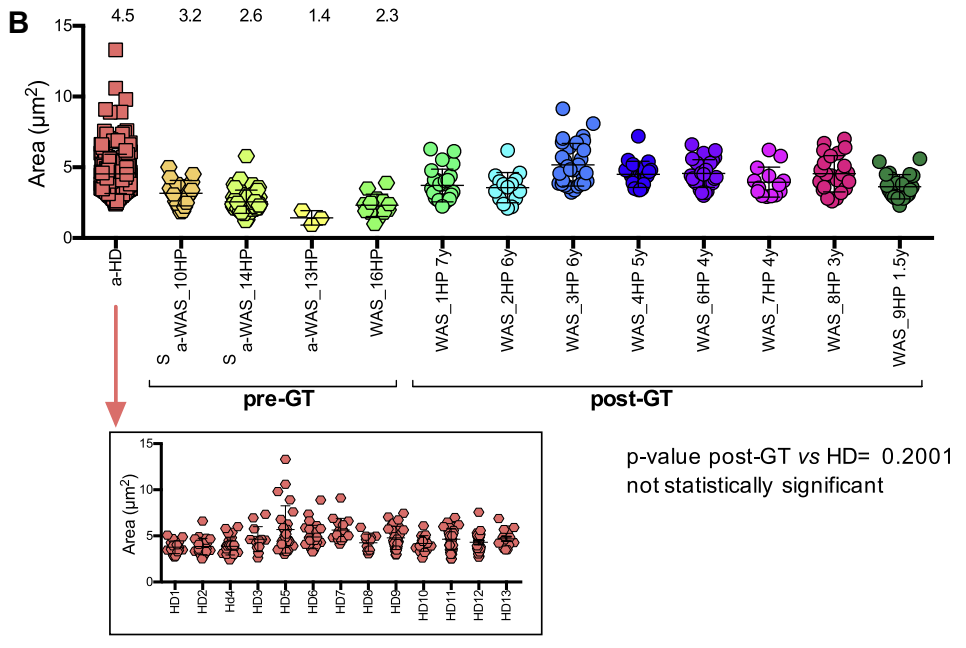
As previously described,[22] pellets of the purified fraction of platelets were collected from HDs and patients with WAS before and after GT and stored at −80°C. Detailed descriptions of proteomic analysis, data processing, and interaction network reconstruction are described in the Methods section in this article's Online Repository.

Statistical analysis

All results are expressed as means ± SDs, unless stated otherwise. Statistical tests used are specified in the Methods section in this article's Online Repository.



Perimeter (log transformation)	Estimate	Std.Error	*p*-value
Intercept	1.4769	0.0362	<0.0001
WAS Group (ref. HD)	-0.0775	0.0584	0.2001



Area (log transformation)	Estimate	Std.Error	*p*-value
(Intercept)	2.1942	0.0181	<0.0001
WAS Group (ref. HD)	-0.0403	0.0285	0.1748

FIG 3. Recovery of platelet *(PLT)* size after GT. Size of platelets isolated from patients before and after GT (WAS_1HP to WAS_9HP) was measured by using TEM. Size is expressed as area (in square micrometers; **A**) or perimeter (in micrometers; **B**). Each *dot* represents a single platelet. Splenectomized untreated patients with WAS are indicated with *S*; adult patients are indicated with *a*. Each HD analyzed is reported in the box. Median values of area and perimeter are reported above HDs and untreated patients with WAS. Linear mixed-effects models have been applied to evaluate whether patients with WAS after GT and HDs, measured several times, were different in terms of platelet area and perimeter. Logarithmic transformation has been applied to both outcomes. Along with the fixed effect of group, subject-specific random effects have been specified in the model. For both outcomes, we did not find a significant group effect (see tables below graphs in the figure).

