



- (51) **International Patent Classification:**  
**G01N 33/532** (2006.01) **G01N 33/534** (2006.01)  
**G01N 33/533** (2006.01)
- (21) **International Application Number:**  
PCT/AU2012/000883
- (22) **International Filing Date:**  
25 July 2012 (25.07.2012)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**  
61/511,463 25 July 2011 (25.07.2011) US
- (71) **Applicant (for all designated States except US):** **MON-ASH UNIVERSITY** [AU/AU]; Wellington Road, Clayton, Victoria 3800 (AU).
- (72) **Inventors; and**
- (75) **Inventors/Applicants (for US only):** **GARDINER, Elizabeth** [AU/AU]; 1/67 Blackburn Road, Blackburn, Victoria 3130 (AU). **ANDREWS, Robert** [AU/AU]; c/o Monash University, Australian Centre for Blood Diseases, AMREP, Level 6, 89 Commercial Road, Melbourne, Victoria 3004 (AU).
- (74) **Agents:** **HUTCHISON, Jane** et al.; c/o Davies Collison Cave, 1 Nicholson Street, Melbourne, victoria 3000 (AU).

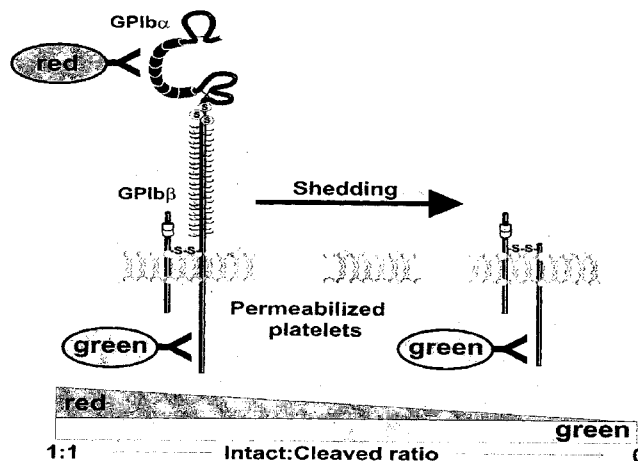
(81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) **Title:** PLATELET ASSESSMENT ASSAYS, DIAGNOSIS AND KITS THEREFOR

**FIGURE 1**

(57) **Abstract:** The specification provides assays and kits for assessing the proteolytic state of test platelets in a biological sample or stratifying subjects with respect to thrombocytopenia, the assay comprising detecting the level of intact platelet membrane receptor (PMR) (a) and detecting the level of total (cleaved and uncleaved) PMR (b) associated with platelets in the sample, and comparing a measure of the relationship between levels (a) and (b) ("test relationship") to a corresponding relationship measure derived from at least one reference biological sample ("reference relationship") selected from a sample obtained from one or more normal (e.g. healthy) reference subjects ("normal reference relationship") or from one or more reference samples having predetermined measured levels of intact and total PMR indicating that the reference subject has supernormal measured levels of cleaved PMR associated with platelets ("supernormal cleavage reference relationship"), wherein the similarity or difference between the test relationship and the reference relationship indicates the proteolytic state of platelets in the test sample. In one embodiment the PMR is GPIbα.

- 1 -

## **TITLE OF THE INVENTION**

**"PLATELET ASSESSMENT ASSAYS, DIAGNOSIS AND KITS THEREFOR"**

## **FIELD OF THE INVENTION**

[0001] This invention relates generally to assays and kits for assessing or monitoring the proteolytic state of platelets in blood samples, such as whole blood or fractions thereof, from a subject. In particular, the present invention enables assays useful in diagnosis, prognosis, monitoring, treatment or stratification of a human or non-human mammalian subject with respect to thrombocytopenia or conditions associated with thrombocytopenia. The present invention has practical uses, *inter alia*, in the treatment and management of thrombocytopenia and may be used in existing or newly developed knowledge-based architecture or platforms associated with pathology services.

## **BACKGROUND OF THE INVENTION**

[0002] Bibliographic details of the publications referred to in this specification are collected at the end of the description.

[0003] Platelets are small, anuclear fragments of megakaryocytes that circulate in the blood and make essential contributions to functions such as blood clotting and wound healing. They play a central role in cardiovascular disease, blood homeostasis, inflammation and tumor metastasis. They are produced by megakaryocytes which are large, polyploid cells that develop in the bone marrow and spleen. Megakaryocytes release platelets into the blood stream where, in humans, they circulate for around 7 to 10 days where they may take part in a haemostatic process or proceed to destruction by the reticuloendothelial system, primarily in the liver and spleen. Like all lineages of blood cells, the steady state number of mature platelets is the result of a balance between their production and destruction. In normal i.e., healthy individuals, precise control of proliferation, differentiation, survival and clearance of these cells ensures maintenance of homeostasis, and reduces the likelihood of haemorrhage should platelet counts fall, or

C:\NRPort\NDCC\EXT\4486876\_3.DOC-7/25/2012

- 2 -

thrombosis resulting from excess platelet production.

**[0004]** If the delicate balance between production and destruction is perturbed, thrombocytopenia (low platelet count) can ensue. Thrombocytopenia is a common problem in the clinic, particularly in haematological and oncological practice, and leads to potentially fatal haemorrhagic episodes. It can occur congenitally, and a number of inherited disorders having been defined, but the majority of thrombocytopenias seen in the clinic are the result of other causes. It can be a major problem for patients undergoing cancer chemotherapy. Acute episodes of cytotoxic drug-related thrombocytopenia, in addition to putting the patient at immediate risk, can force dose modifications or treatment withdrawal. Thrombocytopenia is also frequently encountered in myelodysplastic syndromes (MDS), idiopathic thrombocytopenia purpura (ITP), antiphospholipid antibody syndrome (APS) and thrombotic thrombocytopenia purpura (TTP), and chronic liver disease, and is also associated with viral infections, particularly AIDS and bacterial infection and sepsis. In these more chronic contexts, thrombocytopenia may result from defective platelet production through bone marrow defects, or elevated platelet destruction often as the result of autoimmune reactions.

**[0005]** Treatment for low platelet numbers includes platelet transfusion for subjects with bone marrow defects, and steroid therapy where immune mediated destruction of platelets is indicated, both of which are only short term solutions and carry significant clinical risks. Platelet transfusion, for example, can give rise to immunohaematological side-effects and risk of infection which can be associated with morbidity and mortality. If autoimmune mediated thrombocytopenia is misdiagnosed, patients can be exposed to high levels of steroids or immunosuppressants that will not treat the thrombocytopenia and are likely to provoke significant unwanted side effects. Current methods of distinguishing between thrombocytopenia caused by platelet destruction and thrombocytopenia caused by reduced platelet production involve invasive and time consuming bone marrow biopsy.

**[0006]** There is a need for improved assays and clinical tools that are able to

C:\NRPort\IDCC\DXTV4186876\_3.DOC-7/25/2012

- 3 -

assess the state of circulating platelets and thereby facilitate *inter alia* the diagnosis, treatment and management of thrombocytopenia or other conditions affected by platelet proteolytic state (shedding). There is also a need for assays to qualitatively and quantitatively assess the state of platelets in a biological sample from a subject and in stored blood samples or fractions thereof including platelet fractions.

### SUMMARY OF THE INVENTION

[0007] Receptors spanning the platelet membrane such as the von Willebrand factor receptor GP (glycoprotein) Ib-IX-V complex and GPVI that binds collagen form a unique adhesion-signaling complex which acts synergistically to initiate platelet adhesion, activation and integrin dependent aggregation in haemostasis and thrombosis (Gardiner *et al.*, *Journal of Thrombosis and Haemostasis* 5: 1530-1537, 2007 incorporated herein in its entirety by reference).

[0008] GPIb is a heterodimeric transmembrane protein. As shown in Figure 1, the receptor comprises a large GPIb $\alpha$  chain disulfide linked to a smaller GPIb $\beta$  chain. The  $\alpha$ -chain comprises an N-terminal ectodomain and a C-terminal cytoplasmic domain. Ectodomain shedding is mediated by metalloproteinases which cleave the ectodomain close (proximal) to the platelet membrane. As shown in Figure 2, ectodomain portions of GPV and GPVI are also shed. For human GPIb $\alpha$  the cleavage site is G465V, for human GPV the cleavage site is P494V, and for human GPVI the cleavage site is P243Q (See Table 5). The residual portion of GPIb $\alpha$  that is retained after shedding, is associated with the platelet membrane and is disulfide linked to GPIb $\beta$ . The residual portion comprises a small, unshed ectodomain portion N-terminal to the G465V cleavage site and a cytoplasmic portion domain C-terminal to the G465V cleavage site.

[0009] In a broad embodiment, the present invention enables assays for evaluating the proteolytic status of platelets by assessing the proteolytic status of a shed platelet membrane receptor (referred to herein as "PMR") including one of GPIb $\alpha$ , GPV and GPVI.

- 4 -

**[0010]** Some aspects of the method are predicated, in part, upon the determination by the inventors that a measure of the proportion of intact receptor out of the total amount of receptor, whether intact or cleaved, provides an improved and informative assessment of platelet state in healthy and thrombocytopenic subjects. Surprisingly, in one embodiment, a statistically significant reduction in intact GPIb $\alpha$  relative to total GPIb $\alpha$ , was observed in subjects with idiopathic thrombocytopenia (ITP), antiphospholipid antibody syndrome (APS) and thrombotic thrombocytopenia purpura (TTP), compared to healthy controls. The assay indicated an increased shedding of the GPIb $\alpha$  ectodomain of GPIb $\alpha$  that was independent of platelet numbers. In contrast, the measured levels of platelet bound GPIb $\alpha$  ectodomain alone or the levels of the cytoplasmic domain of GPIb $\alpha$  alone were uninformative in this respect. In some embodiments, the proteolytic state of GPIb $\alpha$  and at least one of GPV and GPVI is assessed.

**[0011]** Specifically, and in an exemplary embodiment, the ratio of the measured level of GPIb $\alpha$  ectodomain (Glycocalicin) (whose presence on the surface of a platelet indicates that the GPIb $\alpha$  protein is intact (not shed or cleaved) ("intact")), to the measured level of the residual C-terminal domain of GPIb $\alpha$  (which is physically associated with (bound to) platelets both before and after cleavage and shedding of the ectodomain N-terminal to the G465V cleavage site ("total")) was found to be significantly different between healthy donors and thrombocytopenic subjects. In this embodiment, the thrombocytopenic subjects had a form of thrombocytopenia which is associated with supernormal peripheral platelet destruction. As shown in the Examples, a high ratio or range of ratios, e.g., 0.9-1.0 or 0.85-1.0 (arbitrary values) of intact to total GPIb $\alpha$  was found in healthy subjects. A lower ratio or range of ratios, such as 0.8 and below or 0.85 and below, was found in samples subjected to cleavage agents or in subjects with thrombocytopenias characterized by elevated platelet clearance. Thresholds can be determined by the skilled addressee using recognized procedures. For the avoidance of doubt, there are various ways of representing the information generated by the subject assay, whether as a ratio, percentage, proportion of receptors or proportion of platelets,

C:\NR\PortB\DOCC\DX1448676\_1.DOC-7/23/2012

- 5 -

amount, or level of intact to total receptor, or total to intact receptor or, by inference, cleaved to total, or total to cleaved, and any such permutations are *mutatis mutandis* encompassed.

