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References

Huseman, D., Gellermann, J., Vollmer, I., Ohde, I., Devaux, S., Ehrich, J.H. & Filler, G. (1999) Long-term prognosis of hemolytic uremic syndrome and effective renal plasma flow. *Pediatric Nephrology*, **13,** 672–677.

Kennedy, A.S., Lewis, Q.F., Scott, J.G., Kremer Hovinga, J.A., Lammle, B., Terrell, D.R., Vesely, S.K. & George, J.N. (2009) Cognitive deficits after recovery from thrombotic thrombocytopenic purpura. *Transfusion*, **49,** 1092–1101.

O'Regan, S., Blais, N., Russo, P., Pison, C.F. & Rousseau, E. (1989) Hemolytic uremic syndrome: glomerular filtration rate, 6 to 11 years later measured by 99mTc DTPA plasma slope clearance. *Clinical Nephrology*, **32,** 217–220.

Small, G., Watson, A.R., Evans, J.H. & Gallagher, J. (1999) Hemolytic uremic syndrome: defining the need for long-term follow-up. *Clinical Nephrology*, **52,** 352–356.

Spizzirri, F.D., Rahman, R.C., Bibiloni, N., Ruscasso, J.D. & Amoreo, O.R. (1997) Childhood hemolytic uremic syndrome in Argentina: long-term follow-up and prognostic features. *Pediatric Nephrology*, **11,** 156–160.

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Restored platelet function after romiplostim treatment in a patient with immune thrombocytopenic purpura

Whilst the precipitating aetiology of immune thrombocytopenic purpura (ITP) remains unclear, the predominant cause of the thrombocytopenia is the presence of circulating anti-platelet autoantibodies that coat platelets, leading to platelet destruction and clearance, primarily in the spleen. Traditional clinical management of patients with ITP, including treatment with corticosteroids, intravenous immunoglobulins, splenectomy, rituximab, and cyclophosphamide, aims to curb platelet destruction (Nurden *et al*, 2009a). Newer reagents approved for trial to treat ITP patients, such as thrombopoietin mimetics, romiplostim (Kuter *et al*, 2008) and eltrombopag, act primarily in the bone marrow to stimulate thrombopoiesis. Whilst neither plasma thrombopoietin levels nor platelet production kinetics are markedly altered in ITP patients, ITP anti-platelet autoantibodies interfere with megakaryocyte proliferation and platelet production *in vitro* (Chang *et al*, 2003). Here we describe a significant improvement to the nature and function of platelet immunoreceptor tyrosine-based activation motif (ITAM) receptors in an ITP patient with an autoantibody to platelet glycoprotein (GP)VI receiving romiplostim.

Restored aggregation response to collagen in patient platelets

Previously, platelets in platelet rich plasma (PRP) isolated from the patient diagnosed with ITP did not aggregate in response to GPVI agonists, collagen and collagen-related peptide, or Fc receptor (FcγRIIa) engagement (Gardiner *et al*, 2008a). A normal response to collagen was achieved in PRP isolated from the ITP patient after 6 months of treatment with romiplostim (3 μg/kg romiplostim weekly for 6 months with normalisation of platelet count) (Fig. 1A). Responses to epinephrine (7 μmol/l), arachadonic acid (1 mmol/l) and ADP (4 μmol/l) were normal (data not shown).

Previously, we demonstrated that a 55-kDa GPVI ectodomain fragment could be cleaved from platelets treated with GPVI agonists or FcγRIIa-activating antibodies, leaving an *c.*10-kDa remnant containing the cytoplasmic tail and transmembrane domains of GPVI associated with the platelet membrane (Gardiner *et al*, 2008b). Resting platelets isolated from healthy donors contain no detectable 10-kDa remnant fragment, although we previously demonstrated cleaved forms of GPVI and FcγRIIa receptors on circulating platelets from the ITP patient (Gardiner *et al*, 2008a). Washed platelets isolated from the ITP patient after 6 months of treatment with romiplostim or from a healthy donor (control) were lysed and levels of GPVI and FcγRIIa were examined by Western blot. Levels of intact GPVI and FcγRIIa in patient platelet lysates were equivalent to levels found in healthy donor platelets as determined by Western blotting with antibodies raised against the cytoplasmic tails of GPVI or FcγRIIa (Fig. 1B) consistent with the restored aggregation response to collagen (Fig. 1A) in the patient platelets. The remnant 10-kDa membrane-associated fragment of GPVI was no longer evident in lysed platelets from the ITP patient (Fig. 1B) implying that autoantibody-induced activation of platelet receptor shedding pathways was markedly attenuated in the ITP patient after romiplostim treatment.