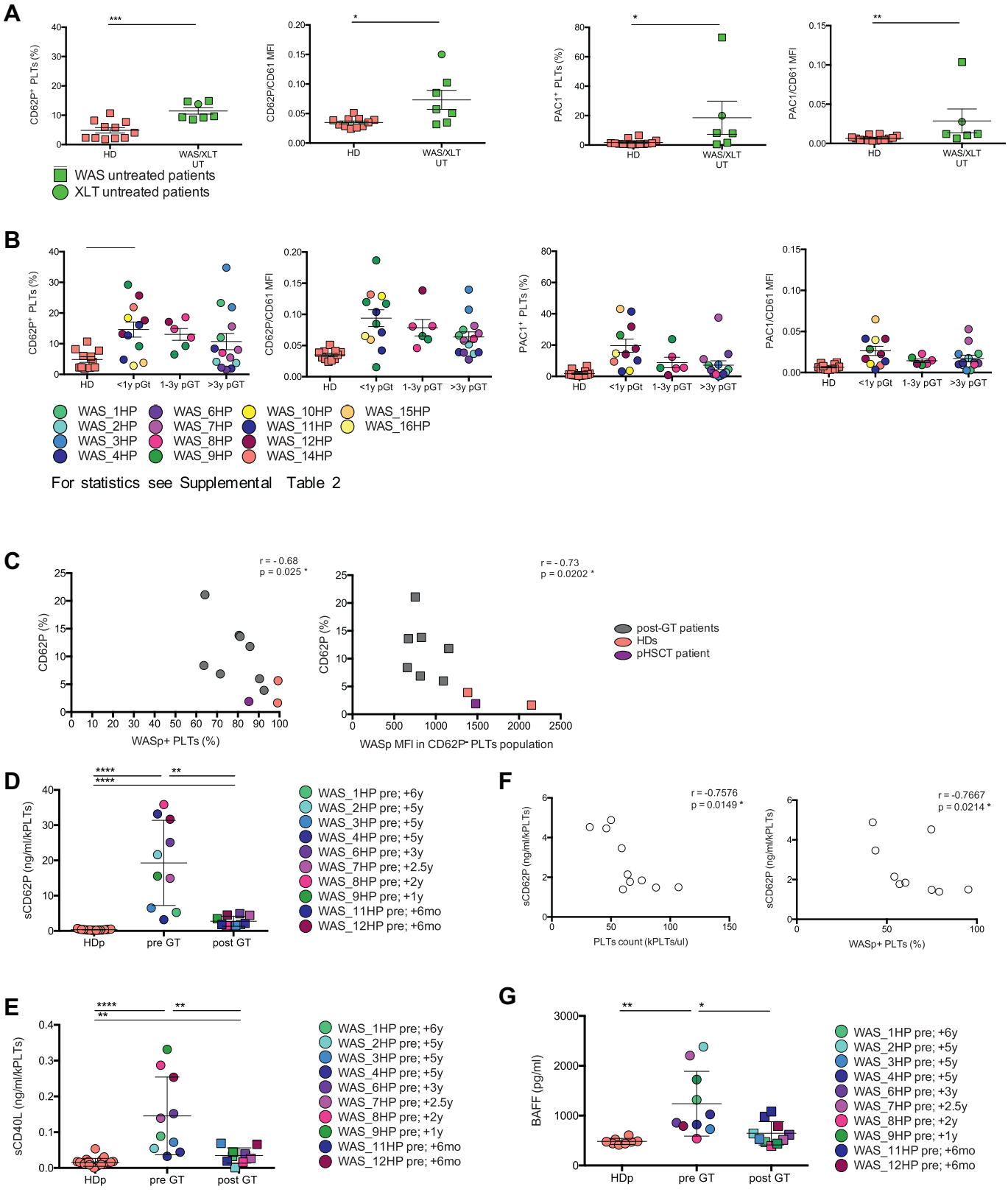


FIG 4. Expression of platelet *(PLT)* activation markers in patients with WAS before and after GT. **A** and **B,** Analysis of activation marker surface expression in platelets at steady state in untreated patients with WAS/XLT (Fig 4, *A*) or post-GT patients (Fig 4, *B*). CD62P or PAC1 expressions are reported as percentages on total platelets or as MFI ratio. **C,** Correlation between percentages of $CD62P^+$ platelets and percentages of $WASp^+$ platelets *(left panel)* and WASp MFI *(right panel)*, respectively. **D,** Levels of sCD62P in plasma of

RESULTS

WASp expression ameliorates the platelet compartment

We analyzed platelet phenotype and function in a cohort of patients with WAS/XLT, including patients who received either LV GT combined with reduced-intensity conditioning or HSCT.

Platelet counts measured in 8 patients (WAS_1HP to WAS_9HP) treated with GT (see Table E1) increased after treatment in all patients but remained less than normal values (Fig 1, *A*). Importantly, mean platelet volume, which was not measurable in untreated patients, was in the normal range at the last follow-up (FU) after GT in all patients analyzed (7.4-10.9 fL; Fig 1, *B*).

The percentage of $WASp^+$ platelets ranged between 3% and 28% before GT (F. Ferrua and M. P. Cicalese et al, unpublished data) and increased up to a median of 76.1% in patients after GT with an FU of less than 2 years and 91.2% in patients with an FU of longer than 2 years (Fig 1, *C*). In line with this, WASp MFI was calculated in the whole platelet population and still remains very low in samples isolated from patients after GT at up to 2 years of FU compared with control subjects (Fig 1, *C*), suggesting a suboptimal expression of the protein in this cellular subset.

Restoration of platelet ultrastructure and size after GT

To evaluate ultrastructural morphology, we performed TEM analysis on the whole platelet population isolated from HDs and patients with WAS (4 before and 8 after GT). Of note, post-GT samples are composed of a heterogeneous population of $WASp^+$ and $WASp^-$ platelets.

We analyzed the distribution of α-granules, glycogen granules, vacuoles, the open canalicular system (OCS), and mitochondria in platelets isolated from patients with WAS and HDs (Fig 2, *A*). Platelets isolated from pre-GT patients showed severe alterations in ultrastructure, with a reduction in granule content, as already reported,[25] and an increased number of vacuoles and enlargement of the OCS (Fig 2, *B*). The features have been described as signs of platelet activation.[26] Conversely, normal granule content and OCS structure were observed in platelets obtained from all post-GT patients (Fig 2, *C*).

Whole-mount TEM showed a reduction of electron-dense granule (δ-g) content in patients with WAS (mean, 3 δ-g/platelet) compared with the reference range for HDs (4-8 δ-g/platelet).[27] The number of granules normalized after GT (mean, 5.6 δ-g/platelet; see Fig E1 in this article's Online Repository at www.jacionline.org).

