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# Flow studies on human GPVI-deficient blood under coagulating and noncoagulating conditions

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## Key Points

- GPVI regulates aggregation and PS exposure on collagen and noncollagen surfaces under flow.
- The estimated frequency of the (c.711\_712insA) variant in GP6 in Chile is 2.9%.

The role of glycoprotein VI (GPVI) in platelets was investigated in 3 families bearing an insertion within the *GP6* gene that introduces a premature stop codon prior to the transmembrane domain, leading to expression of a truncated protein in the cytoplasm devoid of the transmembrane region. Western blotting and flow cytometry of *GP6*<sup>hom</sup> (homozygous) platelets confirmed loss of the full protein. The level of the Fc receptor  $\gamma$ -chain, which associates with GPVI in the membrane, was partially reduced, but expression of other receptors and signaling proteins was not altered. Spreading of platelets on collagen and von Willebrand factor (which supports partial spreading) was abolished in *GP6*<sup>hom</sup> platelets, and spreading on uncoated glass was reduced. Anticoagulated whole blood flowed over immobilized collagen or a mixture of von Willebrand factor, laminin, and rhodocytin (noncollagen surface) generated stable platelet aggregates that express phosphatidylserine (PS). Both responses were blocked on the 2 surfaces in *GP6*<sup>hom</sup> individuals, but adhesion was not altered. Thrombin generation was partially reduced in *GP6*<sup>hom</sup> blood. The frequency of the *GP6*<sup>het</sup> (heterozygous) variant in a representative sample of the Chilean population (1212 donors) is 2.9%, indicating that there are ~4000 *GP6*<sup>hom</sup> individuals in Chile. These results demonstrate that GPVI supports aggregation and PS exposure under flow on collagen and noncollagen surfaces, but not adhesion. The retention of adhesion may contribute to the mild bleeding diathesis of *GP6*<sup>hom</sup> patients and account for why so few of the estimated 4000 *GP6*<sup>hom</sup> individuals in Chile have been identified.

## Introduction

Glycoprotein VI (GPVI) is a member of the immunoglobulin receptor superfamily and a major signaling receptor in platelets for collagen, fibrin, and fibrinogen.<sup>1</sup> GPVI is associated with the Fc receptor (FcR)  $\gamma$ -chain in the membrane. Clustering of GPVI leads to Src family kinase-mediated phosphorylation of a conserved immunoreceptor tyrosine-based activation motif in the FcR  $\gamma$ -chain and binding of the tyrosine kinase Syk through its tandem SH2 domains. This initiates a signaling cascade that culminates in activation of phospholipase C- $\gamma$ 2 (PLC- $\gamma$ 2) and Ca<sup>2+</sup> mobilization, integrin  $\alpha$ IIb $\beta$ 3 activation, granule secretion, and phosphatidylserine (PS) exposure, providing a surface for thrombin generation.<sup>2</sup>

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Several unrelated patients homozygous for an adenine insertion (c.711\_712insA) in exon 6 of the *GP6* gene have been identified in Chile. The insertion generates a premature stop codon prior to the transmembrane region, leading to expression of a truncated protein of ~50 kDa that is retained in the cytosol. In 2013, Matus et al<sup>3</sup> described the phenotype of 5 *GP6*<sup>hom</sup> individuals, 3 females (aged 22, 11, and 5 years) and 2 males (aged 23 and 12 years). All of the individuals had a normal platelet count, and 4 had a mild bleeding diathesis, with the fifth, the 5-year-old girl, being asymptomatic. Symptoms started in early childhood and consisted of mild mucosal and skin bleeding. The Ivy bleeding time, recorded for 2 individuals, was 9 and 13 minutes, thus similar or above the normal level (9.5 min) and consistent with a mild bleeding diathesis. All hemostatic parameters and plasma von Willebrand factor (VWF) activity were within the normal range.<sup>3</sup>

The lack of GPVI surface expression on *GP6*<sup>hom</sup> platelets was shown by flow cytometry using an antibody against the extracellular region of GPVI.<sup>3</sup> The expression of the truncated protein in the cytosol was shown using immunofluorescence in permeabilized platelets and by western blotting. *GP6*<sup>hom</sup> platelets failed to aggregate or secrete <sup>14</sup>C-serotonin in response to the GPVI agonists collagen, convulxin, and C-reactive protein (CRP), whereas their response to arachidonic acid and adenosine 5'-diphosphate was unaltered.<sup>3</sup> *GP6*<sup>het</sup> relatives did not exhibit signs of bleeding<sup>3</sup> and displayed normal aggregation and secretion upon stimulation with collagen and convulxin.<sup>3</sup> In 2 *GP6*<sup>het</sup> individuals, the response to CRP was reduced, which is in accordance with a previously reported effect in heterozygous mouse platelets.<sup>4</sup> The loss of response to CRP in the *GP6*<sup>het</sup> individuals is likely due to loss of avidity, whereas for collagen, this is masked by the presence of integrin  $\alpha 2\beta 1$ .

Since 2013, additional *GP6*<sup>hom</sup> individuals have been identified in Chile, bringing the total to 9 from 8 unrelated families. With no consanguinity and the locations of the families being geographically disperse over several hundred kilometers, the heterozygous (carrier) frequency for c.711\_712insA may be relatively high in Chile. These are the only identified individuals worldwide with a homozygous variant that prevents surface expression of GPVI and as such represent a unique resource to study the role of the immunoglobulin receptor in platelet activation.

In this study, we have measured platelet activation on collagen and noncollagen (a mixture of rhodocytin, laminin, and VWF) surfaces under static and flow conditions in 3 *GP6*<sup>het</sup> and 4 *GP6*<sup>hom</sup> individuals from 3 unrelated families. The results show that GPVI is critical for spreading under static conditions on a variety of surfaces and for aggregation and PS exposure, but not for adhesion on collagen and noncollagen surfaces under flow. Given the unique appearance of *GP6*<sup>hom</sup> patients in the Chilean population, we have sequenced exon 6 of 1212 DNA samples representative of the Chilean population<sup>5</sup> and determined a carrier frequency of 2.9% for this variant.