[0012] Proteolytic state of platelets is determined in the assays as a measure of the proportion of platelets in a sample that have an intact PMR or a measure of this proportion of PMR that is intact out of the total number of detected receptors. Healthy subjects (for example, those subjects not exhibiting thrombocytopenia) have very low levels of cleaved PMR although this increases with the age of the platelets in circulation or storage. Thrombocytopenic subjects have low levels of platelets but if their thrombocytopenia is due to bone marrow deficits then the platelets in circulation or stored are not characterised by supernormal levels of cleaved PMR as determined herein. On the other hand, as determined herein, subjects with increased levels of platelet clearance exhibit increased levels of PMR cleavage. Their proportion of intact to total PMR is reduced to less than 90%, less than 85%, less than 80%, less than 75%, or even less than 70% in some subjects. This proportion is expressed as a test relationship and compared to a control or a corresponding reference relationship to determine the significance of the test value obtained. Typically, diagnostic threshold values are obtained by analysis of large numbers of test and reference subjects and illustrative values are provided herein.

[0013] The present assays provide a significant advance over less informative prior art methods of non-invasively assessing platelets and thrombocytopenia. The present assays permit qualitative and quantitative assessments of proteolytic status of platelets which are independent of the platelet number and independent of GPIIb/IIIa surface levels. This may be used to assess whether a subject has thrombocytopenia as a result of elevated platelet clearance or as a result of decreased platelet production. The present assay provides an early indication of a subject's response to treatment for thrombocytopenia. In a further embodiment, the subject assays facilitate stratification of thrombocytopenia as thrombocytopenia including defects in platelet production and/or

C:\NKP\PMR\10000001\14480876\_1.DOC - 11/27/2012

- 6 -

thrombocytopenia including altered platelet function such as enhanced platelet activation/destruction/clearance.

[0014] Although the invention has been illustrated using the human GPIIb/IIIa platelet receptor as an exemplary PMR, the skilled person will appreciate that other similar cleaved platelet receptors may be assayed using the same approach to provide useful information concerning the proteolytic cleavage state of platelets. In some embodiments, the proteolytic state of GPIIb/IIIa and at least one of GPVI and GPIb is assessed. ITP, APS and TTP are illustrative examples of conditions associated with elevated levels of platelet clearance, however any such condition is encompassed.

[0015] Accordingly, in one broad embodiment, the present invention enables an assay for assessing the proteolytic state of test platelets in a biological sample. In some embodiments, the assays comprise detecting the level of intact platelet membrane receptor (PMR) associated with platelets (a), and the level of total (cleaved and uncleaved) PMR (b) associated with platelets in the sample, and comparing a ratio or other measure of the relationship between levels (a) and (b) (a "test relationship") to a corresponding ratio or relationship measure derived from at least one reference biological sample (a "reference relationship") selected from a sample obtained from one or more normal (e.g. healthy) reference subjects ("normal reference relationship") or from one or more reference samples having predetermined levels of intact and total PMR indicating that the reference sample has supernormal levels of cleaved PMR associated with platelets ("supernormal cleavage reference relationship"), wherein the similarity or difference between the test and reference relationships indicates the proteolytic state of platelets in the test sample.

[0016] In an illustrative embodiment, once the test relationship has been established, a comparison between the test relationship and typically a pre-determined reference relationship is used to assess the proteolytic state of the test platelets. Accordingly, in one embodiment, a similarity between the test relationship and the normal reference relationship or a difference between the test relationship and the

C:\NRPort\NDCCDXT\4486876\_1.DOC-7/25/2012

- 7 -

supernormal cleavage reference relationship indicates the absence of supernormal levels of proteolytic cleavage in test platelets. In another embodiment, a similarity between the test relationship and the supernormal cleavage reference relationship or a difference between the test relationship and the normal reference relationship indicates the presence of supernormal levels of proteolytic cleavage in test platelets.

[0017] In some further embodiments, the assay comprises (i) contacting test platelets with an agent capable of permeabilizing platelets. This permeabilization step is used when the PMR antigen binding molecule that binds the residual domain of the PMR, binds to a cytoplasmic region of the residual domain. In some embodiments, the assays comprise (i) or (ii) contacting test platelets with a PMR antigen-binding molecule that binds to the ectodomain (that extracellular portion of the PMR which is shed from the surface of the platelet after cleavage of the receptor ("shed") close to the platelet membrane) of PMR on the surface of platelets to provide a measure of the level of intact PMR receptor; (iii) contacting test platelets with a PMR antigen-binding molecule that binds to the residual domain (that portion of the receptor retained after ectodomain shedding) of PMR associated with platelets after ectodomain cleavage to provide a measure of the level of total (cleaved and uncleaved) PMR receptor ("total"); (iv) comparing a ratio or other measure of the relationship between the measured level in (ii) and (iii) ("test relationship") to a corresponding relationship measure from at least one reference sample ("reference relationship") selected from a sample obtained from one or more normal (e.g. healthy) reference subjects ("normal reference relationship") or from one or more reference subjects having predetermined measured levels of intact and total PMR indicating that the reference subject has supernormal measured levels of cleaved PMR associated with platelets ("supernormal cleavage reference relationship"), wherein the similarity or difference between the test and reference relationships indicates the proteolytic state of platelets in the test sample. In some embodiments, the antigen-binding molecule that binds to the residual domain, binds to the cytoplasmic domain of PMR. In some embodiments, platelets are permeabilized with, for example, a mild

C:\NRPort\NDCC\DX\4486876\_3.DOC-7/25/2012

- 8 -

detergent to allow intracellular access of antibodies to the cytoplasmic domain, if required. In some embodiments, the antigen binding molecule is/are labeled with a detectable substance.

**[0018]** There are various approaches to assessing the test and reference relationship values. In some embodiments, a similarity between the test relationship and the normal reference relationship or a difference between the test relationship and the supernormal cleavage reference relationship indicates the absence of supernormal levels of proteolytic cleavage in test platelets, and wherein a similarity between the test relationship and the supernormal cleavage reference relationship or a difference between the test relationship and the normal reference relationship indicate the presence of supernormal levels of proteolytic cleavage in test platelets.

**[0019]** In some embodiments, the test platelets are stored platelets, such as platelets in stored blood or stored platelet concentrates.

**[0020]** In some embodiments, the biological sample is whole blood. In other embodiments, blood is fractioned or processed to remove one or more cellular components other than platelets.

**[0021]** In some embodiments, the assay further comprises contacting platelets with an RNA-staining molecule and measuring the level of RNA in test platelets as an indication of platelet age.

**[0022]** In one particular embodiment, the PMR receptor is GPIb $\alpha$ . For human GPIb $\alpha$  the ectodomain is N-terminal to G465V or the equivalent amino acid in a homolog of GPIb $\alpha$ . The residual domain of GPIb $\alpha$  is C-terminal to G465V or the equivalent amino acid in a GPIb $\alpha$  homolog.

**[0023]** In another embodiment, the PMR receptor is GPV. For GPV, the ectodomain is N-terminal to P494V or the equivalent amino acid in a GPV homolog. The residual domain of GPV is C-terminal to P494V or the equivalent amino acid in a GPVI homolog.

**[0024]** In another embodiment, the PMR receptor is GPVI. For GPVI, the

C:\NR\Port6\DOC\CDXT\4486876\_3.DOC-7/25/2012

- 9 -

ectodomain is N-terminal to P243Q or the equivalent amino acid in a GPVI homolog. The residual domain of GPVI is C-terminal to P243Q or the equivalent amino acid in a GPVI homolog.

**[0025]** In another aspect, the subject assay is provided for use in the diagnosis, prognosis, monitoring, treatment or management of conditions associated with thrombocytopenia or for use in the management of stored blood products.

**[0026]** In an exemplified embodiment, the assay is suitable for and employs flow cytometry as the detection system. In these embodiments, the antigen binding molecule that binds to the ectodomain of PMR on the surface of platelets is labelled with a first fluorescent or other detectable molecule, and the antigen binding molecule that binds to the residual domain of PMR receptor associated with platelets after receptor cleavage is labelled with a second fluorescent or other detectable molecule. In accordance with this embodiment, test platelets are subjected to flow cytometry to detect and provide a measure of the level of intact PMR receptor or proportion of platelets with intact PMR receptor ("intact") and to detect and provide a measure of the level of total (cleaved and uncleaved) PMR receptor or the proportion of platelets with cleaved and uncleaved PMR receptor ("total").

**[0027]** Accordingly, in one embodiment, the present invention provides a flow cytometric assay for assessing the proteolytic state of test platelets in a biological sample, the assay comprising (i) contacting test platelets with an agent capable of permeabilizing platelets, (ii) contacting test platelets with a PMR antigen-binding molecule that binds to the ectodomain of PMR on the surface of platelets wherein the antigen binding molecule is labelled with a first fluorescent or other detectable molecule to provide a measure of the level of "intact" PMR receptor; (iii) contacting test platelets with a PMR antigen-binding molecule that binds to the residual domain of PMR associated with platelets after ectodomain cleavage and wherein the antigen-binding molecule is labelled with a second fluorescent or other detectable molecule to provide a measure of the level of total (cleaved and uncleaved) PMR receptors ("total"); (iv) subjecting labelled test platelets to

- 10 -

flow cytometry and (v) comparing a measure of the relationship between the detected levels in (ii) and (iii) ("test relationship") to a corresponding measure of the relationship between the detected levels in (ii) and (iii) from at least one reference sample ("reference relationship") selected from a sample obtained from one or more normal (e.g. healthy or non-thrombocytopenic) reference subjects ("normal reference relationship") or from one or more reference subjects having predetermined detected levels of intact and total PMR indicating that the reference subject has supernormal measured levels of cleaved PMR associated with platelets ("supernormal cleavage reference relationship"), wherein the similarity or difference between the test and reference relationships indicates the proteolytic state of platelets in the test sample.

**[0028]** In some embodiments, wherein the first and second fluorescent or other detectable molecules are different. Where more than one PMR is assessed, each PMR may have one or more unique detectable molecules.

**[0029]** In some embodiments, the assays further comprise contacting platelets with an RNA-staining fluorescent molecule and measuring the level of RNA in test platelets.

**[0030]** In other embodiments, the test and reference relationships are compared for two or more PMR.

**[0031]** There are a range of other detection systems known in the art which may be employed and all such detection systems are encompassed by the present disclosure.