Platelet area and perimeter were also measured by using TEM images. Statistical analyses were not applied because of the low number of untreated patients with WAS analyzed. At a descriptive level, median values of platelet perimeter (in micrometers) and area (in square micrometers; Fig 3, *A* and *B*) were lower in non-splenectomized untreated patients (WAS13_HP and WAS_16HP) compared with those in control subjects. Platelets isolated from splenectomized untreated patients (WAS10_HP and WAS_14HP) were more similar to those from control subjects, which is in line with literature describing amelioration of platelet size and counts after splenectomy.[13,28] Linear mixed-effects models have been applied to evaluate whether patients with WAS after GT and HDs, measured several times at different time points, were different in terms of platelet area and perimeter. Logarithmic transformation has been applied to both outcomes to linearize the relationship. Along with the fixed effect of group, subject-specific random effects have been specified in the model. For both outcomes, we found that all patients with WAS after GT were comparable with HDs (no significant group effect, Fig 3).

Normalization of hyperactivated phenotype in post-GT patients

To assess whether dysmorphic platelet ultrastructure could be associated with dysregulated activation at steady state, we evaluated the expression of P-selectin (CD62P) and the activated form of integrin αIIbβ3 (PAC1). Levels of both activation markers were greater in platelets isolated from untreated patients with WAS than those from HDs (Fig 4, *A*), confirming our previous findings in a smaller cohort of patients.[22] Post-GT patients were analyzed at different time points after treatment (see Table E1) and clustered into 3 groups according to the duration of FU.

Linear mixed-effects models were applied for each parameter and analyzed to assess differences between patient groups and HDs. In particular, a random intercept model has been specified by including subject-specific random effects in the model. Logarithmic transformation was applied to all outcomes, except the MFI of CD62P.

Only patients with less than 1 year of FU had significantly greater percentages of $CD62P^+$ compared with HDs ($P = .0184$). Conversely, the proportion of $PAC1^+$ platelets and the MFI of both markers were significantly greater in all patients analyzed when compared with those of control subjects (Fig 4, *B*, and see Table E2 in this article's Online Repository at www.jacionline.org). However, expression of these markers on platelets isolated from patients with FUs of longer than 3 years were lower compared with that seen in patients with shorter FUs, suggesting a progressive amelioration of the phenotype.

Because of the low number of biological samples, data obtained from patients with an FU period of between 1 and 3 years were not statistically analyzed. At a descriptive level, mean values of CD62P and PAC1 expression were intermediate between those of the group with shorter and the group with longer FUs. Interestingly, the percentage of $CD62P^+$ platelets found at steady state in post-GT patients inversely correlated with the

patients before and after GT. **E,** Correlations between sCD62P plasma levels and total platelet counts *(left panel)* or percentages of $WASp^+$ platelets *(right panel)* are reported. **F** and **G,** Levels of sCD40L (Fig 4, *F*) or BAFF (Fig 4, *G*) in plasma of patients before and after GT are shown. In Fig 4, *D-F,* pre-GT samples are depicted as *circles,* and post-GT samples are depicted as *squares.* For statistics, in Fig 4, *A, D, F,* and *G,* the Mann-Whitney unpaired *t* test or parametric unpaired *t* test versus HDs and the paired *t* test for pre-GT versus post-GT was used (multiplicity correction on *P*-value has been applied). In Fig 4, *B,* linear mixed-effects models have been applied to evaluate whether patients with FUs of less than 1 year or longer than 3 years differed from HDs (details are provided in Table E2); logarithmic transformation has been applied to all the outcomes, except the MFI of CD62P. In Fig 4, *C* and *E,* Spearman correlation was used. $*P < .05$, $**P < .005$, $***P < .001$, and $****P < .0001$.