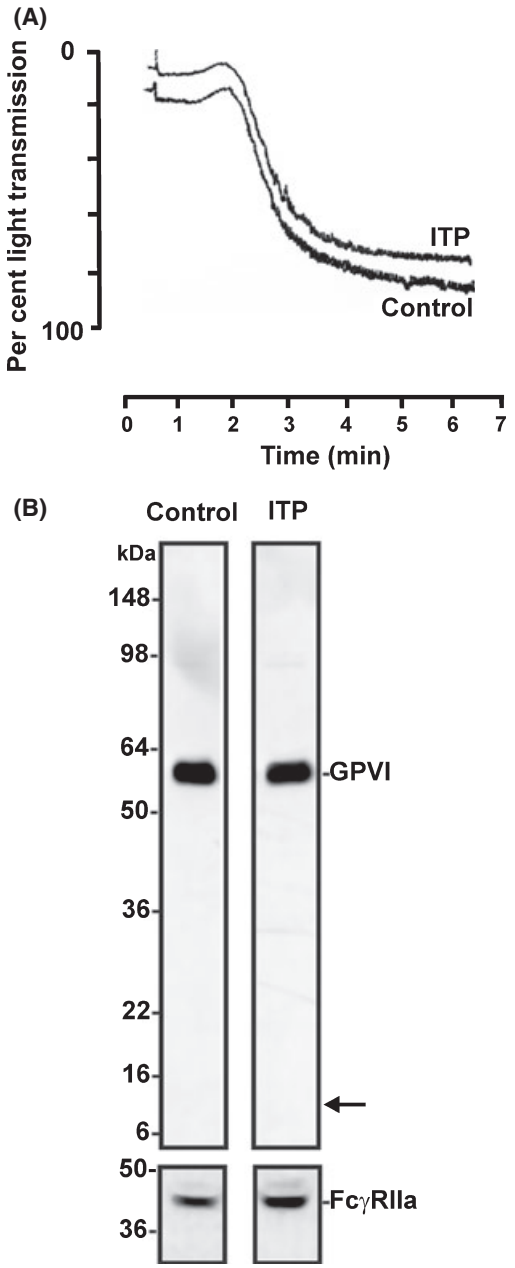

Fig 1. Circulating platelets isolated from an ITP patient respond to collagen and show no evidence of GPVI shedding and cleavage of FcγRIIa. (A) Aggregation of platelets in citrated PRP isolated from a healthy donor control or the patient diagnosed with ITP in response to 5 μg/ml collagen. (B) Equivalent amounts of washed platelets (3×10^8/ml) that had been isolated from either a control or the ITP patient and lysed in sodium dodecyl sulphate (SDS)-containing buffer were examined using 5–20% polyacrylamide/SDS gels and Western blot with antibodies directed against the cytoplasmic tail of GPVI or FcγRIIa that detect both full length and cleaved forms of each receptor. Bound antibody was detected using a horseradish peroxidase-conjugated anti-rabbit secondary antibody and enhanced chemiluminescence. All lanes within each figure came from the same experiment, and the same gel/Western blot. Data are representative of two identical experiments performed on separate days. The arrow indicates the position to where a 10-kDa GPVI cytoplasmic tail remnant would migrate.