## Methods

### Ethical statement

Experiments involving blood samples and DNA from Chilean patients were approved by the Ethical Scientific Committee at the Pontificia Universidad Catolica de Chile and were conducted in accordance with the guidelines of the National Commission on

Science and Technology of Chile. Informed consent was obtained according to the guidelines of the local ethics committee and complied with the ethical principles according to the Declaration of Helsinki. The experiments on mice were performed in line with UK Home Office approval (see supplemental Materials and methods).

### Patients

The major patient bleeding features, including a Bleeding Assessment Tool,<sup>6</sup> are reported in supplemental Table 1.

### Reagents and samples

The list of reagents including the control and GPVI-null mouse platelets are reported in supplemental Materials and methods.

### Blood withdrawal and platelet preparation

Blood was taken with 3.2% sodium citrate (ratio 1:9) as the anticoagulant. Blood was taken from 3 families with a total of 4 *GP6*<sup>hom</sup> and 3 *GP6*<sup>het</sup> individuals. In 2 individuals, blood was drawn on 2 occasions separated by 9 months. All family members had been genotyped for a predicted adenine insertion between positions 711 and 712 of exon 6. Blood was taken from 4 healthy, unrelated individuals on the same experimental days. Washed platelets were separated from plasma by centrifugation, resuspended in a modified Tyrode's buffer, and recentrifuged before final suspension (see supplemental Materials and methods).

### Western blotting

Platelets were mixed with sodium dodecyl sulfate reducing sample buffer and proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. The antibodies and concentrations are described in supplemental Materials and methods.

### Platelet spreading

Glass coverslips were coated with collagen (10  $\mu\text{g}/\text{mL}$ ) and VWF (100  $\mu\text{g}/\text{mL}$ ) overnight at 4°C and blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 60 min at room temperature before washing with PBS. Washed platelets were spread on coated and uncoated coverslips for 45 minutes at 37°C. The coverslips were washed in PBS, and adherent platelets were fixed in 10% formalin, permeabilized with 0.1% Triton X-100 (in PBS), and stained with Alexa Fluor 488-Phalloidin. Platelets were imaged on a Zeiss LSM7 microscope.

Platelet measurements including area were calculated using a semiautomated machine learning-based workflow<sup>7</sup> and implemented using the open source software KNIME<sup>8</sup> and ilastik.<sup>9</sup> First, a pixel classifier was trained within ilastik to produce binary segmentations. The classifier was then run on the full data set within KNIME and touching platelets were identified manually by clicking on their center. A watershed transformation was then used to extract cellular boundaries facilitating the calculation of per-platelet measurements including area and circularity. Any objects  $< 1 \mu\text{m}^2$  were discarded. The measurements for each platelet were then used to train a random forest classifier to automatically group platelets into predefined subtypes, specifically nonspread, partially spread, and fully spread.

## Flow studies

Thrombus formation was assessed in whole blood.<sup>10</sup> Glass coverslips were coated with collagen (50  $\mu\text{g}/\text{mL}$ ) or a combination of human VWF (50  $\mu\text{g}/\text{mL}$ ), laminin (100  $\mu\text{g}/\text{mL}$ ), and rhodocytin (250  $\mu\text{g}/\text{mL}$ ) before blocking with 1% bovine serum albumin in *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.45). Citrated whole blood, recalcified with  $\text{CaCl}_2$  (7.5 mM) and  $\text{MgCl}_2$  (3.75 mM), in the presence of PPACK was perfused for 4 minutes at  $1000 \text{ s}^{-1}$  and then labeled by perfusion with AF647-Annexin-A5 (1:200) in a modified *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.45) supplemented with  $\text{CaCl}_2$  (2 mM) and heparin (1 U/mL) for 1.5 minutes. Phase-contrast and fluorescence images were taken for analysis of surface area coverage of adherent platelets and PS exposure. For studies under coagulating conditions,<sup>11</sup> citrated whole blood without PPACK was perfused over slides coated with collagen (50  $\mu\text{g}/\text{mL}$ ) and recombinant tissue factor (500 pM), and simultaneously, PS exposure was labeled with AF647-Annexin-A5. Image analysis was performed by using predefined scripts<sup>12,13</sup> in the open source software Fiji,<sup>14</sup> and contraction was assessed by visual inspection compared with representative images.<sup>12</sup>

## Thrombin generation

Thrombin generation in platelet-rich plasma (PRP) was assessed by continuous assessment of thrombin generation using a calibrated automated thrombogram as described.<sup>15</sup> In brief, the PRP platelet count was adjusted to  $150 \times 10^9/\text{L}$  with autologous platelet-poor plasma. PRP (80  $\mu\text{L}$ ) was supplemented with tissue factor (20  $\mu\text{L}$ ) and fluorogenic substrate plus  $\text{CaCl}_2$  (20  $\mu\text{L}$ ) to trigger thrombin generation. The resultant curves were analyzed, and results are expressed as endogenous thrombin potential (ETP).

## Exon 6 genotyping

Genomic DNA was extracted from the whole blood of 1235 individuals, representative of the Chilean population as previously described.<sup>5</sup> PCR amplification was performed across the *GP6* exon 6 variant region using the following primers: 3'-CTCAAAGGGGAATGGAGATA-5' and 5'-AAGAGAGAGCTCCGTCTCAC-3' (as used in Matus et al<sup>3</sup>). DNA sequencing was performed using the BigDye Terminator sequencing kit v3.1 and run on the 3730 DNA analyzer. Sequencing results were analyzed using SnapGene Software.

## Statistics

Data are represented as mean  $\pm$  standard deviation (SD). Statistical analysis was performed using GraphPad Prism v8 software (San Diego, CA). Significance was determined using unpaired Student *t* test or a 1-way ANOVA with Tukey posttest, as appropriate. Differences with  $P < .05$  were considered significant.