**[0032]** The antigen binding molecules and/or assays of the present invention may be provided in kit format. In one embodiment, the invention provides a kit for assessing the proteolytic state of test platelets in a biological sample, the kit comprising: (i) a PMR antigen-binding molecule that binds to the ectodomain of PMR, wherein the antigen-binding molecule is either immobilized (bound) to one or more test portions and/or contained within a conjugate portion; and/or (ii) a PMR antigen-binding molecule that binds to the residual domain of PMR associated with platelets after ectodomain cleavage, wherein the antigen-binding molecule is either immobilized (bound) to one or more test

C:\NRPort\INDC\DXTW448676\_3.DOC-7/25/2012

- 11 -

portions and/or contained within a conjugate portion and (iii) instructions for using the device for assessing the proteolytic status of platelets.

[0033] In some embodiment, the PMR receptor is GPIb $\alpha$ .

[0034] In a particular embodiment, the PMR receptor is GPV.

[0035] In a particular embodiment, the PMR receptor is GPVI.

[0036] In some embodiments, the GPIb $\alpha$  is assessed together with one or two of GPV and GPVI.

[0037] In another aspect the invention provides a kit as described herein for use in the diagnosis, prognosis, monitoring, treatment or management of conditions associated with thrombocytopenia or for use in the management of stored blood products.

[0038] In some embodiments, the PMR is GPV.

[0039] In a particular embodiment, the PMR is GPVI.

[0040] In some embodiments, the PMR is GPIb $\alpha$  and the assay further comprises each step wherein the PMR is GPV and/or further comprises each step wherein the PMR is GPVI.

[0041] In some embodiments, the assay comprises detecting (a) and (b) to establish a test relationship for GPIb $\alpha$ , GPV and GPVI and determining a reference relationship for GPIb $\alpha$ , GPV and GPVI.

[0042] In another embodiment, the present invention contemplates an antigen-binding molecule that is immuno-interactive with the residual domain of GPIb $\alpha$  associated with the platelet after proteolytic cleavage and shedding of the ectodomain. In some embodiments, the invention provides and an antigen-binding molecule that is immuno-interactive with the ectodomain or residual domain of PMR for use in the assays of the present invention. In a related aspect, the present invention provides a solid or semi-solid support comprising at least one antigen-binding molecule that specifically binds to the ectodomain or residual domain of one or more PMR as, broadly described herein, immobilized thereon. In some embodiments, the solid or semi-solid assays comprise a spatial array of antigen-binding molecule immobilized thereon suitable for

C:\NRPort\HDC\DX\TV4486876\_3.DOC-7/25/2012

- 12 -

detecting ratios of intact:total PMR.

[0043] In one embodiment, the assays of the present invention distinguish between forms of thrombocytopenia characterized by platelet destruction such as immune mediated thrombocytopenias, where the ratio or value (test relationship) of intact to total GPIb $\alpha$  in circulating platelets indicates whether thrombocytopenia results substantially from platelet destruction or substantially from reduced platelet production. Accordingly, in one embodiment, the present assays are useful for stratifying subjects in distinguishing between (i) thrombocytopenia associated with increased clearance in particular immune mediated thrombocytopenias, and (ii) bone-marrow deficiencies resulting in thrombocytopenia, and thus in the management, treatment or prophylaxis of thrombocytopenia.

[0044] In some embodiments, the present invention relates to a method of treatment or prophylaxis of a subject comprising assessing a test sample from the subject for platelet state as described herein and then providing at least one therapy to the subject or conducting further tests and therapies depending upon the results of the assay. Representative examples of therapies include steroid, anti-inflammatory or immunoglobulin therapy, platelet transfusion, plasma exchange, as appropriate.

[0045] In some embodiments, assays for differentially treating a population with respect to thrombocytopenia are provided. In some embodiments, the assays comprise (i) detecting (a or ta) the level of an intact PMR and detecting (b or tb) the level of total (cleaved or uncleaved) PMR associated with platelets in a test biological sample. In some embodiments, the methods comprise (ii) comparing a measure of the relationship between levels (a) and (b) (the "test" relationship) to a control. In an illustrative example, the test relationship is ta/tb. In an illustrative embodiment, the PMR is GPIb $\alpha$ . In some embodiments, the methods further comprise comparing a measure of the relationship between levels (a) and (b) from the test sample to a corresponding measure of the relationship between levels (a or ra) and (b or rb) derived from a reference biological sample (the "reference" relationship e.g., ra/rb). In some embodiments, subjects in the

C:\NR\Portb\IDCC\DX\T44\K676\_1.DOC-7/25/2012

- 13 -

population are stratified with respect to the proteolytic state of their platelets determined using the test relationship. In some embodiments, subjects receive different treatment regimes and/or monitoring regimes based upon the proteolytic state of the platelets determined using the test relationship. In an illustrative example, subjects identified as having an elevated level of PMR cleavage/shedding may receive an appropriate steroid therapy.

**[0046]** In some embodiments, step (ii) comprises comparing a measure of the relationship between levels (a) and (b) ("test relationship") to a corresponding relationship measure derived from at least one reference biological sample ("reference relationship") selected from a sample obtained from one or more normal (e.g. healthy) reference subjects ("normal reference relationship") or from one or more reference samples having predetermined measured levels of intact and total PMR indicating that the reference subject has supernormal measured levels of cleaved PMR associated with platelets ("supernormal cleavage reference relationship"), wherein the similarity or difference between the test relationship and the reference relationship indicates the proteolytic state of platelets in the test sample.

**[0047]** In some embodiments, the assay comprises (i) contacting test platelets with an agent capable of permeabilizing platelets, (ii) contacting test platelets with a PMR antigen-binding molecule that binds to the ectodomain of PMR on the surface of platelets to provide a measure of the level of intact PMR; (iii) contacting test platelets with a PMR antigen-binding molecule that binds to the residual domain of PMR associated with platelets after ectodomain cleavage to provide a measure of the level of total (cleaved and uncleaved) PMR ("total"); (iv) comparing a measure of the relationship between the measured levels in (ii) and (iii) ("test relationship") to a corresponding relationship measure from at least one reference sample ("reference relationship") selected from a sample obtained from one or more normal (e.g. healthy) reference subjects ("normal reference relationship") or from one or more reference subjects having predetermined measured levels of intact and total PMR indicating that the reference

C:\NRPort\IDCC\DXTW\186876\_3.DOC-7/25/2012

- 14 -

subject has supernormal measured levels of cleaved PMR associated with platelets ("supernormal cleavage reference relationship"), wherein the similarity or difference between the test and reference relationships indicates the proteolytic state of platelets in the test sample.

[0048] In some embodiments, a similarity between the test relationship and the normal reference relationship or a difference between the test relationship and the supernormal cleavage reference relationship indicates the absence of supernormal levels of proteolytic cleavage in test platelets, and wherein a similarity between the test relationship and the supernormal cleavage reference relationship or a difference between the test relationship and the normal reference relationship indicate the presence of supernormal levels of proteolytic cleavage in test platelets.

[0049] In some embodiments, the biological sample is whole blood.

[0050] In some embodiments, the assays further comprise contacting platelets with an RNA-staining molecule and measuring the level of RNA in test platelets.

[0051] In an illustrative embodiment the PMR is GPIb $\alpha$ .

[0052] In some embodiments, the PMR is GPIb $\alpha$  and the assay further comprises steps wherein the PMR is GPV and/or wherein the PMR is GPVI.

[0053] In further embodiments, the antigen binding molecule that binds to the ectodomain of PMR on the surface of platelets is labelled with a first fluorescent or other detectable molecule, wherein the antigen binding molecule that binds to the residual domain of PMR associated with platelets after receptor cleavage is labelled with a second fluorescent or other detectable molecule, and wherein test platelets are subjected to flow cytometry to provide a measure of the level of intact PMR or proportion of platelets with intact PMR ("intact") and a measure of the level of total (cleaved and uncleaved) PMR or the proportion of platelets with cleaved and uncleaved PMR ("total").

[0054] In an illustrative example, subjects identified as having an elevated bend of PMR cleavage may receive an appropriate steroid therapy.

[0055] In an illustrative embodiment, the invention enables an assay to monitor

C:\NR\Portb\NDCC\DXTH\486876\_3.DOC-7/25/2012

- 15 -

the response of a thrombocytopenic subject to therapy such as steroid or anti-inflammatory therapies. As shown in the Examples, the test relationship, in this case GPIb $\alpha$  intact:total ratio provided an early marker of platelet status and platelet recovery during treatment. Accordingly, the subject assays are useful for indicating whether further similar treatment is warranted or that a different treatment such as platelet transfusion is warranted.

[0056] Thus, in some embodiments, the assays facilitate the identification of appropriate treatment protocols and are also instructive in pharmacotranslational studies and in the clinical management of patients.

[0057] In other embodiments, the present assay may be used to guide drug discovery and testing for agents that modulate platelet survival, production or clearance, or platelet function in a subject or blood product, as appropriate.

#### BRIEF DESCRIPTION OF THE DRAWINGS

[0058] Figure 1 is a pictorial representation of a flow cytometry assay to assess the level of intact GPIb $\alpha$  (red fluorochrome) and total GPIb $\alpha$  (green fluorochrome) in a circulating platelet population. The ratio of red to green fluorescence reflects the proteolytic status of GPIb $\alpha$ , and therefore platelet age and extent of platelet activation and destruction.

[0059] Figure 2 is a pictorial representation of platelet membrane receptors GPIb $\alpha$ , GPV and GPVI which undergo shedding *in vivo*. The cleavage sites for each shed receptor have been identified in humans and antibodies or other antigen binding molecules are routinely generated against shed ectodomain or residual (cytoplasmic) domains (see Shen *et al.*, *Blood* 95: 903-910, 2000; Gardiner *et al.*, 2007 (*supra*)).

[0060] Figure 3 is a graphical representation of technical data showing that GPIb $\alpha$  intact:total ratio is a marker for ITP thrombocytopenia and further provides an early marker for platelet recovery during steroid or other therapy.

[0061] Figure 4 is a graphical representation of data showing the temporal

C:\NP\Portb\DC\DX\448676\_3.DOC-7/25/2012

- 16 -

relationship between GPIb $\alpha$  index (Intact/Total) and platelet count in ITP-C subject shown to be responsive to steroids after one month.

[0062] Figure 5 is a graphical representation of data showing the temporal relationship between GPIb $\alpha$  index (Intact/Total) and platelet count in ITP-X subject non responsive to steroids, treated with azathioprine at arrow.

[0063] Figure 6 is a graphical representation of data showing the temporal relationship between GPIb $\alpha$  index (Intact/Total) and platelet count in ITP-V subject treated with thrombopoietin (TPO) mimic, Romiplostim.

[0064] Figure 7 is a graphical representation of data showing the temporal relationship between GPIb $\alpha$  index (Intact/Total) and platelet count in ITP-J subject treated with steroids, then the TPO mimic Romiplostim at arrow.