Levels of GPVI are normal on patient platelets

In our previous report, levels of intact GPVI on the ITP patient platelets were estimated to be 10–20% of levels on control platelets as detected by flow cytometry using any of 1A12, 4B8 or 1G5 monoclonal anti-GPVI antibodies (Gardiner *et al*, 2008a) (Table I). After romiplostim treatment, levels of GPVI as well as GPIbα and αIIbβ3 (data not shown) were similar to those observed on healthy donor platelets by flow cytometry (Table I). This is in agreement with the observed normal aggregatory response to GPVI agonists and immunoblots detecting a single band corresponding to intact GPVI (Fig. 1). Romiplostim treatment may directly influence expression of GPVI. Kanaji *et al* (2005) reported that thrombopoietin initiated the receptor-mediated demethylation of a cytosine-phosphate-guanosine-rich island within the promotor region of the *GP6* gene in megakaryocytes, upregulating expression of GPVI. Analysis of surface levels of GPVI in a cohort of patients before and after treatment with romiplostim may confirm this.

Levels of soluble GPVI in patient plasma remain elevated

We recently established a sandwich enzyme-linked immunosorbent assay (ELISA) to measure levels of the shed GPVI ectodomain in human citrated-plasma samples (Al-Tamimi *et al*, 2009). Using our assay, the level of soluble GPVI in plasma from healthy donors was *c.*15 ng/ml. Previously, plasma from the ITP patient contained *c.*150 ng/ml soluble GPVI and in this study, levels of soluble GPVI were shown to be *c.*127 ng/ml after romiplostim treatment. Using an ELISA similar to that used to measure anti-GPVI autoantibody in a patient with lupus nephritis (Nurden *et al*, 2009b), we noted that levels of circulating anti-GPVI autoantibody were still detectable after romiplostim treatment (Table I). The explanation for the elevated level of soluble GPVI remains unclear, but may reflect the increased platelet count, an underlying low level of GPVI shedding and a potential prolonged clearance time for soluble plasma GPVI due to its *O*-linked carbohydrate mucin core. Currently, there is no information regarding the stability of soluble GPVI in plasma; however, other members of the immunoglobulin family of adhesion receptors, including vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 are shed from both leucocytes and endothelial cells by metalloproteinases. Soluble ICAM-1 displays resistance to proteolysis by plasmin and proteases present in inflamed synovium in plasma and plasma-soluble VCAM-1 and soluble ICAM-1 display prolonged elevation in inflammatory and myocardial diseases (reviewed in Garton *et al*, 2006). It is possible that soluble GPVI also exhibits an extended lifetime in plasma.

The relatively high level of soluble GPVI in plasma may also reflect platelet ITAM receptor engagement by the patient's anti-GPVI antibody in bone marrow. It is unclear

Table I. Platelet and plasma indicators in a patient with ITP.

	Pre-romiplostim treatment	Romiplostim treatment for 6 months
Platelet count* $\times 10^9$/l	70	>200
Collagen-dependent aggregation	No	Yes
Levels of GPVI on platelets by flow cytometry†	7·9 ± 1·00 (control 97·8 ±1·47)	74·2 ± 13·54 (control 79·1 ± 5·42)
Presence of cleaved GPVI by Western blot	Yes	No
Plasma soluble GPVI‡ (ng/ml)	148 ± 0·4 (control 15 ± 7)	127·2 ± 4·16 (control 26·8 ± 1·55)
Anti-platelet antibody§	Positive	Positive
Anti-GPVI IgG in plasma¶ (μg/ml)	1·216 ± 0·183	2·566 ± 0·199

*Patient platelet count was as low as 2×10^9/l prior to prednisolone treatment.
†Representative geomean fluorescence intensity ± standard deviation (SD) for 1 of 3 anti-GPVI monoclonal antibodies. GPVI levels on healthy donor platelets measured using the same antibody on the same day are in parentheses.
‡Average of triplicate measurements by ELISA (ng/ml ± SD). GPVI levels in plasma from healthy donors isolated on the same day are in parentheses.
§Measured by MAIPA (monoclonal antibody immobilisation of platelet antigens) using patient platelets and serum.
¶Measured by indirect sandwich ELISA using a polyclonal anti-GPVI antibody to capture saturating amounts of GPVI ectodomain followed by incubation with serial dilutions of patient or control plasma and detection using a horseradish peroxidase-conjugated anti-human IgG and enhanced chemiluminescence (Nurden *et al*, 2009b). Data were corrected for background, and relative light units compared with signal from standard amounts of control IgG.