## Results

### *GP6*<sup>hom</sup> donors have a reduced level of the FcR $\gamma$ -chain

The presence of a premature stop in *GP6* results in the formation of a truncated protein of  $\sim 50$  kDa, which lacks the transmembrane and is retained in the cytosol.<sup>3</sup> Consistent with this, the expression of full-length GPVI was reduced and abolished in *GP6*<sup>het</sup> and *GP6*<sup>hom</sup> individuals, respectively, as shown by western blotting with

an antibody against the cytoplasmic tail of GPVI (Figure 1A). Flow cytometry revealed that surface expression of GPVI was reduced and abolished in *GP6*<sup>het</sup> and *GP6*<sup>hom</sup> individuals, respectively (not shown), as previously described.<sup>3</sup> The level of the FcR  $\gamma$ -chain was reduced by 40% to 65% relative to controls in 3 *GP6*<sup>hom</sup> individuals (Figure 1B). The stimulation of whole tyrosine phosphorylation, including Syk (525/526), LAT (200), and PLC $\gamma$ 2 (1217), by collagen was abolished in *GP6*<sup>hom</sup> platelets (Figure 1C). The levels of various membrane proteins (GPIb $\alpha$ , GPIIb, and CLEC-2), signaling proteins (Syk, LAT, Btk, RhoA, and PLC $\gamma$ 2), and  $\alpha$ -tubulin were similar among control, *GP6*<sup>het</sup>, and *GP6*<sup>hom</sup> individuals (Figure 1A-E).

These results confirm the previous reports of reduction and loss of full-length GPVI in *GP6*<sup>het</sup> and *GP6*<sup>hom</sup> platelets and demonstrate that the level of the FcR  $\gamma$ -chain is also reduced. This decrease was not anticipated, as the level has been reported to be unchanged in *GP6*<sup>hom</sup> mouse platelets.<sup>16</sup> However, on repeating these studies, we observed a small reduction ( $\sim 15\%$ ) in *GP6*<sup>hom</sup> mice platelets, which may be due to increased degradation (supplemental Figure 1). It is unclear to what extent the decrease in the FcR  $\gamma$ -chain contributes to the clinical phenotype of the patients.

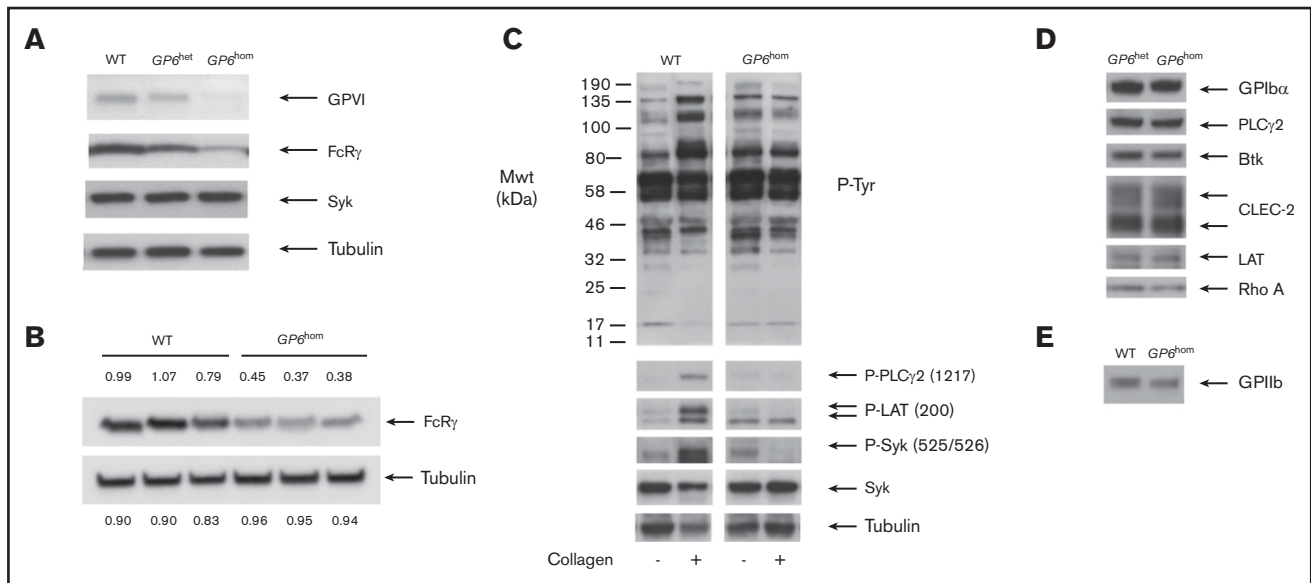
### Spreading on collagen, VWF, and glass

Adhesion and spreading were investigated under static conditions on surfaces coated with collagen, VWF, and glass (uncoated), with the latter serving as a representative charged surface. The majority of platelets from controls generated filopodia and lamellipodia on collagen and on glass (Figure 2A-B). The small number of partially and nonspread platelets on both surfaces is likely to represent cells that have recently adhered (Figure 2A). In contrast, only one-third of platelets underwent partial spreading on VWF (Figure 2A). Spreading of *GP6*<sup>hom</sup> platelets on collagen and VWF was abolished and markedly reduced on uncoated glass, whereas adhesion was not altered on all 3 surfaces (Figure 2A-B), indicating involvement of additional receptors, such as integrin  $\alpha 2\beta 1$  for collagen and GPIb-IX-V and integrin  $\alpha \text{IIb}\beta 3$  for VWF. Spreading, but not adhesion, was reduced in *GP6*<sup>het</sup> platelets on all 3 surfaces, although for collagen, this was less marked than for *GP6*<sup>hom</sup> platelets (Figure 2B). These results demonstrate that GPVI is essential for spreading, but not adhesion, of platelets on collagen, VWF, and a charged surface.