#### DETAILED DESCRIPTION OF THE INVENTION

[0065] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, preferred methods and materials are described. For the purposes of the present invention, the following terms are defined below.

[0066] The articles "a" and "an" are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article. By way of example, "an element" means one element or more than one element.

[0067] By "antigen-binding molecule" is meant a molecule that has binding affinity for a target antigen. As used herein, the target antigen is a PMR, including the shed ectodomain portion thereof or the residual (including cytoplasmic) portion thereof as described elsewhere herein. It will be understood that this term extends to immunoglobulins, immunoglobulin fragments and non-immunoglobulin derived protein or protein frameworks that exhibit antigen-binding activity. As used herein, the term

C:\NRP\mb\IDCC\DX\TV4486876\_3.DOC-7/23/2012

- 17 -

"binds specifically," "specifically immuno-interactive" and the like when referring to an antigen-binding molecule refers to a binding reaction which is determinative of the presence of an antigen in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated assay conditions, the specified antigen-binding molecules bind to a particular antigen and do not bind in a significant amount to other proteins or antigens present in the sample. Specific binding to an antigen under such conditions may require an antigen-binding molecule that is selected for its specificity for a particular antigen. For example, antigen-binding molecules can be raised to a selected protein antigen, which bind to that antigen but not to other proteins present in a sample. A variety of immunoassay formats may be used to select antigen-binding molecules specifically immuno-interactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immuno-interactive with a protein. See Harlow and Lane, *"Antibodies, A Laboratory Manual"*, Cold Spring Harbor Publications, New York, 1988 for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity. Reference herein to "immuno-interactive" includes reference to any interaction, reaction, or other form of association between molecules and in particular where one of the molecules is, or mimics, a component of the immune system.

[0068] The term "biological sample" or "sample" as used herein refers to a sample comprising platelets that may be extracted, substantially untreated (anticoagulant may be present), diluted or concentrated or derived from a subject. The sample includes a biological fluid containing platelets such as, without limitation, whole blood or fraction thereof including platelets, purified or concentrated platelets.

[0069] Samples include blood or blood fractions from subjects who are thrombocytopenic and therefore have a low number of platelets in the blood or fraction. As used herein, the components of the test or reference samples including platelets, and detection marker-antigen complexes and components thereof may be moved relative to each other by capillary flow or diffusion through chromatographic material. The material

C:\NRPort\BDD\CDXT\486876\_1\_DOC-7/25/2012

- 18 -

may be functionalized or coated to permit, for example, cross-linking of reagents. Methods for immobilizing antibodies or other binding agents to solid supports are well known in the art and are described for example in US Patent No. 4,168,146, Cautrecases, *J. Biol. Chem.* 245: 3059, 1970. Materials contemplated for use herein include inorganic materials such as silica, glass, polymeric material such as cellulose, starch, dextrose, agarose, special fibrous paper (filter/chromatography paper) nitrocellulose, cellulose acetate, PVC, polyacrylamide, polysaccharide, polyacrylate, polyethylsulphonate, polyethylene and the like. "Chromatographically active" components are simply those capable of flow through all or part of the immunochromatographic device. Chromatographic assays are particularly sophisticated and a large number of different formats are available which are tailored to the particular reagents and instruments and the outcomes required in any particular investigation. "Rapid" assays, using chromatographic principles, are tailored for accuracy, speed and ease of use. Immunoassay or enzyme-based chromatographic assays are particularly preferred and these are described in Wild D, *"The Immunoassay Handbook"*, Nature Publishing Group, 2001 and by reference to U.S. Patent Nos. 4,016,043; 4,590,159; 5,266,497; 4,962,023; 5,714,389; 5,877,028, 5,922,537, 6,168,956 and 6,548,309, 6,180,417, and 5,266,497 incorporated herein and information disclosed by references cited therein. Various modifications of immunochromatographic methods are described in Published U.S. Patent Application Nos. 20010006821, 20040087036 and 20040214347 which are incorporated herein in their entirety. Immunogold filtration methods for multiple analyte analyses are described in Published US Patent Application No. 20030165970 incorporated herein.

**[0070]** Throughout this specification, unless the context requires otherwise, the word "comprise," and variations such as "comprises" and "comprising" will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. Thus, use of the term "comprising" and the like indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be

C:\WP\PortB\DOC\DX\44\66\76\_3.DOC-7/25/2012

- 19 -

present. By "consisting of" is meant including, and limited to, whatever follows the phrase "consisting of". Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

[0071] Reference to a "control" broadly includes data that the skilled person would use to facilitate the accurate interpretation of technical data.

[0072] In some embodiments, a "difference" between test and reference values includes at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or 90%, or even an at least about 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900% or 1000% increase or decrease between values.

[0073] In some embodiments, a "similarity" between test and reference values includes no more than about 20%, 18%, 16%, 14%, 12%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% or 0.1% difference between values.

[0074] In an illustrative example, the ratio of intact:total GPIIb $\alpha$  (or total to intact GPIIb $\alpha$ ) from test platelets or a test subject are compared to reference ratios(s) in one or more cohorts (populations/groups) of reference subjects whose platelet status is established (pre-determined). In some assays, reference subjects include normal subjects with normal platelets or thrombocytopenic subjects with functionally or proteolytically normal platelets. In some embodiments, the reference sample may be a sample from the test subject taken at an earlier time point. Thus, a temporal change in test and reference ratios or relationship values can be used to monitor changes in platelet state.

[0075] In some embodiments, a reference subject is a group of reference subjects. The measured level or ratio in a reference subject group may be a mean value or a

C:\NRPort\BDD\CDXT\4486476\_3.DOC-7/25/2012

- 20 -

preselected level, threshold or range of levels or ratios that define, characterize or distinguish a particular group. In some embodiments, thresholds may be selected that provide an acceptable ability to distinguish between platelet states. In some embodiments, a threshold or range is selected, above which (or below which) the test is considered to be "positive" and below which the test is considered to be "negative".

[0076] In some embodiments, ratio thresholds are selected to discriminate between subjects (such as between thrombocytopenic subjects) with at least about 60%, 65%, or 70% accuracy.

[0077] Reference herein to "derived from" means that the sample is obtained from a particular source but not necessarily directly from that source.

[0078] The reference herein to "platelet proteolytic state", "platelet state" the "state of platelets" or the "state of test platelets" and the like includes proteolytic or shedding status (loss or otherwise of PMR ectodomain N-terminal to ectodomain cleavage site) which is associated with the risk or rate of platelet clearance, platelet activation or functional status (e.g., in relation to thrombus formation, thrombocytic activity, or wound healing capability). In some embodiments, the state of platelets includes an indication of the proportion of platelets having shed PMR ectodomains or the surface density of intact PMR. When combined with repeat or serial testing, the assay also provides and the platelet state includes an indication of the rate of receptor shedding or rate of change of receptor shedding in test platelets from a test subject, or in stored platelets.

[0079] As used herein reference to "detecting", "evaluating", "enumerating", "measuring" includes assessing the level of "intact" and "total" PMR or more particularly, the ratio of "intact" and "total" PMR by assessing the reporter signal from a detectable marker directly or indirectly attached to the "intact" PMR binding agent (antibody or fragment) or "total" PMR-binding agent (antibody or fragment thereof). Assessment may be by instrument or by visual comparison, typically with pre-determined controls.

[0080] By "detection marker" or "reporter molecule" "detection substance" is

C:\NR\Portb\DOC\DXTH486876\_3.DOC-7/25/2012

- 21 -

meant a molecule or particle which, by its chemical nature, provides an analytically identifiable signal which allows the detection and quantification of antigen bound antibody detection may be qualitative or quantitative. By qualitative is meant for example, that the user may observe a similarity or difference between test and reference ratios in order to assess the outcome of the test/method. Antigen binding molecules may be labeled with the same or different reporter molecules. Fluorophores are particularly useful (see Table 6). Other labels include luminescence and phosphorescence as well as infrared dyes. As will be well recognized, a wide variety of different reporter systems employing detectable molecules (substances) are available. Those allowing rapid visual detection are clearly the most useful in the context of point of care diagnostics.

[0081] The term "normal" is used to broadly refer to the phenotype of platelet proteolytic state that is characteristic of most of the members of the species occurring naturally. However, as thrombocytopenia caused by loss of platelet production involves platelets with substantially non-supernormal levels of proteolytic cleavage, platelets from such subjects are also considered normal and provide an example of normal platelets in accordance with the present invention. "Supernormal" merely refers to more than normal.

[0082] "Stratification" or "stratify" includes identification, diagnosis, clarification, monitoring and/or determination of thrombocytopenia by evaluating the state of platelets in a subject. The process involves identifying an increased or supra normal level of platelet membrane receptor shedding or decreased platelet shedding or normal levels of platelet shedding of platelets in a test subject. Increased shedding is indicative *inter alia* that a subject relative to a normal subject has increased platelet clearance such as is found in immune mediated thrombocytopenia.

[0083] In some embodiments, decreased shedding or improved platelet status is indicated by an increased PMR intact:total value which is at least 10%, 11%, 12%, 13%, 14%, 15%, 16%, 17%, 18%, 19%, 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% more than pre-treatment values. In some embodiments, subjects are monitored and treatment is

C:\NR\PortB\DC\CDXT\486876\_3.DOC-7/25/2012

- 22 -

maintained until the PMR intact to total percentage value reaches at least 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91% or 90%, of the level from a corresponding reference percentage from a normal control subject. In some embodiments, decreased shedding is indicated by a PMR intact:total value of that varies from a corresponding value or a low level of platelet shedding from a normal reference sample by no more than about 20, 18, 16, 14, 12, 10, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1% or 0.1%. Other assessment methods will be readily apparent to the skilled addressee. In some embodiments, the referenced relationship value is 1 or 1:1 or 100%.

[0084] The terms "subject" or "individual" or "patient", used interchangeably herein, refer to any subject, particularly a vertebrate subject, and even more particularly a mammalian subject, for whom therapy or prophylaxis is desired. Suitable vertebrate animals that fall within the scope of the invention include, but are not restricted to, primates, livestock animals (e.g., sheep, cows, horses, donkeys, pigs), laboratory test animals (e.g., rabbits, mice, rats, guinea pigs, hamsters), companion animals (e.g., cats, dogs) and captive wild animals (e.g., foxes, deer, dingoes) provided the subject has essentially anuclear platelets. The aforementioned terms do not imply that symptoms are present.

[0085] The present invention contemplates the use of antigen-binding molecules that are specifically immuno-interactive with different domains of a PMR polypeptide for assessing platelet state in a test sample. In some embodiment, the antigen-binding molecule is a whole polyclonal antibody. Such antibodies may be prepared, for example, by injecting the GPIb domain of interest (antigen) or peptide portions thereof into a production species, which may include mice or rabbits, to obtain polyclonal antisera. Methods of producing polyclonal antibodies are well known to those skilled in the art. Exemplary protocols which may be used are described for example in Coligan *et al.*, "Current Protocols in Immunology", John Wiley & Sons, Inc, 1991 and Ausubel *et al.*, "Current Protocols in Molecular Biology" John Wiley & Sons Inc, in particular Section III of Chapter 11, 1994-1998.