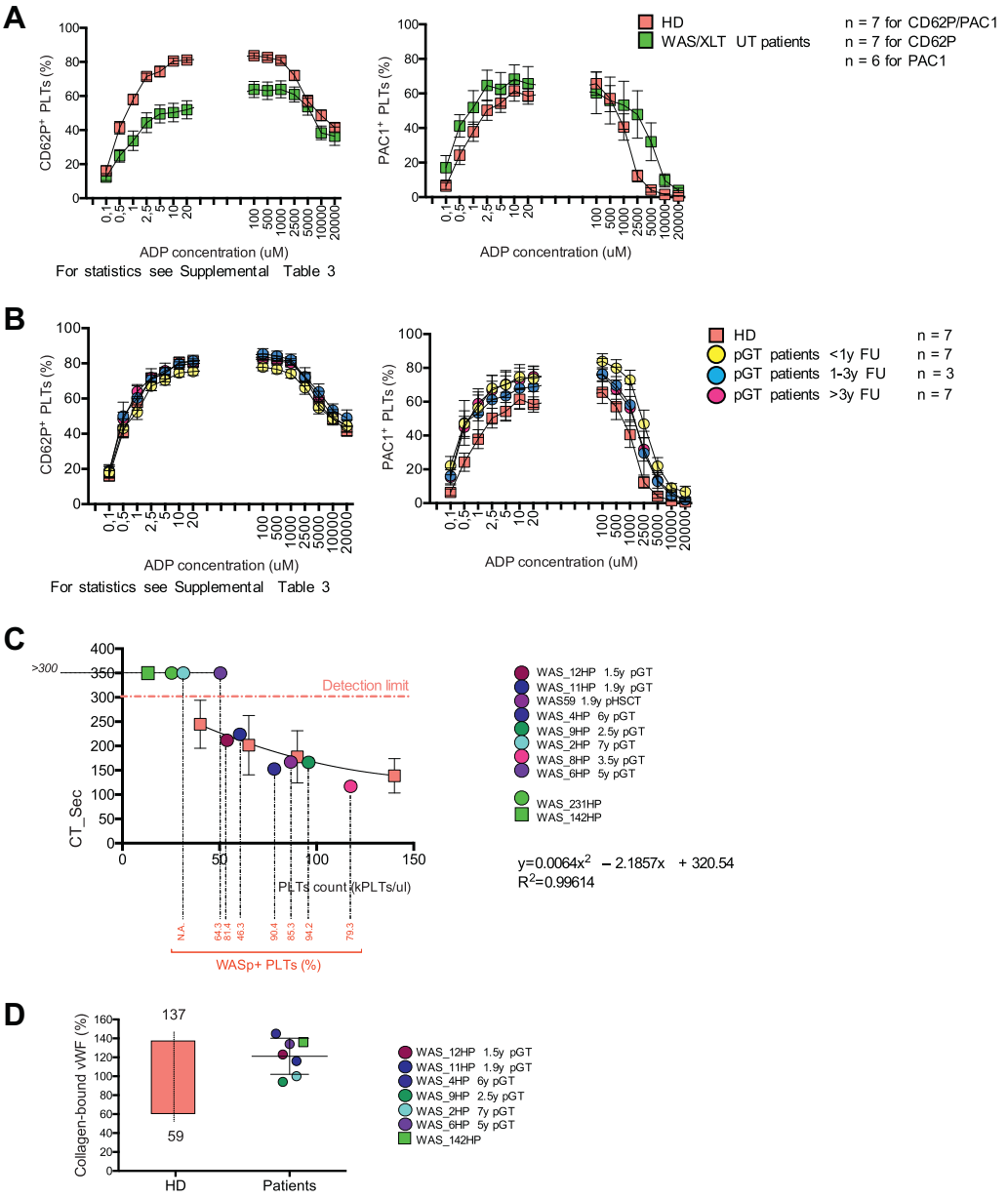


FIG 5. Platelet *(PLT)* functionality recovers after GT. **A** and **B,** Platelet activation in untreated patients with WAS/XLT (Fig 5, *A*) and post-GT patients (Fig 5, *B*) is reported as the percentage of $CD62P^+$ platelets *(left panels)* or $PAC1^+$ platelets *(right panels)* after ADP stimulation (mean ± SEM). **C,** CTs, as measured by using the PFA-100 device, are reported for different platelet counts from HDs (pink curve) as the standard curve. In the graph each patient analyzed is shown along with the frequency of $WASp^+$ platelets (in red), when available. *N.A.*, Not available. **D,** Plasma level of collagen-bound vWF (as a percentage) evaluated by using an ELISA in patients who underwent the PFA-100 test. Normal ranges for HDs are reported. For statistics, in Fig 5, *A* and *B*, linear mixed-effects models to evaluate the effect of group and of ADP concentration, along with their interaction, have been applied. We allow for a different dose-response relationship depending on the concentration level itself by defining a binary variable indicating whether the ADP dose was low (range, 0.1-20 μm) or high (range, 100-20,000 μm). A 3-way interaction model including subject-specific random effects (Fig 5, *A*) and nested random effects (Fig 5, *B*), thus accounting for subject-specific effect and for multiple evaluations of the same subject at different FU visits, was applied (details are provided in Table E3).

percentage of $WASp^+$ platelets and WASp MFI (Fig 4, *C*) in platelets.

Consistently, all patients showed significantly lower plasma levels of sCD62P after GT versus before GT (Fig 4, *D*). This decrease inversely correlates with platelet counts and WASp expression (Fig 4, *E*). Post-GT patients also had lower plasma concentrations of sCD40L compared with pre-GT patients (Fig 4, *F*) and normalization of serum BAFF levels, 2 factors implicated in autoreactive B-cell survival and autoimmunity (Fig 4, *G*).

TABLE I. Samples analyzed by proteomics

	Patient ID	FU time point	Platelets (× 10^6)
Patients	WAS_2HP	Pre-GT	4.2
		5 y post-GT	70.7
	WAS_3HP	Pre-GT	7
		5 y post-GT	46.7
	WAS_4HP	Pre-GT	1
		4 y post-GT	96.8
HDs	1	—	104
	2	—	37
	3	—	176

GT corrects both platelet activation and aggregation on stimulation

Activation and aggregation were tested in untreated and GT-treated patients with WAS to evaluate whether GT restores platelet function along with phenotype. It should be noted that because of technical limitations, we were not able to sort $WASp^+$ and $WASp^-$ platelets without affecting viability and function because all samples from post-GT patients contained a heterogeneous population of $WASp^+$ and $WASp^-$ platelets.

To evaluate platelet activation in response to agonist stimulation, we used increasing ADP doses. Linear mixed-effects models were applied to evaluate the effect of group (patients with WAS vs HDs) and that of the ADP concentration, along with their interaction, on both CD62P and PAC1 expression. Cubic transformed and raw data were considered when modeling the 2 outcomes, respectively. Moreover, we allowed for a different dose-response relationship depending on the concentration level itself by defining a new binary variable, indicating whether the ADP dose was low (range, 0.1-20 μm) or high (range, 100-20,000 μm). Hence we finally specified a model with a 3-way interaction, including subject-specific random effects.