### Platelet aggregation under noncoagulating flow conditions

Experiments were undertaken to evaluate the role of GPVI in adhesion and aggregation of noncoagulated blood under arterial shear. Coagulation was inhibited by citrate and PPACK. Whole blood was flowed over collagen and noncollagen (mixture of VWF, laminin, and rhodocytin) surfaces at a shear rate of  $1000 \text{ s}^{-1}$ , and platelet adhesion and aggregation were analyzed along with contraction score. The contraction score was determined based on a predefined set of example images and depicts the tightness of the aggregate.<sup>10,17</sup>

Blood from *GP6*<sup>het</sup> donors form robust, large aggregates on collagen with a similar surface area coverage and contraction score to that of controls, although PS exposure is reduced by  $\sim 50\%$  (Figure 3A). In contrast, only single platelets adhere and small aggregates form on collagen in *GP6*<sup>hom</sup> blood (Figure 3A). This is accompanied by a reduction in abrogation of PS exposure, although adhesion is not altered (Figure 3A). This demonstrates a critical role



**Figure 1. Protein expression and tyrosine phosphorylation in control and  $GP6^{hom}$  platelets.** (A) Western blot showing levels of GPVI, FcR $\gamma$ , Syk, and tubulin (loading control) in control,  $GP6^{het}$ , and  $GP6^{hom}$  individuals. (B) Densitometry reading of the level of FcR $\gamma$  and tubulin (loading control) in a  $GP6^{hom}$  individual relative to a control (the  $GP6^{hom}$  individual is not related to the donor in panel A). (C) Collagen (30  $\mu$ g/mL) stimulation of tyrosine phosphorylation in a control and  $GP6^{hom}$  individual (90-second stimulation). (D) Western blot of representative proteins from  $GP6^{het}$  and  $GP6^{hom}$  donors. (E) Western blot of GPIIb from control and  $GP6^{hom}$  donors. Results are representative of 3 (A-C) or 4 (D-E) unrelated  $GP6^{hom}$  individuals. Mwt, molecular weight; WT, wild type.

of GPVI in aggregation and PS exposure, but not in adhesion, on a collagen surface.

A similar degree of surface coverage to that on collagen was observed for whole blood on a mixture of VWF, laminin, and rhodocytin (Figure 3B). However, in contrast to the collagen surface, coverage consisted of smaller aggregates and single platelets, resulting in a lower contraction score. The proportion of PS-positive platelets was  $\sim$ 50% of that observed on collagen (Figure 3B). The surface coverage of blood from  $GP6^{het}$  and  $GP6^{hom}$  donors was similar to that of controls on the noncollagen surface, although the number of single platelets was increased, notably in  $GP6^{hom}$  blood (Figure 3B). The exposure of PS was abrogated on both surfaces (Figure 3B). Thus, GPVI is also important for aggregation and PS exposure, but not adhesion, on a noncollagen surface.

To investigate whether the adhesion of  $GP6^{hom}$  platelets on collagen is through the second platelet receptor for collagen, integrin  $\alpha 2\beta 1$ , experiments were performed using the blocking monoclonal antibody (mAb) 6F1.<sup>18</sup> mAb 6F1 abrogated adhesion of  $GP6^{hom}$  blood on collagen (Figure 3Ci), but also partially reduced adhesion on the noncollagen surface (Figure 3Cii). Since there are no reports of binding of VWF, laminin, or rhodocytin to integrin  $\alpha 2\beta 1$ , this may be due to steric hindrance.

These results demonstrate a critical role for GPVI in supporting platelet aggregation and PS exposure in the absence of coagulation on collagen and noncollagen surfaces, with the adhesion of  $GP6^{hom}$  platelets on collagen being mediated by integrin  $\alpha 2\beta 1$ .

### Platelet aggregation under coagulating flow conditions

Additional flow studies were performed at the same arterial shear rate (1000  $s^{-1}$ ) in the presence of recombinant tissue factor and

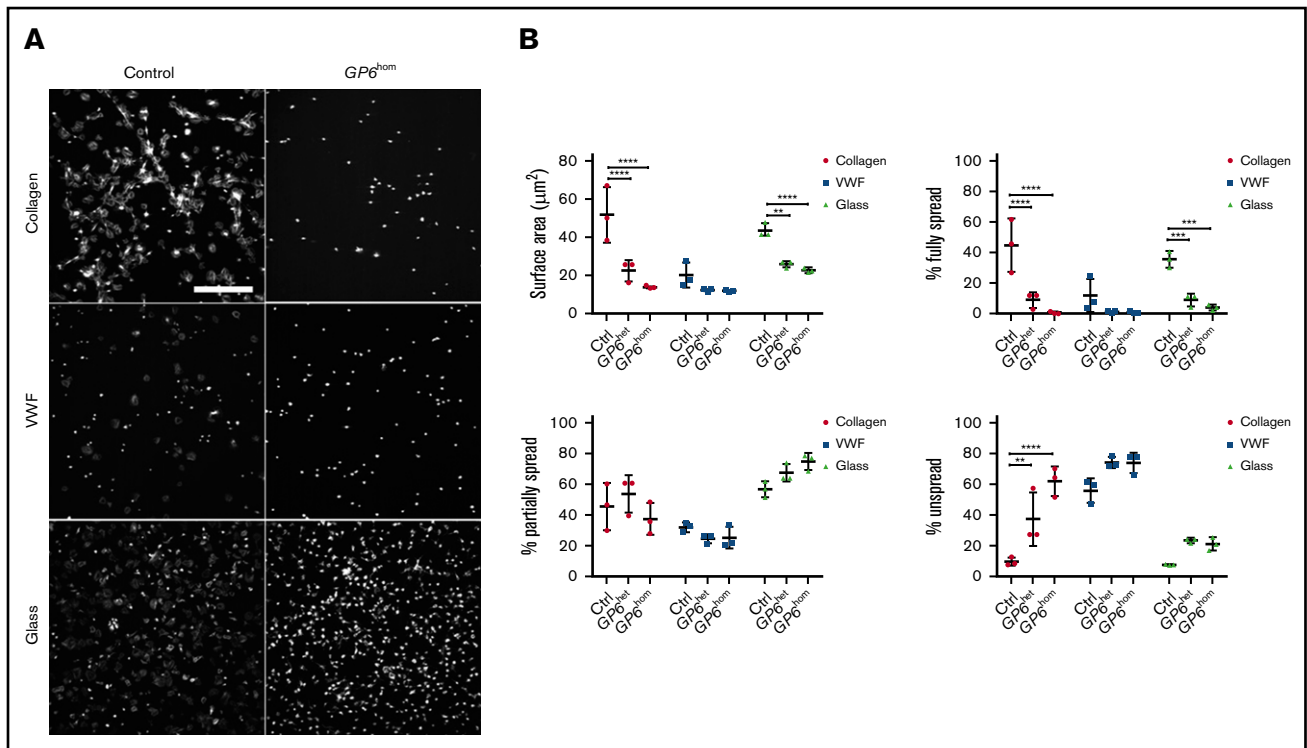
$Ca^{2+}$  (without PPACK) to determine the role of GPVI in the presence of thrombin and fibrin. Under these conditions, fibrin strands were seen to develop in control blood from platelet aggregates on collagen (Figure 4), suggesting that they are catalyzed by platelet-dependent coagulation, as described previously.<sup>11</sup> In contrast, there was no detectable fibrin formation on the noncollagen surface (not shown), and further studies were not performed on this surface.