C:\NP\Portb\NDCC\DXTV4486876\_3.DOC-7/25/2012

- 23 -

**[0086]** In lieu of polyclonal antisera obtained in a production species, monoclonal antibodies may be produced using the standard method as described, for example, by Köhler and Milstein, *Nature* 256: 495-497, 1975, or by more recent modifications thereof as described, for example, in Coligan *et al.*, 1991 (*supra*) by immortalizing spleen or other antibody producing cells derived from a production species which has been inoculated with one or more antigens.

**[0087]** The invention also contemplates as antigen-binding molecules Fv, Fab, Fab' and F(ab')<sub>2</sub> immunoglobulin fragments. Alternatively, the antigen-binding molecule may comprise a synthetic stabilized Fv fragment. Exemplary fragments of this type include single chain Fv fragments (sFv, frequently termed scFv) in which a peptide linker is used to bridge the N terminus or C terminus of a V<sub>H</sub> domain with the C terminus or N-terminus, respectively, of a V<sub>L</sub> domain. ScFv lack all constant parts of whole antibodies and are not able to activate complement. ScFvs may be prepared, for example, in accordance with methods outlined in Kreber *et al.*, *J. Immunol. Methods*, 201(1): 35-55. Alternatively, they may be prepared by methods described in U.S. Patent No 5,091,513, European Patent No 239,400 or the articles by Winter and Milstein, *Nature* 349: 293, 1991 and Plückthun *et al.*, In *Antibody engineering: A practical approach*, 203-252, 1996. In another embodiment, the synthetic stabilized Fv fragment comprises a disulfide stabilized Fv (dsFv) in which cysteine residues are introduced into the V<sub>H</sub> and V<sub>L</sub> domains such that in the fully folded Fv molecule the two residues will form a disulfide bond between them. Suitable methods of producing dsFv are described for example in (Glockscuther *et al.* *Biochem.* 29: 1363-1367; Reiter *et al.*, *J. Biol. Chem.* 269: 18327-18331, 1994; Reiter *et al.*, *Biochem.* 33: 5451-5459, 1994; Reiter *et al.*, *Cancer Res.* 54: 2714-2718, 1994; Webber *et al.*, *Mol. Immunol.* 32: 249-258, 1995.

**[0088]** Phage display and combinatorial methods for generating antigen-binding molecules are known in the art (as described in, e.g., Huse *et al.*, *Science* 246: 1275-1281; 1989; Griffiths *et al.*, *EMBO J* 12: 725-734, 1993; Hawkins *et al.*, *J Mol Biol* 226: 889-896, 1992; Clackson *et al.*, *Nature* 352: 624-628, 1991; Gram *et al.*, *PNAS* 89: 3576-

C:\NR\PortB\NDCC\DXTV446876\_1.DOC-7/25/2012

- 24 -

3580, 1992; Garrad *et al.*, *Bio/Technology* 9: 1373-1377, 1991; Hoogenboom *et al.*, *Nuc Acid Res* 19: 4133-4137, 1991; and Barbas *et al.*, *PNAS* 88: 7978-7982, 1991).

[0089] Detection of antigen binding molecules can be facilitated by coupling (e.g., physically linking) them to a detectable substance (i.e., antibody labeling). Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase,  $\beta$ -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin (a list of suitable fluorophores is provided in Table 6); an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin, and examples of suitable radioactive material include  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{35}\text{S}$  or  $^3\text{H}$ . The label may be selected from a group including a chromogen, a catalyst, an enzyme, a fluorophore, a chemiluminescent molecule, a lanthanide ion such as Europium ( $\text{Eu}^{34}$ ), a radioisotope and a direct visual label. In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like.

[0090] Monoclonal and/or polyclonal antibodies to the ectodomain or residual domain of GPIIb $\alpha$ , GPVI and GPV are known (Gardiner *et al.*, 2007 (*supra*); Shen *et al.*, 2000 (*supra*), incorporated herein their entirety by reference). Illustrative antibodies binding to the intact GPIIb $\alpha$  ectodomain include the CDR sequences underlined in Table 4.

[0091] A large number of enzymes useful as labels is disclosed in United States Patent Specifications U.S. 4,366,241, U.S. 4,843,000, and U.S. 4,849,338. Enzyme labels useful in the present invention include alkaline phosphatase, horseradish peroxidase,

C:\NRP\BMDCC\DX\4166876\_3.DOC-7/25/2012

- 25 -

luciferase,  $\beta$ -galactosidase, glucose oxidase, lysozyme, malate dehydrogenase and the like. The enzyme label may be used alone or in combination with a second enzyme in solution.

[0092] Consistent with the present invention, the proteolytic state of platelets and levels of PMR domains can be assayed using any suitable method known in the art. Antibody-based techniques include immunohistological and immunohistochemical methods for measuring the level of a protein of interest in a tissue sample. Specific recognition may be provided, for example, by a primary antibody (polyclonal or monoclonal) and a secondary detection system is used to detect presence (or binding) of the primary antibody. Detectable labels can be conjugated to the secondary antibody, such as a fluorescent label, a radiolabel, or an enzyme (e.g., alkaline phosphatase, horseradish peroxidase) which produces a quantifiable, e.g., colored, product. In another suitable method, the primary antibody itself can be detectably labeled. As a result, immunohistological labeling of a tissue section is provided. In some embodiments, a protein extract is produced from a biological sample (e.g., tissue, cells) for analysis. Such an extract (e.g., a detergent extract) can be subjected to western-blot or dot/slot assay of the level of the protein of interest, using routine immunoblotting methods (Jalkanen *et al.*, *J. Cell. Biol.* 101: 976-985, 1985; Jalkanen *et al.*, *J. Cell. Biol.* 105: 3087-3096, 1987).

[0093] Other useful antibody-based methods include immunoassays, such as the enzyme-linked immunosorbent assay (ELISA) and the radioimmunoassay (RIA). For example, an antigen-specific monoclonal antibody, can be used both as an immunoabsorbent and as an enzyme-labeled probe. The amount of such protein present in a sample can be calculated by reference to the amount present in a standard preparation using a linear regression computer algorithm (see Lacobilli *et al.*, *Breast Cancer Research and Treatment* 11: 19-30, 1988).

[0094] Flow cytometry using commercial or microfabricated flow cytometry without limitation to any particular flow cytometer and/or biosensor methods are also contemplated. Different assays have different requirements for agents, washing steps,

C:\WP\Port1\DOC\DX\T4486876\_3.DOC-7/25/2012

- 26 -

incubation periods and the like and these are known to the skilled addressee. Although the invention has been illustrated using a flow cytometry based format, the broader aspects of the invention are in no way limited to this format. Fluorescence based detection systems are particularly suitable for flow cytometric analysis. Other methods of analyzing fluorescence emissions are contemplated such as 2-photon and 3-photon time resolved fluorescence spectrometry, fluorescence lifetime imaging and fluorescence resonance energy transfer as known in the art. Any optical means for identifying labels may be used. Electromagnetic scattering from light and X-rays may be employed.

**[0095]** Accordingly, in one illustrative embodiment, the assay is a flow cytometry based assay. Flow cytometry is a powerful tool for characterizing and enumerating cells. The flow cytometer detects and counts individual platelets passing in a stream through a laser beam. By examining a large number of cells, flow cytometry can provide quantitative data on the percentage of cells bearing different molecules. In accordance with the present assay, platelets are contacted with a first binding agent that binds to the residual domain of the PMR receptor and a second binding agent that binds to the ectodomain of the PMR. These agents are conveniently labelled with different detection markers such as fluorescent or chemiluminescent dyes. In other embodiments, one or more further binding agents that bind to the receptor domain or to a binding agent directly or indirectly bound to the receptor domain may be employed. In these cases, the second or subsequent binding agent is typically labelled with a detection or detectable marker to facilitate evaluating the level of the two forms of the receptor (intact or cleaved) associated with test platelets in the test sample.

**[0096]** The suspended mixture of labelled cells is then forced through an aperture, creating a fine stream of liquid containing cells spaced singly at intervals. As each cell passes through a laser beam it scatters the laser light, and any dye molecules bound to the cell will be excited and will fluoresce. Sensitive photomultiplier tubes detect both the scattered light, which gives information on the size and granularity of the cell, and the fluorescence emissions, which give information on the binding of the labelled binding

C:\NR\Portb\NDCC\DX\4486876\_3.DOC-7/25/2012

- 27 -

agents and hence on the characteristics of each platelet in the population. Where two or more binding agents are used, each coupled to a different fluorescent dye, then the data may be displayed in the form of a two-dimensional scatter diagram or as a contour diagram, where the fluorescence of one dye-labelled binding agent is plotted against that of a second, with the result that a population of cells labelling with one binding agent can be further subdivided on the basis of its reactivity with another binding agent.

[0097] Immunoassays are another particularly useful format of assay for the present invention. A wide range of immunoassay techniques are available, such as those described in Wild D, 2001 (*supra*). Reference may also be made to Chapter 11 of Ausubel (Ed), "*Current Protocols in Molecular Biology*", 5<sup>th</sup> Edition, John Wiley & Sons, Inc, NY, 2002. For example, the enzyme-linked immunosorbent assay (ELISA) and radio-immunoassay (RIA) are routinely used in laboratories. Arrays and high throughput screening methods are also employed. These methods generally require a high level of skill in laboratory techniques.

[0098] A wide range of immunoassay techniques are available that providing qualitative or quantitative results. These include both single-site and two-site or "sandwich" assays of the non-competitive types, as well as in the traditional competitive binding assays. Sandwich assays are among the most useful and commonly used assays and are favored for use in the present invention. A number of variations of the sandwich assay technique exist, and all are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an antibody is immobilized on a solid or semi-solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex, a second antibody specific to the antigen, labelled with a detection molecule capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal produced by the detectable marker

- 28 -

(reporter molecule). The results may be qualitative or quantitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of antigen. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody.

**[0099]** In a typical forward sandwich assay, a first antibody having specificity for the PMR domain antigen is either covalently or passively bound to a solid or semi-solid support. The support is typically glass or a polymer, the most commonly used polymers being nitrocellulose, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride, polypropylene or mixture or derivatives of these. The solid supports may be in the form of tubes, beads, discs or microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well-known in the art and generally consist of cross-linking covalently binding or physically adsorbing the polymer-antibody complex to the solid surface which is then washed in preparation for the test sample.