In terms of CD62P expression, platelets isolated from both untreated patients with WAS/XLT and HDs showed a significantly larger response when stimulated with high compared with low doses of ADP ($P < .0001$). Additionally, we found a positive effect of ADP concentration on marker expression within the low dose ranges (≤20 μmol/L) versus a slightly negative effect at greater doses (≥100 μmol/L). A negative group effect on CD62P expression was observed in patients with WAS compared with control subjects, and only very high doses of ADP were able to induce expression of CD62P more comparable to those seen in healthy control subjects (significant 3-way interaction term, see Table E3 in this article's Online Repository at www.jacionline.org). Conversely, no significant group effect was found in PAC1 expression, the value of which was found to be comparable in patients with WAS and control subjects. We only found a positive effect of the ADP concentration at low doses, which became slightly negative for high ADP dose ranges (Fig 5, *A*, and see Table E3).

Significantly greater expression of CD62P on platelet surfaces with high compared with low ADP doses was also found in post-GT patients ($P < .0001$). Conversely to patients with WAS/XLT, post-GT patients showed comparable levels of CD62P on platelet surfaces. When analyzing PAC1 percentages, only patients with FUs of less than 1 year had significantly greater values of the outcome than HDs ($P = .0491$); this effect is greater at high concentrations ($P = .0079$). For both outcomes, we found a positive effect of ADP concentration at lower ranges and a slightly negative effect at high ranges (Fig 5, *B*, and see Table E3). In these models nested random effects were specified to account for subject-specific effect and for the fact that some subjects were evaluated multiple times at different FU visits. Cubic transformation was applied to model data on PAC1 expression, whereas raw data were used as input for the linear mixed-effects model for CD62P.

Platelet aggregation was tested *in vitro* by using the PFA-100 System, which mimics a vascular injury and measures platelet adhesion and aggregation in terms of capillary tube closure times (CTs; in seconds) using collagen/ADP cartridges. A standard reference curve was generated from HD samples (n = 18), plotting platelet counts versus CTs on collagen/ADP stimulation (Fig 5, *C*). Seven post-GT patients and 1 post-HSCT patient with WAS were tested. Platelet aggregation in response to ADP was comparable with that seen in HDs in the majority of the patients (Fig 5, *C*, and see Table E4 in this article's Online Repository at www.jacionline.org). A prolonged CT (>300 seconds, machine detection limit) was observed with samples from 2 post-GT patients with very low platelet counts, although no significant bleeding occurred in these patients after GT. Conversely, samples from the 2 untreated patients with WAS/XLT analyzed showed disturbed aggregation in response to ADP with a CT of greater than 300 seconds (Fig 5, *C*, and see Table E4).

Plasma levels were measured to exclude that CT values could have been influenced by vWF concentration. All patients analyzed with the PFA-100 system, either untreated or after GT, had vWF plasma levels within the normal range for HDs (Fig 5, *D*), suggesting that differences in platelet aggregation were not due to abnormal glycoprotein Ib/vWF–dependent signaling.

Analysis of platelet proteomic profiles

Proteomic analysis was performed on 3 patients before and 4/5 years after GT in parallel with 3 adult HDs to identify putative molecular pathways causative of altered platelet phenotype and function in the absence of WASp (Table I).

More than 1315 proteins were identified (see Table E5 in this article's Online Repository at www.jacionline.org), and 372 were shared among the 3 analyzed groups (corresponding to 28.3% of total proteins; see Fig E2, *A* and *B*, in this article's Online Repository at www.jacionline.org). Linear discriminant analysis (LDA) was applied to identify differentially expressed proteins; 255 of 1315 proteins presenting an F-ratio of 3.5 or greater and a *P* value of .05 or less were extracted as descriptors (see Table E6 in this article's Online Repository at www.jacionline.org). Hierarchical clustering of identified descriptors showed that post-GT patients had an intermediate profile between HDs and pre-GT patients in terms of protein composition and expression (see Fig E2, *C*).

Selected descriptors were plotted into a *Homo sapiens* protein-protein interaction network to evaluate differences in protein pathway expression between patients and control subjects (see Fig E3 in this article's Online Repository at www.jacionline.org). The majority of identified protein networks were downregulated in platelets isolated from samples from pre-GT patients compared with control samples and ameliorated after GT. We