Large platelet aggregates/thrombi are formed when control blood is flowed over collagen under coagulating conditions (Figure 4). PS exposure is also observed and was similar in magnitude to that on collagen in the absence of coagulation (compare Figures 3A and 4). A similar degree of thrombus formation was observed with  $GP6^{het}$  and  $GP6^{hom}$  blood, but fibrin formation was reduced and abolished, respectively (Figure 4). PS was not altered in  $GP6^{het}$  blood but was abrogated in  $GP6^{hom}$  blood (Figure 4).

These results demonstrate a critical role for GPVI in supporting PS exposure and fibrin formation under coagulating conditions, but not in thrombus formation on collagen at arterial shear.

### Thrombin generation is reduced in $GP6$ -deficient platelets

Using the calibrated automated thrombogram (also known as the CAT assay), we observed a trend toward a reduction in thrombin generation in PRP isolated from  $GP6^{hom}$  individuals, depicted as a reduced peak height and ETP (Figure 5A-C). However, this trend was not seen in other parameters (eg, time to peak), and the ETP was not significantly different, suggesting that there is only a slight decrease in the total amount of thrombin formed in  $GP6^{hom}$  platelets. This partially resembles the results using GPVI-deficient platelets (3 patients with immune thrombocytopenia and 1 compound heterozygote with 2 GPVI variants) and healthy PRP



**Figure 2. Platelet spreading on collagen, VWF, and glass.** (A) Human platelets ( $2 \times 10^7/\text{mL}$ ) were spread on coated and uncoated (not blocked) coverslips as described in "Methods." The images are representative of 3 control and 3  $GP6^{\text{hom}}$  patients. (B) The graphs illustrate quantification of surface area and the percentage of fully, partially spread, or nonspread platelets (control = 3,  $GP6^{\text{het}}$  = 2,  $GP6^{\text{hom}}$  = 3). Measurements are the mean of 48 882 platelets in a replicate (7 fields of view per replicate), and the figures are representative of part of a field of view. Significance was measured using 2-way ANOVA.  $**P < .01$ ,  $***P < .001$ ;  $****P < .0001$ . The surface area in the field of view is  $213 \times 213 \mu\text{m}$ . Scale bar,  $50 \mu\text{m}$ .

pretreated with GPVI-blocking Fab 9012. Mammadova-Bach et al<sup>19</sup> reported a significant decrease in peak height in GPVI-deficient patients, while our results only show a trend toward a decrease. This difference is likely to be due to the partial nature of the decrease and the low number of patient samples and the variation in response between donors. A similar level of reduction in thrombin generation was seen in PRP from  $GP6^{\text{het}}$  relative to  $GP6^{\text{hom}}$  individuals.

### Frequency of the $GP6$ c.711\_712insA variant in the Chilean population

To investigate the frequency of the c.711\_712insA  $GP6$  variant in the Chilean population, sequencing of  $GP6$  exon 6 was performed on 1235 DNA samples. Samples represent mixed Chilean Latinos with Mapuche Native American ancestry, as described previously.<sup>5</sup> Of these, 23 samples gave sequencing traces that were of too poor quality to analyze and were excluded. Of the remaining 1212 samples, a total of 36 were found to be heterozygous for the c.711\_712insA variant (supplemental Figure 2), equivalent to 2.9% of the total analyzed. No  $GP6^{\text{hom}}$  samples were identified.