**[0100]** An aliquot of the sample to be tested is then added to the solid phase complex and incubated for a period of time sufficient (e.g. 2-40 minutes or overnight if more convenient) and under suitable conditions (e.g. from room temperature to about 37°C including 25°C) to allow binding of any subunit present in the antibody. Following the incubation period, the antibody subunit solid phase is washed and incubated with a second antibody specific for a portion of the antigen. The second antibody is linked to a detectable marker which is used to indicate the binding of the second antibody to the antigen. An alternative method involves immobilizing the target molecules in the biological sample and then exposing the immobilized target to specific antibody which may or may not be labelled with a detectable marker.

**[0101]** Depending on the amount of target and the strength of the signal from the detectable marker, a bound target may be detectable by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary

C:\NR\Perb\NDCCDXTV4486876\_3.DOC-7/25/2012

- 29 -

complex. The complex is detected by the signal emitted by the reporter molecule. Biosensor devices may be employed to detect and report on antigen-antibody interactions, such as those between the ectodomain of GPIb or GPV or GPVI and an antibody or other binding agent that binds to this domain, and the residual domain of GPIb $\alpha$ , GPV or GPVI and an antibody or antigen binding fragment or other binding agent which binds to this domain. Biosensor devices are described, for example, in U.S. Patent Publication No. 20100006452.

**[0102]** In other embodiments, the method is a liquid phase method. In one example of a liquid phase immunoassay, the sample is contacted with an agent capable of binding the antigen and a detector agent comprising a visually detectable agent such as colloidal gold or silver labelled. The test sample is applied by flowing onto a defined zone of an insoluble porous support film having a pore size impassable to a complex formed between the antigen, if present, with the binding substance and the detector substance, but passable to the binding substance and detector substance while remaining uncomplexed in the absence of the desired antigen. If the antigen is present in the test specimen, the detector substance binds with the antigen and the binding substance to form a visually inspectable complex on the surface of the porous support film. After application of the test sample to the porous support, the surface of the porous support is visually inspected for color to determine the presence and quantity or the absence of the antigen being assayed.

**[0103]** In another assay, magnetic antibodies that bind to antigen are used to tag the antigens and a high  $T_c$  superconducting quantum interference device is used to measure the amount of free and bound antibody and hence the presence or level of antigens.

**[0104]** A liposome immunomigration, liquid-phase competition strip immunoassay is, for example, described in Glorio-Paulet *et al.*, *J Agric Food Chem* 48 (5): 1678-1682, 2000. Biosensor devices may be employed to detect and report on antigen-antibody interactions such as those between the cytoplasmic domain of GPIb and

- 30 -

an antibody or other binding agent that binds to this domain, and the ectodomain of GPIb $\alpha$  and an antibody or antigen binding fragment or other binding agent which binds to this domain. Biosensor devices are described for example in US Patent Publication No. 20100006452.

**[0105]** A variety of methods have been developed in the art which require little skill and are rapid to perform, and which are therefore suitable for the detection of specific antigens, at the point of care. In particular, lateral flow, dipstick and capillary tube and biosensor-based kits are available in the art.

**[0106]** In some embodiments, the detection marker is a colloidal particle or microparticle. Colloidal metal and metalloid particles include those comprising gold, silver, platinum, iron, copper, selenium; metal complexes such as cyclopentadienylmanganese(I) tricarbonyl, gold cluster; and microparticles such as latex and dyed latex particles.

**[0107]** Additionally, recent developments in the field of protein capture arrays permit the simultaneous detection and/or quantification of a large number of proteins. For example, low-density protein arrays on filter membranes, such as the universal protein array system (Ge, *Nucleic Acids Res.* 28(2): e3, 2000) allow imaging of arrayed antigens using standard ELISA techniques and a scanning charge-coupled device (CCD) detector. Immuno-sensor arrays have also been developed that enable the simultaneous detection of clinical analytes.

**[0108]** In certain embodiments, the techniques used for detection of PMR domains in platelets will include internal or external standards to permit quantitative or semi-quantitative determination of those products, to thereby enable a valid comparison of the level in a biological sample with the corresponding level in a reference sample or samples. Such standards can be determined by the skilled practitioner using standard protocols. In specific examples, absolute values for the level of each domain.

**[0109]** The present invention further provides a system where data on which ratio of intact to total PMR are provided by a client server to a central processor which

C:\NRPort\NDCC\DX\44\6\676\_1.DOC-7/25/2012

- 31 -

analyses and compares to a control and optionally considers other information such as patient age, sex, weight and other medical conditions and then provides a report, including, for example, a platelet proteolytic state, intact:total PMR ratio or platelet maturity profile.

[0110] Hence, knowledge-based computer software and hardware also form part of the present invention. In particular, the assays of the present invention may be used in existing or newly developed knowledge-based architecture or platforms associated with pathology services. For example, results from the assays are transmitted *via* a communications network (e.g. the internet) to a processing system in which an algorithm is stored and used to generate a predicted posterior probability value which translates to the index of disease probability which is then forwarded to an end user in the form of a diagnostic or predictive report.

[0111] The assays may, therefore, be in the form of a kit or computer-based system which comprises the reagents necessary to detect the intact to total GPIb ratio and the computer hardware and/or software to facilitate determination or determination and comparison and transmission of reports to a clinician.

[0112] In an illustrative example, the present invention contemplates a method of allowing a user to determine the platelet proteolytic state of a subject *per se* and/or with respect to thrombocytopenia, the method including receiving data in the form of a ratio of intact:total PMR ratio or other relationship value, *via* a communications network, the method includes conduct of the assay as described herein then transferring data from the user *via* a communications network; (b) processing the subject data *via* multivariate analysis to provide, for example, a platelet proteolytic state value; (c) determining the status of the subject in accordance with the results of the value in comparison with predetermined values; and (d) transferring an indication of the status of the subject to the user *via* the communications network reference to the multivariate analysis includes an algorithm which performs the multivariate or univariate analysis function. In some embodiments, the method includes, the method including receiving data in the form of a

C:\NR\Portb\NDCCDXT\4486876\_3.DOC-7/23/2012

- 32 -

ratio of intact:total PMR ratio or other relationship value, *via* a communications network, the method includes conduct of the assay as described herein then transferring data from the user *via* a communications network.

[0113] Conveniently, the method generally further includes: (a) having the user determine the data using a remote end station; and (b) transferring the data from an end station to a base station *via* the communications network.

[0114] The base station can include first and second processing systems, in which case the method can include: (a) transferring the data to the first processing system; (b) transferring the data to the second processing system; and (c) causing the first processing system to perform the multivariate analysis function to generate the disease index value.

[0115] The method may also include: (a) transferring the results of the multivariate analysis function to the first processing system; and (b) causing the first processing system to determine the status of the subject. In this case, the method also includes at least one of: (a) transferring the data between the communications network and the first processing system through a first firewall; and (b) transferring the data between the first and the second processing systems through a second firewall.

[0116] The second processing system may be coupled to a database adapted to store predetermined data and/or the multivariate analysis function, the method include: (a) querying the database to obtain at least selected predetermined data or access to the multivariate analysis function from the database; and (b) comparing the selected predetermined data to the subject data or generating a predicted probability index. The second processing system can be coupled to a database, the method including storing the data in the database. Thus, the base station attempts to identify individuals having similar parameter values to the test subject and once the status has been determined on the basis of that identification, the base station provides an indication of the diagnosis to the end station.

[0117] The method can also include having the user determine the data using a secure array, the secure array of elements capable of determining the level of lipid

C:\NRPort\NDCCDXT\4486876\_3.DOC-7/23/2012

- 33 -

analytes and having a number of features each located at respective position(s) on the respective code. In this case, the method typically includes causing the base station to: (a) determine the code from the data; (b) determine a layout indicating the position of each feature on the array; and (c) determine the parameter values in accordance with the determined layout, and the data.

[0118] The method can also include causing the base station to: (a) determine payment information, the payment information representing the provision of payment by the user; and (b) perform the comparison in response to the determination of the payment information.

[0119] The use of numerical values in the various ranges specified in this application, unless expressly indicated otherwise, are stated as approximations as though the minimum and maximum values within the stated ranges were both preceded by the word "about". In this manner, slight variations above and below the stated ranges can be used to achieve substantially the same results as values within the ranges. Also, the disclosure of these ranges is intended as a continuous range including every value between the minimum and maximum values.

[0120] In order that the invention may be readily understood and put into practical effect, particular preferred embodiments will now be described by way of the following non-limiting examples.

#### EXAMPLE 1

*In one embodiment, a flow cytometry-based assay of platelet state has been developed:*

[0121] A flow cytometry-based method has been developed for simultaneously analyzing intact/cleaved GPIb $\alpha$  in permeabilized platelets in whole blood. A rabbit polyclonal or mouse monoclonal anti-GPIb $\alpha$  cytoplasmic domain antibodies (Green) and a rabbit polyclonal or mouse monoclonal anti-GPIb $\alpha$  ectodomain antibody (AK2; Red) are used to assess the amount of intact receptor relative to the total amount of tail (intact plus remnant) by flow cytometry (Figure 1).

C:\NRPort\NDC\DX\4186\76\_3.DOC-7/25/2012

- 34 -

[0122] In accordance with the present invention, and in some embodiments, dual-labeling is used involving phycoerythrin (PE)-labeled antibody against the GPIb $\alpha$  ectodomain, platelets are rapidly fixed and washed by centrifugation then permeabilized with 0.1-0.5% (w/v) saponin (ThromboFix) in buffer containing Alexa-488-labeled anti-GPIb $\alpha$  cytoplasmic tail IgG. Platelets are then washed in permeabilization buffer to remove any unbound cytoplasmic tail IgG and levels of respective antibody are assessed by flow cytometry, using the forward and side scatter characteristics of platelets and confirmed using antibodies against other platelet receptors ( $\alpha_{IIb}\beta_3$ , PECAM-1). The level of Alexa-488 staining reflects the detected total amount of expressed GPIb $\alpha$ , while levels of PE staining reflect the detected amount of the ectodomain portion of GPIb $\alpha$  (intact GPIb $\alpha$ ).

## EXAMPLE 2

### *The GPIb $\alpha$ intact:total ratio provides a sensitive measure of enhanced GPIb proteolysis in disease states*

[0123] The assay described herein has been verified in platelets from 20 healthy donors and 11 patients with thrombocytopenia, by testing untreated platelets or platelets treated with agents known to induce proteolysis of GPIb $\alpha$  in permeabilized versus non-permeabilized platelets, in washed platelets versus whole blood, etc. Flow cytometry data correlate with the degree of shedding assessed by Western blotting platelets from the same samples. An index reporting on the normal range for GPIb $\alpha$  molecular status has been developed from data obtained from 20 healthy donors at the Australian Centre for Blood Diseases donor database.