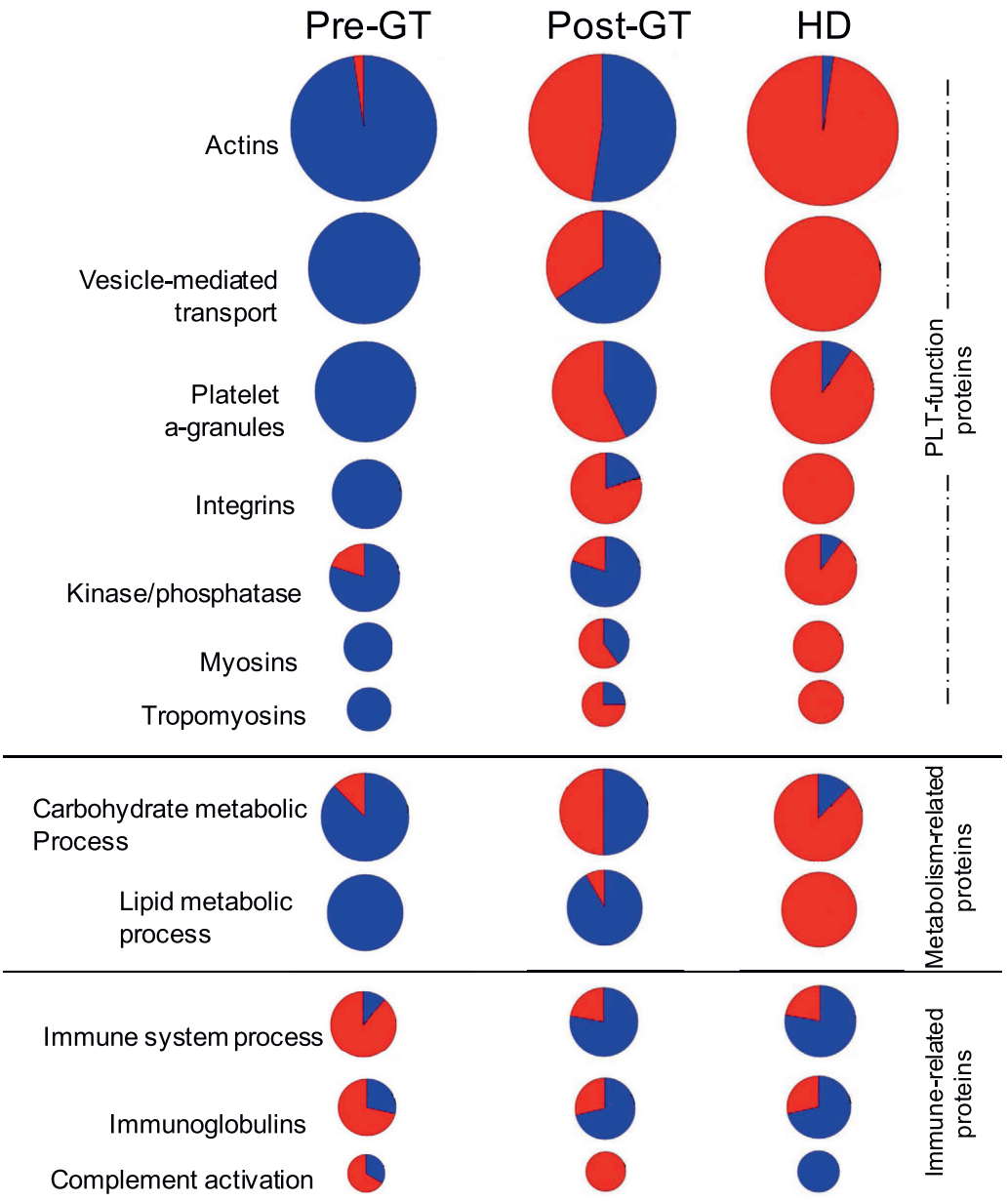


FIG 6. Protein expression per functional cluster in HDs and patients with WAS before and after GT. Enrichment of main functional categories of low (blue) and high (red) abundant proteins (see Fig E2) in pre-GT patients, post-GT patients, and HDs. Bubble size is indicative of the number of proteins involved in each pathway. Proteins have been classified into 3 groups: platelet function proteins, metabolism-related proteins, and immune-related proteins.

focused our analysis on 3 main areas: platelet function, platelet metabolism, and immune-related proteins (Fig 6).

All proteins involved in cytoskeletal rearrangements, comprising actin and its interactors (actinin, dynactin, nonmuscle myosin, tropomyosin, Arpc2/3 complex, cofilin, vinculin, and filamin A), were downregulated in pre-GT samples, whereas their expression was partially restored in post-GT patients.

In comparison with pre-GT samples, samples isolated from post-GT patients showed an increased expression of (1) membrane integrins and their regulators (integrin-linked protein kinase and fermitin family homolog 3), (2) proteins involved in wound healing and platelet plug formation (PECAM1, TREML1, GPIBB, vWF, and PF4), and (3) proteins implicated in vesicle transport, especially clathrin-mediated transport (CLTC, PICALM, and AP1B1).

A second major group of proteins found to be downregulated in pre-GT patients and partially restored after GT treatment were linked to lipid and carbohydrate metabolism (LDHB, ALDOA, GAPDH, HK1, and PYGB), fuel for glycolysis, and mitochondrial oxidative phosphorylation. Downregulation of proteins involved in lipid metabolism in pre-GT samples can lead to (1) decreased processing of fatty acids and fuel for mitochondrial respiration and (2) lower production of platelet function mediators, such as thromboxane A_2 and phosphatidylinositol.