what impact the platelet autoantibody has on shedding activity in the bone marrow of the patient under romiplostim treatment and whether such treatment leads to increased shedding activity under conditions of accelerated platelet production. Nishikii *et al* (2008) observed metalloproteinase-dependent cleavage of platelet receptors GPIbα, GPV and GPVI in murine embryonic stem cells grown in thrombopoietin-containing culture medium, however our data indicates improved platelet GPVI function and no evidence of increased shedding of GPVI on circulating platelets (that is, no detectable 10-kDa remnant fragment). Acquired tolerance of anti-platelet autoantibodies may be an important additional consequence of romiplostim treatment in patients with ITP.

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References

Al-Tamimi, M., Mu, F.T., Moroi, M., Gardiner, E.E., Berndt, M.C. & Andrews, R.K. (2009) Measuring soluble platelet glycoprotein VI in human plasma by ELISA. *Platelets*, **20,** 143–149.

Chang, M., Nakagawa, P.A., Williams, S.A., Schwartz, M.R., Imfeld, K.L., Buzby, J.S. & Nugent, D.J. (2003) Immune thrombocytopenic purpura (ITP) plasma and purified ITP monoclonal autoantibodies inhibit megakaryocytopoiesis in vitro. *Blood*, **102,** 887–895.

Gardiner, E.E., Al-Tamimi, M., Mu, F.T., Karunakaran, D., Thom, J.Y., Moroi, M., Andrews, R.K., Berndt, M.C. & Baker, R.I. (2008a) Compromised ITAM-based platelet receptor function in a patient with immune thrombocytopenic purpura. *Journal of Thrombosis and Haemostasis*, **6,** 1175–1182.

Gardiner, E.E., Karunakaran, D., Arthur, J.F., Mu, F.T., Powell, M.S., Baker, R.I., Hogarth, P.M., Kahn, M.L., Andrews, R.K. & Berndt, M.C. (2008b) Dual ITAM-mediated proteolytic pathways for irreversible inactivation of platelet receptors: De-ITAM-izing FcγRIIa. *Blood*, **111,** 165–174.

Garton, K.J., Gough, P.J. & Raines, E.W. (2006) Emerging roles for ectodomain shedding in the regulation of inflammatory responses. *Journal of Leukocyte Biology*, **79,** 1105–1116.

Kanaji, S., Kanaji, T., Jacquelin, B., Chang, M., Nugent, D.J., Komatsu, N., Moroi, M., Izuhara, K. & Kunicki, T.J. (2005) Thrombopoietin initiates demethylation-based transcription of *GP6* during megakaryocyte differentiation. *Blood*, **105,** 3888–3892.

Kuter, D.J., Bussel, J.B., Lyons, R.M., Pullarkat, V., Gernsheimer, T.B., Senecal, F.M., Aledort, L.M., George, J.N., Kessler, C.M., Sanz, M.A., Liebman, H.A., Slovick, F.T., de Wolf, J.T.M., Bourgeois, E., Guthrie, T.H., Jr, Newland, A., Wasser, J.S., Hamburg, S.I., Grande, C., Lefrère, F., Lichtin, A.E., Tarantino, M.D., Terebelo, H.R., Viallard, J.-F., Cuevas, F.J., Go, R.S., Henry, D.H., Redner, R.L., Rice, L., Schipperus, M.R., Guo, D.M. & Nichol, J.L. (2008) Efficacy of romiplostim in patients with chronic immune thrombocytopenic purpura: a double-blind randomised controlled trial. *The Lancet*, **371,** 395–403.

Nishikii, H., Eto, K., Tamura, N., Hattori, K., Heissig, B., Kanaji, T., Sawaguchi, A., Goto, S., Ware, J. & Nakauchi, H. (2008) Metalloproteinase regulation improves *in vitro* generation of efficacious platelets from mouse embryonic stem cells. *Journal of Experimental Medicine*, **205,** 1917–1927.