[0124] The proteolytic status of GPIb $\alpha$  is sensitive to shedding *in vitro* as the GPIb $\alpha$  intact:total ratio (arbitrary units) in platelets from 20 healthy donors ( $0.92 \pm 0.11$ ) was significantly reduced by 10-min treatment of platelets in plasma with a reagent (NEM) that directly induces GPIb $\alpha$  shedding ( $0.67 \pm 0.05$ ;  $n=11$ ,  $P < 0.001$ ). This indicated the GPIb $\alpha$  intact:total ratio provides a sensitive measure of enhanced proteolysis in

C:\NRPort\IDCCDXT\4486876\_3.DOC-7/25/2012

- 35 -

disease states, supported by preliminary studies in patients with thrombocytopenia

[0125] First, as proof-of-concept that the proteolytic status of GPIb $\alpha$  could be measured in clinical samples from thrombocytopenic patients, and that GPIb $\alpha$  proteolytic status changes depending on the cause of the thrombocytopenia, 11 patients with subnormal platelet count ( $<100 \times 10^9/l$ ) due to various causes were initially analyzed. Table 1 shows that the GPIb $\alpha$  intact:total staining ratio fell below the normal range in ITP ( $n=7$ ), antiphospholipid antibody syndrome (APS;  $n=1$ ) or thrombotic thrombocytopenia purpura (TTP;  $n=3$ ).

[0126] Table 2 shows that there was no significant correlation between GPIb $\alpha$  intact:total ratio and platelet count, GPIb $\alpha$  surface levels or platelet volume in the Thrombocytopenia group.

[0127] As shown in Table 2, there was a lack of any significant correlation between GPIb $\alpha$  intact:total ratio and platelet count, platelet size (mean platelet volume, range 8.5-10.5 units) or GPIb $\alpha$  surface levels in the thrombocytopenia group. This supports the value of the ratio as an independent measure of platelet quality. In contrast, there was a significant relationship between GPIb $\alpha$  surface levels and platelet count (Table 2) suggesting that unlike the GPIb $\alpha$  ratio, GPIb $\alpha$  surface levels alone do not adequately measure platelet quality. Temporal analysis of individual APS and TTP patients at 1-2 day intervals following treatment with steroids or plasma-exchange, respectively, resulted in recovery of platelet count from  $<20$  to  $>150 \times 10^9/l$  beyond days 5-7, whereas the GPIb $\alpha$  intact:total ratio normalized ( $>0.9$ ) 2-3 days earlier than full recovery of platelet count.

[0128] The unknown relative contribution of platelet consumption and platelet production to thrombocytopenia is an ongoing clinical problem. The present invention provides, *inter alia*, a new clinical tool for monitoring platelet function and survival in thrombocytopenic samples that will aid clinical decision-making in the setting of thrombocytopenia.

**EXAMPLE 3*****GPIb $\alpha$  (Intact:Total) is a reliable early marker for evaluating ITP and an early marker for evaluating response to treatment***

[0129] Gardiner *et al.*, *Blood* 111: 165-74, 2008a showed that engagement of another platelet Fc receptor, Fc $\gamma$ RIIa, by anti-platelet antibodies induced ectodomain shedding of GPVI, generating soluble GPVI (sGPVI) in plasma. However, apart from a single individual with an anti-GPVI auto-antibody (Gardiner *et al.*, *Journal of Thrombosis and Haemostasis*, 6: 1175-1182, 2008b), it is not known whether other anti-platelet auto-antibodies associated with idiopathic thrombocytopenic purpura (ITP) affect GPVI and/or GPIb $\alpha$  surface expression.

[0130] Platelet receptor expression and platelet parameters were assessed in patients with idiopathic thrombocytopenia (ITP) before and during steroid treatment.

[0131] The diagnosis of ITP was based on clinical and laboratory data according to consensus guidelines (Neunert *et al.*, *Blood* 117: 4190-4120, 2011). Flow cytometry (Gardiner *et al.*, 2008b (*supra*)) and sGPVI ELISA (Al-Tamimi *et al.*, *Platelets* 20: 143-149, 2009) were used to assess: 1) whether patients with ITP had dysregulated expression or shedding of GPVI or GPIb $\alpha$ , and 2) changes in platelet receptor expression after treatment. The absolute-immature platelet fraction (A-IPF) based on thiazole orange (TO)-staining of platelet mRNA was calculated as % platelets with mRNA above threshold x platelet count (as described by Barsam *et al.*, *Blood* 117(21): 5723-32, 2011 incorporated herein by reference in its entirety).

[0132] Unlike platelet count, A-IPF or surface levels, GPIb $\alpha$  Intact:Total is independent of:

Platelet count  $r^2 = 0.021$   $P=0.815$

GPIb $\alpha$  surface level  $r^2 = 0.507$   $P=0.117$ .

[0133] As shown in Table 3 (updated in Table 7) and Figure 3, GPVI shedding (decreased GPVI surface expression and increased sGPVI in plasma) is associated with ITP, consistent with GPVI shedding *in vivo* following engagement of Fc $\gamma$ RIIa by anti-

C:\NRPort\BIDCDXT\4486876\_3.DOC-7/25/2012

- 37 -

platelet antibodies (Gardiner *et al.* 2008a (*supra*)). This has previously been shown for an ITP patient with an anti-GPVI IgG (Gardiner *et al.* 2008b (*supra*)).

[0134] The proteolytic status of GPIb $\alpha$  (Intact:Total) is a reliable early marker for evaluating response to treatment in ITP patients.

[0135] GPIb $\alpha$  Intact:Total provides an early marker for thrombocytopenia and an early marker for evaluating the response to treatment with steroid therapy or other therapies.

#### EXAMPLE 4

##### *Current diagnostic methods for monitoring platelets*

[0136] Existing methods for analyzing platelets or platelet receptors in thrombocytopenia and other haematological defects include estimating the number of platelets hemocytometrically.

[0137] Anuclear platelets contain RNA and "reticulated" or immature platelets (reticulocytes) stain positively with thiazole orange (TO) whereas the percentage of reticulated platelets (%RP) decreases the platelets age. The age of a population of platelets has been widely assessed according to the research literature using thiazole orange (TO), a membrane-permeable nucleic acid-specific fluorescent dye. In healthy individuals, %RP is ~6% of the total platelet population, but is increased to ~70% in patients with ITP, and increases to 28% during the recovery phase of chemotherapy-related thrombocytopenia. A recent study (Barsam *et al.*, 2011 (*supra*)) describes the use of the Sysmex automated system to analyze the immature platelet fraction in the clinical setting, by examining 8 ITP patients pre- and post-treatment. The limitation of this approach is the lack of standardization, TO staining is highly variable between individuals, may be disadvantageously sensitive to the time between sample collection and analysis, is dependent on platelet size and does not discriminate between different causes of thrombocytopenia.

- 38 -

[0138] In another approach, the glyco-calicin index (GCI) is determined. The soluble plasma GPIb $\alpha$  concentration normalized for platelet count is inversely correlated with %RP in ITP, consistent with increased GPIb $\alpha$  shedding during platelet destruction. This method lacks sensitivity because background levels of glyco-calicin are very high (1-3  $\mu$ g/ml). The GCI is not routinely measured clinically, and does not accurately report on the quality of circulating platelets at the time of measurement.

[0139] In a further approach, GPIb $\alpha$  surface levels are determined. However, GPIb $\alpha$  surface expression decreases as normal platelets age in the circulation, and also varies with platelet count and size. Thus, changes due to disease are not distinguishable, nor can it be determined whether altered levels are due to shedding or other causes. The surface expression levels will also not be sensitive to whether the cause of increased shedding is successfully treated, whereas measuring proteolytic status as determined herein is independent of platelet count and GPIb $\alpha$  expression levels.

## EXAMPLE 5

### *Platelet PMR intact/total ratio and platelet parameters in patients with idiopathic thrombocytopenia (ITP) before and during steroid treatment*

[0140] The GPIb $\alpha$  index (Intact/total) and platelet counts were determined in subjects (Subjects C, X, V and J) with chronic ITP before and after various treatment protocols. The results are represented graphically in Figure 4 to Figure 7. Subject C was thrombocytopenic and platelet counts gradually increased over time during steroid therapy (after one month). However, the GPIb $\alpha$  index was approximately normal from week 1 to week 20. Subject X was non-responsive to steroids in terms of platelet counts and exhibited low platelet counts. The GPIb $\alpha$  index level was initially low, fluctuated, but increased prior to azathioprine treatment notwithstanding decreasing platelet counts. Upon treatment with azathioprine platelet levels increased and the GPIb $\alpha$  index increased to a normal level (0.9). Subject V displayed a rising GPIb $\alpha$  index on Romiplostin. For Subject J, an improvement in platelet quality (normalisation of the GPIb $\alpha$  Intact:Total

C:\NR\Portb\NCC\DX\4486876\_3.DOC-7/25/2012

- 39 -

ratio) precedes improvement in platelet count (see Figure 7). The proteolytic status of GPIb $\alpha$  (Intact:Total) is a reliable early marker for evaluating response to treatment in ITP patients. Particularly in patients who have a low platelet count such as below 100, or about  $100 \times 10^6/l$ .

[0141] The disclosure of every patent, patent application, and publication cited herein is hereby incorporated herein by reference in its entirety.

[0142] The citation of any reference herein should not be construed as an admission that such reference is available as "Prior Art" to the instant application.

[0143] Many modifications will be apparent to those skilled in the art without departing from the scope of the present invention.

**TABLE 1: Summary of clinical data for Example 2**

|                                                           | Healthy donors                   | Thrombocytopenia                | P value <sup>2</sup> | Significant? |
|-----------------------------------------------------------|----------------------------------|---------------------------------|----------------------|--------------|
| <i>n</i>                                                  | 20                               | 11                              |                      |              |
| <b>Platelet count (range)</b>                             | 383 $\pm$ 18<br>(225-527)        | 59 $\pm$ 10<br>(19-137)         | <0.0001              | Yes          |
| <sup>1</sup> <b>Surface GPIb<math>\alpha</math></b>       | 940 $\pm$ 95                     | 669 $\pm$ 119                   | 0.094                | No           |
| <sup>1</sup> <b>Total GPIb<math>\alpha</math></b>         | 283.5 $\pm$ 19                   | 303.5 $\pm$ 31                  | 0.552                | No           |
| <b>GPIb<math>\alpha</math> intact/total ratio (range)</b> | 0.925 $\pm$ 0.01<br>(0.816-0.98) | 0.722 $\pm$ 0.09<br>(0.72-0.96) | 0.006                | Yes          |
| <b>Mean platelet volume (fL)</b>                          | 8.2 $\pm$ 0.12                   | 9.4 $\pm$ 0.36                  | 0.216                | No           |

<sup>1</sup> Geomean values for PE-AK2 (intact GPIb $\alpha$ ) and Alexa<sup>488</sup>-GPIb $\alpha$  tail (total GPIb $\alpha$ ).

<sup>2</sup> P value calculated using unpaired, two-tailed t test.