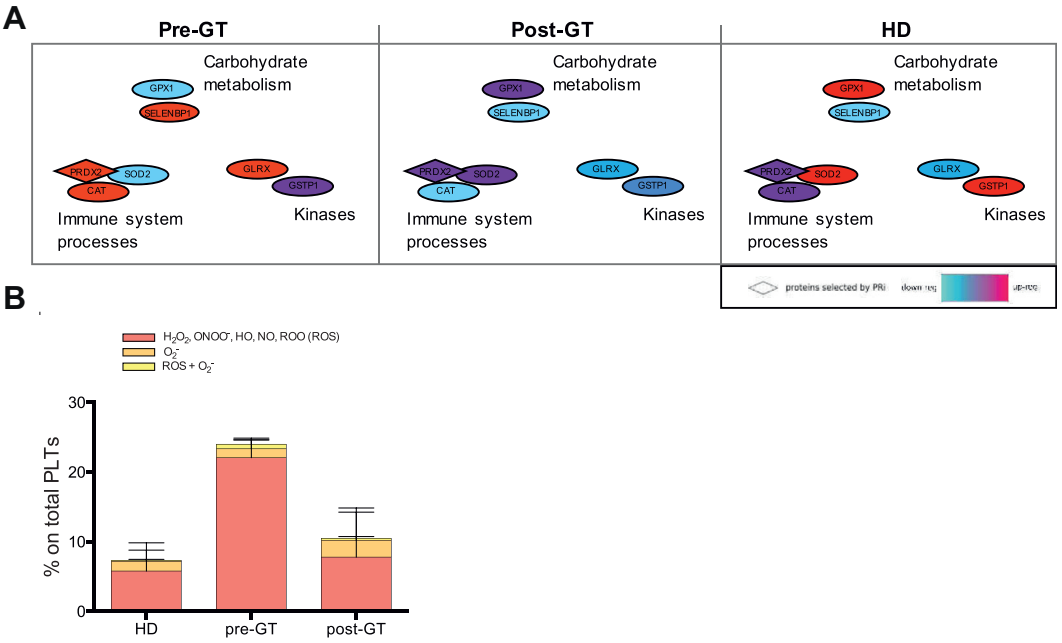


FIG 7. Oxidative stress and ROS in HDs and patients with WAS before and after GT. **A,** Schematic representation of expression of proteins involved in ROS regulation as identified with proteomics. Color code (based on normalized SpC) indicates protein expression levels in HD, pre-GT, and post-GT samples. **B,** Flow cytometric evaluation of ROS production in resting platelets *(PLTs)* in HDs (n = 5), untreated patients with WAS/XLT (n = 2), and post-GT patients (n = 6).

Conversely, pre-GT samples showed overexpression of immune-related proteins, such as different variable regions of immunoglobulin heavy and light chains; constant regions of IgM, IgG_4, and IgG_2 heavy chain; and complement factors.

Using the proteome remodeling index (see Figs E3 and E4 in this article's Online Repository at www.jacionline.org), we identified proteins of which the perturbed expression in pre-GT samples (higher or lower compared with control values) was restored to normal levels in post-GT patients (see Table E7 in this article's Online Repository at www.jacionline.org); these descriptors have been filtered for statistical significance and reported in the protein-protein interaction network (see Fig E3). The majority of the proteins that showed normalized expression after GT were cytoskeleton-related proteins, such as actin B and its regulators, integrins, and surface glycoproteins.

To evaluate whether the dysregulated protein expression found in the absence of WASp could correspond with a reversion in the topological importance of proteins within different networks, we identified the most important hubs present in platelets isolated before and after GT (see Table E8 in this article's Online Repository at www.jacionline.org). The term "hubs" refers to proteins with greater numbers of protein connections within the cell, suggesting their topological and biological relevance.[29] Almost all the hubs identified in HDs were also found in post-GT samples but not in pre-GT samples; conversely, all the hubs found in pre-GT samples were not present in HDs or post-GT samples. The results suggest that the protein expression changes observed in $WASp^-$ platelets are associated with dysregulation of cellular and signal transduction. Indeed, we observed a different pattern of proteins involved in signal transduction in WASp-deficient platelets comprising signal transducer and activator of transcription 3 (STAT3) and high-mobility group box 1 (HMGB1) instead of GRB2, LYN kinase, and integrin-linked protein kinase,[30,31] which are found in HD samples.

ROS signaling is dysregulated in the absence of WASp

Using proteomics, we identified several proteins involved in regulation of reactive oxygen species (ROS) production and degradation (Fig 7, *A*, and see Table E6). In pre-GT samples different scavenger enzymes, such as glutathione peroxidase 1 (GPX1), glutathione-S-transferase P1 member, and superoxide dismutase 2 (SOD2), were downregulated. Of note, lower expression of GPX1 is in line with upregulation of SELENBP1, which has been proposed to negatively regulate GPX1 activity.[32] Conversely, other antioxidant enzymes, such as glutaredoxin, peroxiredoxin-2, and catalase, were found to be overexpressed in pre-GT patients (Fig 7, *A*) and normalized in post-GT samples. In line with the dysregulated expression of ROS-related enzymes, we found that platelets isolated from untreated patients with WAS produce greater quantities of ROS in resting conditions than those from HDs, whereas ROS production in post-GT patients was much lower (Fig 7, *B*).

DISCUSSION

GT has been recently proposed as a potentially curative treatment for a variety of genetic diseases. WAS, a monogenic primary immunodeficiency affecting only hematopoietic cells, was considered an excellent candidate for GT. Although allogeneic HSCT still remains the gold standard treatment, different GT clinical trials with LV vectors are ongoing in Europe (NCT01515462 and NCT01347242) and the United States

(NCT01410825). In the TIGET-WAS Trial (NCT01515462) patients received, after a reduced-intensity conditioning regimen, autologous $CD34^+$ cells transduced with LV carrying the sequence of human WAS cDNA driven by the endogenous promoter.[19] Although platelet counts increase in the majority of patients treated with GT, complete restoration of platelet counts remains an issue. Despite the overall success of HSCT, the standard of care for WAS, reconstitution of platelet numbers, is one of the major challenges. Platelet counts increase significantly or normalize after HSCT compared with GT, but a complete restoration of platelet numbers strictly depends on the degree of myeloid chimerism, even in previously splenectomized patients.[17,18,33,34] Full chimerism has not been obtained in GT trials because of the reduced-conditioning regimen. However, the risk of transplant-related toxicity and the occurrence of clonal dominance have always discouraged the use of a more intensive conditioning regimen to achieve better myeloid engraftment or use of a stronger promoter.[35]

The clinical analysis conducted on the first 8 patients treated in the current TIGET-WAS clinical trial showed a significant increase in platelet counts in all patients at 1 year after GT in the range of mixed-myeloid chimerism after HSCT. This finding was stable over time and accompanied by reduction of bleeding events and platelet transfusion independence (F. Ferrua, M. P. Cicalese, et al, unpublished data). Of note, the presence and/or persistence of anti-platelet autoantibodies has been observed in some treated patients, without any direct correlation with platelet counts (data not shown). However, because the tests used to evaluate the presence of these autoantibodies do not provide a titer or specificity indication, no definitive conclusion regarding the effect of these autoantibodies on platelet reconstitution can be drawn thus far.