Nurden, A.T., Viallard, J.-F. & Nurden, P. (2009a) New-generation drugs that stimulate platelet production in chronic immune thrombocytopenic purpura. *The Lancet*, **373,** 1562–1569.

Nurden, P., Tandon, N., Takizawa, H., Couzi, L., Morel, D., Fiore, M., Pillois, X., Loyau, S., Jandrot-Perrus, M. & Nurden, A.T. (2009b) An acquired inhibitor to the GPVI platelet collagen receptor in a patient with lupus nephritis. *Journal of Thrombosis and Haemostasis*, **7,** 1541–1549.

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Whole-body magnetic resonance imaging, including diffusion-weighted imaging, for diagnosing bone marrow involvement in malignant lymphoma

Accurate detection of bone marrow involvement in patients with malignant lymphoma [Hodgkin lymphoma (HL) and non-Hodgkin lymphoma (NHL)] is of crucial importance because of its prognostic and therapeutic implications (Armitage, 2005; Connors, 2005). Blind bone marrow biopsy (BMB) of the iliac crest is the standard method for bone marrow assessment, but this is an invasive and painful procedure, and has a small risk of (hemorrhagic) complications (Bain, 2006). Recently, whole-body magnetic resonance imaging (MRI), including diffusion-weighted imaging (DWI), has emerged as a potential alternative to computed tomography for the staging of malignant lymphoma (Kellenberger *et al*, 2004; Brennan *et al*, 2005; Kwee *et al*, 2009), including the bone marrow. Of note, DWI is a sequence that is sensitive to the random (Brownian) extra-, intra-, and transcellular motion of water molecules, and provides a high lesion-to-background contrast, which potentially improves detectability of bone marrow lesions (Dietrich *et al*, 2009). If whole-body MRI is accurate in excluding bone marrow involvement, it may spare patients unnecessary BMBs. This study aimed to determine the value of whole-body MRI, including DWI, for diagnosing bone marrow involvement in malignant lymphoma.

This study was approved by the local Institutional Review Board and all patients provided written informed consent. In total, 48 consecutive patients (32 men and 16 women; mean age: 48·4 years; age range: 13–82 years) with newly diagnosed, histologically proven malignant lymphoma (HL: $n = 10$; NHL: $n = 38$) prospectively underwent whole-body MRI using a protocol that was described previously (Kwee *et al*, 2009) and blind BMB of the posterior iliac crest, in a random order, and before start of treatment. BMB results of four patients were reported in a previous publication (Kwee *et al*, 2009). T1-weighted (T1W) and short inversion time inversion recovery (STIR) whole-body MRI were performed in all patients, and whole-body DWI was performed in 44 patients. Unilateral BMB was performed in 40 patients, and bilateral BMB was performed in eight patients. The time interval between whole-body MRI and BMB ranged from 0 to 37 d, with 41 of 48 BMBs (85·4%) being performed before whole-body MRI. A board-certified radiologist (who had 14 years of clinical experience with MRI), blinded to BMB findings, evaluated two separate sets of whole-body MR images: whole-body MRI without DWI (i.e. T1W and STIR only) and whole-body MRI with DWI (i.e. T1W, STIR, and DWI), using previously described criteria for bone marrow assessment (Yasumoto *et al*, 2002). Although the majority of BMBs was performed before whole-body MRI, the observer was aware of this issue. In addition, BMB usually causes a mild signal change at a limited portion of the posterior iliac crest, with a

Table I. Results of whole-body MRI without DWI (T1W and STIR) and whole-body MRI with DWI (T1W, STIR, and DWI) compared to results of BMB regarding the diagnosis of bone marrow involvement (+: positive MRI or BMB; −: negative MRI or BMB).

Whole-body MRI without DWI			Whole-body MRI with DWI		
MRI	BMB	No. of cases	MRI	BMB	No. of cases
+	+	5	+	+	5
+	−	8	+	−	8
−	+	7	−	+	6
−	−	28	−	−	25

MRI, magnetic resonance imaging; DWI, diffusion-weighted imaging; BMB, bone marrow biopsy; T1W, T1-weighted; STIR, short inversion time inversion recovery.