### Discussion

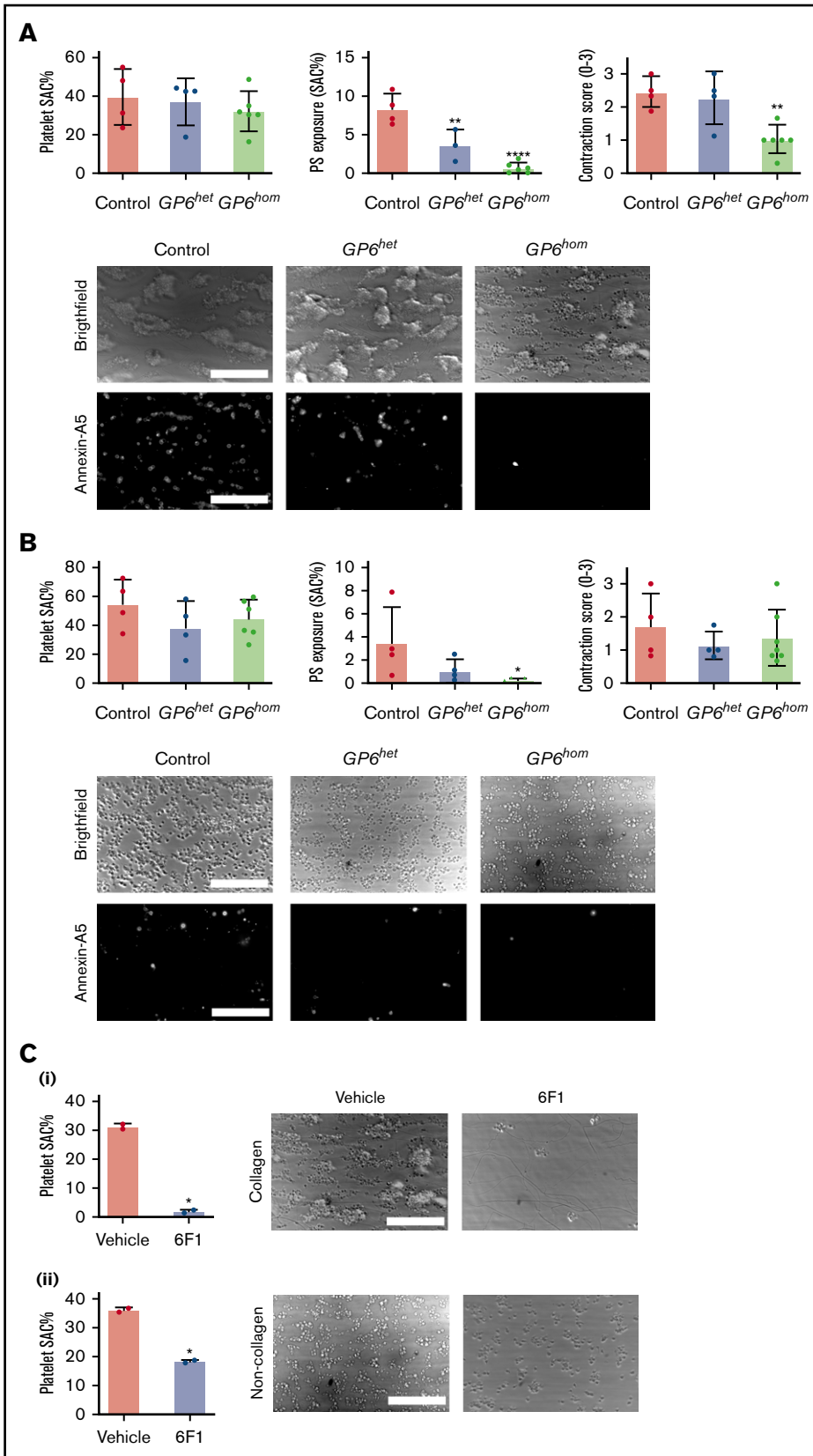
This study demonstrates (1) a critical role for GPVI in aggregation and PS exposure, but not adhesion, in human platelets flowed at arteriolar shear over collagen and noncollagen surfaces under noncoagulating conditions; and (2) a critical role for GPVI in PS exposure, but not thrombus formation, on collagen under

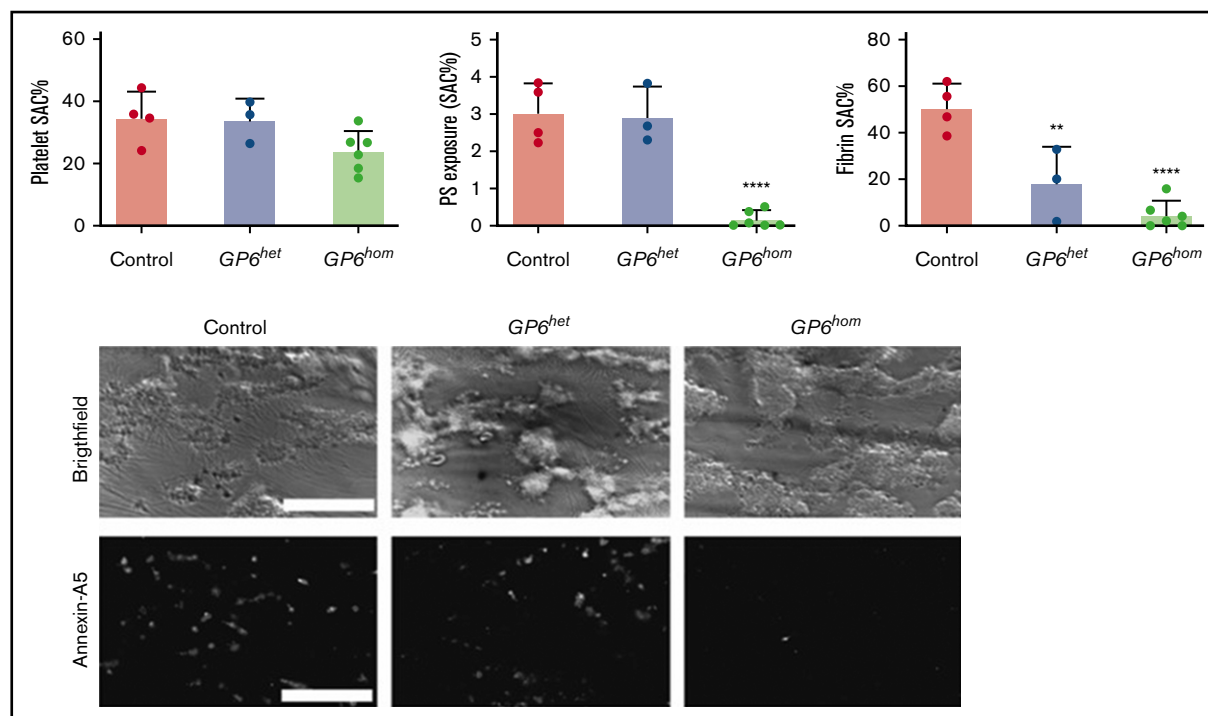
coagulating conditions. In addition, this study shows that spreading of platelets on collagen, VWF, and uncoated glass, as well as thrombin formation, is reduced or abolished in  $GP6^{\text{hom}}$  platelets. Together, these results show that the role of GPVI in supporting aggregation at arteriolar shear extends to noncollagen surfaces and that GPVI is critical for PS exposure and supporting thrombin formation.