Although clinical benefits in terms of immunologic reconstitution have been reported in those patients, no in-depth evaluation of platelet function in GT-treated patients has ever been performed. Furthermore, this analysis could provide relevant information, particularly from the perspective of a future clinical trial in patients with XLT.

Here we demonstrate that patients with untreated WAS have smaller and dysmorphic platelets compared with healthy subjects. Normalization of platelet size and restoration of ultrastructure were found in GT-treated patients.

In terms of phenotype, levels of both CD62P, which is involved in phagocyte-mediated platelet elimination,[36-41] and CD40L, platelets of which are the major source in circulation[42] and which is implicated in autoimmune induction,[43-45] were found to be increased at steady state in pre-GT patients. In line with this, plasma levels of BAFF, a cytokine that sustains autoreactive B cells, were increased before GT and normalized after treatment. Consistent with the greater expression of α-granule–derived activation markers (CD62P and CD40L), also αIIbβ3 integrin activation (PAC1) was increased in patients with untreated WAS and normalized after therapy.

Steady-state hyperactivation in platelets isolated from untreated patients or at early time points after GT might be due to (1) a general inflammatory state consequent to impaired antigen clearance; (2) a decreased ATP level that hampers maintenance of a proper Ca^{2+} and ion balance, thus preventing activation in circulation; (3) increased ROS production, which can in turn activate platelets; and (4) high sCD40L levels in circulation, which can activate platelets in an autoamplification loop.[46] Also, platelets isolated from pre-GT patients had lower metabolic activity, as indicated by downregulation of many proteins involved in carbohydrates and lipid processing. This might lead to decreased ATP production and increased senescence, as also suggested by previously observed greater phosphatidylserine exposure in $WASp^-$ platelets both in murine and human samples.[22,47]

In particular, endogenous platelet-derived ROS or exogenous ROS produced under inflammatory conditions, which can be present in patients with WAS, might influence platelet function[48] by decreasing platelet reactivity to stimuli[49,50] and amplifying the activation loop of sCD40L.[51] Moreover, platelets express several receptors for complement components, mediating activation of both classical and alternative complement pathways that can in turn activate platelets.[52]

Platelet function has been found to be defective in patients with untreated WAS. Using proteomics, we found that $WASp^-$ platelets showed decreased levels of integrins, signal molecules, and vesicle-transport related proteins. Dysregulated expression of these proteins associated with decreased metabolic activity indicates an intrinsic defect of platelet activity and function in the absence of WASp. In line with this, $WASp^-$ platelets do not properly respond to ADP in terms of CD62P expression, reflecting exhaustion, deregulation of vesicles and granule transport, and decreased signal transduction. On the other hand, we observed that inside-out signaling leading to integrin affinity maturation (evaluated by PAC1 binding) occurs normally in pre-GT patients, confirming previous findings.[53] However, the outside-in integrin signal, which triggers intracellular events resulting in platelet aggregation,[54] was found to be defective, corroborating the nonredundant role of WASp in regulating late events of platelet function.[21] In post-GT patients both platelet activation and aggregation were comparable with that in control subjects.

Topological analyses showed that in $WASp^-$ platelets, intracellular signaling mainly involves the proteins STAT3 and HMGB1, which are likely to counteract the downregulation of many molecules involved in classical signal transduction pathways. STAT3 was recently described to act as a protein scaffold in proinflammatory cytokine IL-6–mediated platelet activation, linking platelet hyperactivity to inflammation.[55] HMGB1 has been widely described as a mediator of platelet function[56] inducing a paracrine and autocrine activation loop.[57]

In conclusion, our study demonstrates that $WASp^-$ platelets are dysfunctional and more susceptible to peripheral elimination. The absence of WASp in platelets leads to a general downregulation of protein expression and reorganization of protein hierarchy in signal transduction. These events are associated with the activated phenotype in terms of surface markers at steady state, along with defective platelet function in terms of activation and aggregation after stimulation. The activation state can induce increased senescence, contributing to their peripheral elimination. In line with this, splenectomized patients show increased platelet counts without life-saving treatment. GT is able to restore protein expression and platelet function. Bleeding protection is likely mediated by $WASp^+$ platelets, which, although reduced in number, are able to exert their function according to WASp expression.

Our data highlight novel mechanisms that render WASp-deficient platelets more activated and susceptible to their turnover and elimination. These defects are present in patients with untreated WAS/XLT, irrespective of the type of mutation, confirming that the absence of WASp in platelets is *per se* sufficient to induce thrombocytopenia in all patients and confirming

recent observation indicating life-threating complications in patients with XLT who can easily progress to more severe forms with substantial risk of severe disease-related complications.[23,58] Furthermore, we demonstrate that despite platelet counts remaining less than normal, their ultrastructure, activation, and function dramatically improve after GT treatment, conferring protection from bleeding events and platelet transfusion independence.

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Key messages

- **This first long-term FU study of platelet reconstitution in patients with WAS treated by using LV GT demonstrates protection from bleeding.**
- **GT restores the phenotype, function, and proteomic profile of platelets early after treatment and stably over time.**

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