GPVI has long been recognized as a collagen receptor, but more recently, it has been shown to serve as a receptor for further ligands in the vasculature and vessel wall, including fibrinogen, fibrin, fibronectin, vitronectin, and laminin.<sup>1,19-21</sup> In addition, GPVI has been shown to associate with GPIIb $\alpha$  in the membrane<sup>22</sup> and support thrombus formation and platelet adhesion to immobilized VWF at arterial and venous shear.<sup>23</sup> VWF stimulates tyrosine phosphorylation of the FcR  $\gamma$ -chain,<sup>24,25</sup> and treatment with an anti-GPVI antibody reduces FcR  $\gamma$ -chain and Syk phosphorylation upon ristocetin stimulation.<sup>23</sup> One or more of these interactions could explain the reduction in spreading on VWF and platelet aggregation and PS exposure on a mixture of VWF, laminin, and rhodocytin. Alternatively, the reduction in these responses could be due to the  $\sim 50\%$  reduction in the FcR  $\gamma$ -chain that is seen in  $GP6^{\text{hom}}$  platelets, and this could be tested in transgenic mice that are heterozygous for the FcR  $\gamma$ -chain. Aggregation to botracetin/VWF is abolished in mouse platelets deficient in FcR  $\gamma$ -chain-deficient but not GPVI-deficient mouse platelets.<sup>26</sup>

**Figure 3. GPVI deficiency leads to abolished PS exposure under flow in the absence of coagulation.**

Whole blood from control,  $GP6^{het}$ , and  $GP6^{hom}$  subjects was recalcified in presence of PPACK and perfused over collagen (A) and noncollagen (B) (laminin, VWF, and rhodocytin) surfaces. Surface area coverage (SAC), PS exposure, and contraction score are presented as mean  $\pm$  SD; control = 4,  $GP6^{het}$  = 4, and  $GP6^{hom}$  = 6. \*\*\*\* $P$  < .0001, \*\* $P$  < .01, \* $P$  < .05. Representative bright-field and Alexa Fluor 647-Annexin A5 images are shown. Images were taken at the end point (8 min) after labeling was performed. (C) Whole blood from  $GP6^{hom}$  patients treated with the monoclonal antibody 6F1 (10  $\mu$ g/mL) was recalcified and perfused over collagen (i) and noncollagen (ii) surfaces. Quantification of surface area coverage is presented as mean  $\pm$  SD.  $GP6^{hom}$  = 2. Representative bright-field images are shown. Scale bars, 50  $\mu$ m.



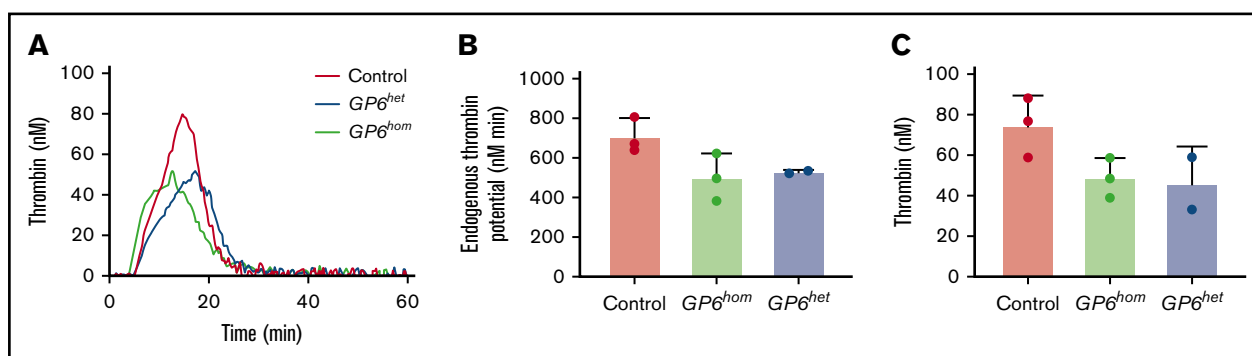


**Figure 4. GPVI deficiency leads to abolished PS exposure under flow.** Whole blood from control,  $GP6^{hom}$ , and  $GP6^{het}$  individuals was recalcified and perfused over a collagen surface cocoated with tissue factor. Quantification of surface area coverage (SAC), PS exposure, and fibrin surface area coverage is shown. Data are shown as mean  $\pm$  SD; control = 4,  $GP6^{het}$  = 3, and  $GP6^{hom}$  = 6. \*\*\*\* $P$  < .0001, \*\* $P$  < .01. Representative bright-field and Alexa Fluor 647-Annexin A5 images taken after 6 minutes of blood perfusion are shown from a control and a  $GP6^{hom}$  subject on a collagen surface. Scale bars, 50  $\mu$ m.

The present study also reports that spreading of  $GP6^{hom}$  platelets is reduced on uncoated glass, suggesting that the GPVI is activated by a charge interaction. Previously, we have shown that GPVI can be activated by a diverse group of charged ligands, including diesel exhaust particles, polysulfated sugars such as fucoidan and dextran sulfate, and histones.<sup>20</sup> These ligands are structurally distinct and have no resemblance to endogenous ligands, consistent with crosslinking of GPVI and platelet activation being mediated by a charge interaction. The role of GPVI in spreading on glass raises the possibility that other endogenous ligands may activate GPVI through a charge interaction, including ligands from the platelet releasate, and this

could potentially also contribute to the reduction in aggregation and PS exposure.

The role of GPVI in supporting platelet aggregation on collagen in the absence of coagulation is well documented.<sup>27,28</sup> However, it is only recently that conditions have been developed that permit the study of platelet aggregation on collagen in the absence of thrombin inhibition.<sup>11</sup> The observation therefore that GPVI is critical for platelet aggregation and PS exposure under both coagulating and noncoagulating conditions highlights the critical role of the glycoprotein receptor in thrombus formation. The retention of adhesion and formation of small aggregates on the 2 surfaces is



**Figure 5. GPVI deficiency leads to a partial reduction in thrombin generation.** Thrombin generation assay was performed in platelets from control,  $GP6^{het}$ , and  $GP6^{hom}$  patients. (A) Thrombin generation curves from a control,  $GP6^{het}$ , and  $GP6^{hom}$  individual are shown. (B) ETP is shown. (C) Peak height is shown. Data are presented as mean  $\pm$  SD; control = 3,  $GP6^{het}$  = 2, and  $GP6^{hom}$  = 3.



explained by the presence of a second receptor for collagen, integrin  $\alpha 2\beta 1$ , and by receptors for rhodocytin (CLEC-2), laminin ( $\alpha 6\beta 1$ ), and VWF (GPIIb-IX-V). The observation that adhesion of  $GP6^{hom}$  platelets is retained on collagen is consistent with previous reports of integrin  $\alpha 2\beta 1$  supporting platelet adhesion to collagen in human platelets under flow.<sup>18,29-33</sup> The retention of adhesion to collagen by integrin  $\alpha 2\beta 1$ , in combination with the vascular wall damage that leads to the exposure of negatively-charged phospholipid on the cell surface thus leading to tissue factor driven thrombin formation, could account for the relatively minor bleeding phenotype of  $GP6^{hom}$  individuals. The role of integrin  $\alpha 2\beta 1$  in supporting adhesion of human platelets in the absence of GPVI is in contrast to mice, in which adhesion to collagen is abolished in the absence of GPVI.<sup>34</sup> This difference could be due to the affinity of nonactivated integrin  $\alpha 2\beta 1$  for collagen, the relative density of GPVI and  $\alpha 2\beta 1$  on the platelet surface, or the difference in size of mouse and human platelets.

The present results demonstrate that the degree of platelet PS exposure is governed by the level of GPVI expression, as a marked defect was seen in  $GP6^{het}$  and  $GP6^{hom}$  individuals under coagulating and noncoagulating conditions. The reduction in platelet procoagulant activity was associated with a slight decrease in thrombin formation. This is in agreement with the results of Mammadova-Bach et al using GPVI-deficient platelets and a GPVI-blocking Fab.<sup>19</sup> GPVI signals synergize with Gq family G protein-coupled receptors to induce PS exposure and a procoagulant surface.<sup>2,35,36</sup>

The potential significance of the present findings should be considered in light of the frequency of the  $GP6$  c.711\_712insA in the Chilean population. The prevalence of the variant in the Chilean cohort was estimated with a carrier status probability of 0.0297 (36/1212). If we consider this observation, and that the allele frequency for the c.711\_712insA variant is in Hardy-Weinberg equilibrium, then the theoretical prevalence of  $GP6^{hom}$  in Chile could be 1/4534 (ie, the product of  $0.0297 \times 0.0297$  with division by 4) or greater. This result, combined with population data from the last national census, suggests that 4079 individuals with  $GP6^{hom}$  could be currently living in Chile, which is more than double the number of registered hemophilia patients. With such a high predicted prevalence, the question remains as to why so few patients have been identified. This may be due to the limited genetic testing that has been performed in patients with a bleeding disorder and the relatively mild bleeding diathesis caused by loss of GPVI. A bleeding assessment tool (BAT) in  $GP6^{hom}$  individuals is shown in supplemental Table 1 and includes an asymptomatic girl (age 12 years) who is the sister of one of the index cases. This suggests that some homozygous carriers of  $GP6$  c.711\_712insA do not present with bleeding issues. The majority of  $GP6^{het}$  individuals do not have signs of excessive bleeding. This further underscores the interest in targeting GPVI as an antiplatelet therapy.

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An important consideration is whether  $GP6^{hom}$  individuals are protected from thrombosis. As yet, the number and age of the  $GP6^{hom}$  individuals is too few and too low to ascertain whether this is the case. With the relatively high carrier frequency (2.9%), there is a chance that the heterozygous variant is being selected for in the Chilean population. This is unlikely to have conferred a selection advantage in the context of atherosclerosis, as this is a relatively modern disease and relatively late in onset. Whether this has conferred an advantage in other thrombotic-related or other conditions is not known. Larger, longer-term screening of  $GP6^{het}$  and  $GP6^{hom}$  individuals and their cardiovascular clinical manifestations will help to inform their propensity for bleeding and thrombosis.

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## Authorship

Contribution: M.N. and G.P. designed and performed experiments, analyzed and interpreted data, and wrote the manuscript; A.D. performed experiments and wrote the manuscript; L.G.Q. performed experiments; J.A.P. designed and performed the spreading analysis and edited the manuscript; N.V.M. designed the genetic analysis and edited the manuscript; E.E.G. provided essential reagents and edited the manuscript; J.W.M.H. edited the manuscript; D.M. diagnosed and recruited patients and edited the manuscript; S.P.W. designed experiments, supervised research, interpreted data, and wrote the manuscript; M.F.B. organized the patient recruitment, coordinated the blood extraction, performed the thrombin generation assay, and participated in the flow cytometry experiments; and J.F.M. and L.A. selected the subjects representative of the Chilean population, drew blood, and extracted leukocyte DNA.

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