**TABLE 2: Summaries of data for Example 2**

|                                      |                           |                 |
|--------------------------------------|---------------------------|-----------------|
| GPIb ratio v platelet count          | $r^2 = 0.0163, P = 0.705$ | No significance |
| GPIb ratio v surface levels          | $r^2 = 0.1876, P = 0.332$ | No significance |
| GPIb ratio v platelet vol            | $r^2 = 0.117, P = 0.45$   | No significance |
| GPIb surface levels v platelet count | $r^2 = 0.5995, P = 0.04$  | Significant     |
| GPIb surface levels v platelet vol   | $r^2 = 0.235, P = 0.18$   | No significance |

C:\WP\PortDCC\DX\T\4486876\_3.DOC-7/25/2012

- 40 -

TABLE 3: Patient data for Example 4

|                                                               | Healthy donors<br>(n = 17) | ITP Patients <sup>b</sup> |                                                |                                                  |
|---------------------------------------------------------------|----------------------------|---------------------------|------------------------------------------------|--------------------------------------------------|
|                                                               |                            | Pre-treatment<br>(n = 9)  | Post-treatment <sup>c</sup>                    |                                                  |
|                                                               |                            |                           | Short term <sup>d</sup><br>(1 week)<br>(n = 8) | Long term <sup>d</sup><br>(> 4 weeks)<br>(n = 4) |
| <sup>e</sup> Platelet count (x 10 <sup>9</sup> /L)<br>(range) | 247 ± 13<br>(137-360)      | 61 ± 9<br>(33-122)        | 120 ± 29<br>(24-268)                           | 187 ± 41<br>(100-300)                            |
| <i>P</i> *                                                    |                            | <0.0001***                | ***                                            | ns                                               |
| Mean platelet vol (fL)<br>(range)                             | 8.45 ± 0.2<br>(7.1-9.4)    | 9.7 ± 0.4<br>(7.5-10.8)   | 8.7 ± 0.3<br>(7.9-10.1)                        | n/a                                              |
| <i>P</i> *                                                    |                            | 0.0084 **                 | ns                                             | -                                                |
| GPVI (GeoMean)                                                | 274.7 ± 26.5               | 137.1 ± 17.51             | 158.5 ± 14.6                                   | 156.1 ± 34.1                                     |
| <i>P</i> *                                                    |                            | 0.0027**                  | *                                              | ns                                               |
| <sup>f</sup> Plasma sGPVI (ng/ml)                             | 19.87 ± 2.97               | 39.1 ± 3.67               | 45.19 ± 5.73                                   | 53.89 ± 4.53                                     |
| <i>P</i> *                                                    |                            | 0.0006***                 | ***                                            | ***                                              |
| GPIbα (GeoMean)                                               | 711.1 ± 37.2               | 638.2 ± 77.15             | 685.8 ± 55.43                                  | n/a                                              |
| <i>P</i> *                                                    |                            | 0.357                     | ns                                             | -                                                |
| <sup>g</sup> GPIbα Intact:Total                               | 0.922 ± 0.01               | 0.848 ± 0.02              | 0.89 ± 0.05                                    | 0.900 ± 0.069                                    |
| <i>P</i> *                                                    |                            | 0.0053**                  | ns                                             | ns                                               |

<sup>a</sup> Healthy Donors – aged 18-42 (59% female)<sup>b</sup> ITP Patients – aged 28-79 (67% female); P values compared to Healthy Donors (ns, not significant).<sup>c</sup> Treatment with steroids (Prednisolone, Dapsone or in combination).<sup>d</sup> Earliest time point was 1 week, latest time point >4 weeks (n/a, not available)<sup>e</sup> Platelet count in platelet-rich plasma, reference range, 150-400 x 10<sup>9</sup>/L<sup>f</sup> Reference values in healthy donors: mean 19 ± 4 ng/mL, range 11-24 ng/mL, n=10<sup>g</sup> Measured using anti-GPIbα cytoplasmic tail IgG that recognizes intact GPIbα and remnant tail (~16 kDa, red.).

C:\WRPort\NDCCDXT\4486876\_3.DOC-7/25/2012

- 41 -

TABLE 4

| SEQ ID NO. |                      | CDR sequence underlined                 |
|------------|----------------------|-----------------------------------------|
| 1          | AK2 Heavy Chain CDR1 | CTVSGFSLIGYSVNWVRQPPGKGL                |
| 2          | AK2 Heavy Chain CDR2 | GLEWLGMIWGDGSTD <del>FNS</del> ALKSRLSI |
| 3          | AK2 Heavy Chain CDR3 | TDTARYYC <del>ARD</del> RRGSHAMDYWGQG   |
| 4          | AK2 Kappa chain CDR1 | RASESVDSYGNSFMH <del>W</del> YQQ        |
| 5          | AK2 Kappa chain CDR2 | LLIYRASNL <del>ES</del> GIPA            |
| 6          | AK2 Kappa chain CDR3 | YCQ <del>Q</del> SNEDPIHVRL             |

TABLE 5: Platelet ectodomain sequences cleaved by ADAMs

| Name                                             | Cut by human<br>rADAM10 | Cut by human<br>rADAM17 | Site                   |
|--------------------------------------------------|-------------------------|-------------------------|------------------------|
| Human GPVI<br>cleavage site is<br>R243Q          | Yes                     | No                      | <sup>240</sup> PAR*QYY |
| Human GPIb $\alpha$<br>cleavage site is<br>G465V | No                      | Yes                     | <sup>462</sup> LRG*VLQ |
| Human GPV<br>cleavage site is<br>P494V           | Yes                     | Yes                     | <sup>491</sup> AQP*VTT |

C:\WP\PortB\NDCCDXT4486876\_1.DOC:7/25/2012

- 42 -

**TABLE 6: List of suitable fluorophores**

| Probe                                 | Ex <sup>1</sup> (nm) | Em <sup>2</sup> (nm) |
|---------------------------------------|----------------------|----------------------|
| <b>Reactive and conjugated probes</b> |                      |                      |
| Hydroxycoumarin                       | 325                  | 386                  |
| Aminocoumarin                         | 350                  | 455                  |
| Methoxycoumarin                       | 360                  | 410                  |
| Cascade Blue                          | 375; 400             | 423                  |
| Lucifer Yellow                        | 425                  | 528                  |
| NBD                                   | 466                  | 539                  |
| R-Phycoerythrin (PE)                  | 480; 565             | 578                  |
| PE-Cy5 conjugates                     | 480; 565; 650        | 670                  |
| PE-Cy7 conjugates                     | 480; 565; 743        | 767                  |
| APC-Cy7 conjugates                    | 650; 755             | 767                  |
| Red 613                               | 480; 565             | 613                  |
| Fluorescein                           | 495                  | 519                  |
| FluorX                                | 494                  | 520                  |
| BODIPY-FL                             | 503                  | 512                  |
| TRITC                                 | 547                  | 574                  |
| X-Rhodamine                           | 570                  | 576                  |
| Lissamine Rhodamine B                 | 570                  | 590                  |
| PerCP                                 | 490                  | 675                  |
| Texas Red                             | 589                  | 615                  |
| Allophycocyanin (APC)                 | 650                  | 660                  |
| TruRed                                | 490, 675             | 695                  |
| Alexa Fluor 350                       | 346                  | 445                  |
| Alexa Fluor 430                       | 430                  | 545                  |
| Alexa Fluor 488                       | 494                  | 517                  |
| Alexa Fluor 532                       | 530                  | 555                  |
| Alexa Fluor 546                       | 556                  | 573                  |
| Alexa Fluor 555                       | 556                  | 573                  |
| Alexa Fluor 568                       | 578                  | 603                  |
| Alexa Fluor 594                       | 590                  | 617                  |
| Alexa Fluor 633                       | 621                  | 639                  |
| Alexa Fluor 647                       | 650                  | 688                  |
| Alexa Fluor 660                       | 663                  | 690                  |
| Alexa Fluor 680                       | 679                  | 702                  |
| Alexa Fluor 700                       | 696                  | 719                  |
| Alexa Fluor 750                       | 752                  | 779                  |

C:\WP\PortB\CC\X134868\0\_3\_DOC-1125\2012

- 43 -

| Probe                       | Ex <sup>1</sup> (nm) | Em <sup>2</sup> (nm) |
|-----------------------------|----------------------|----------------------|
| Cy2                         | 489                  | 506                  |
| Cy3                         | (512); 550           | 570; (615)           |
| Cy3,5                       | 581                  | 596; (640)           |
| Cy5                         | (625); 650           | 670                  |
| Cy5,5                       | 675                  | 694                  |
| Cy7                         | 743                  | 767                  |
| <b>Nucleic acid probes</b>  |                      |                      |
| Hoeschst 33342              | 343                  | 483                  |
| DAPI                        | 345                  | 455                  |
| Hoechst 33258               | 345                  | 478                  |
| SYTOX Blue                  | 431                  | 480                  |
| Chromomycin A3              | 445                  | 575                  |
| Mithramycin                 | 445                  | 575                  |
| YOYO-1                      | 491                  | 509                  |
| SYTOX Green                 | 504                  | 523                  |
| SYTOX Orange                | 547                  | 570                  |
| Ethidium Bromide            | 493                  | 620                  |
| 7-AAD                       | 546                  | 647                  |
| Acridine Orange             | 503                  | 530/640              |
| TOTO-1, TO-PRO-1            | 509                  | 533                  |
| Thiazole Orange             | 510                  | 530                  |
| Propidium Iodide (PI)       | 536                  | 617                  |
| TOTO-3, TO-PRO-3            | 642                  | 661                  |
| LDS 751                     | 543; 590             | 712; 607             |
| <b>Fluorescent Proteins</b> |                      |                      |
| Y66F                        | 360                  | 508                  |
| Y66H                        | 360                  | 442                  |
| EBFP                        | 380                  | 440                  |
| Wild-type                   | 396, 475             | 50, 503              |
| GFpuv                       | 385                  | 508                  |
| ECFP                        | 434                  | 477                  |
| Y66W                        | 436                  | 485                  |
| S65A                        | 471                  | 504                  |
| S65C                        | 479                  | 507                  |
| S65L                        | 484                  | 510                  |
| S65T                        | 488                  | 511                  |
| EGFP                        | 489                  | 508                  |

C:\NRPortb\NDCC\DXTV44\6876\_3.DOC-7/25/2012

- 44 -

| Probe               | Ex <sup>1</sup> (nm) | Em <sup>2</sup> (nm) |
|---------------------|----------------------|----------------------|
| EYFP                | 514                  | 527                  |
| DsRed               | 558                  | 583                  |
| <b>Other probes</b> |                      |                      |
| Monochlorobimane    | 380                  | 461                  |
| Calcein             | 496                  | 517                  |

<sup>1</sup> Ex: Peak excitation wavelength (nm)<sup>2</sup> Em: Peak emission wavelength (nm)

**TABLE 7: Patient data for chronic ITP patients with platelet count <40 when off treatment (Example 5) updated from Example 3**

|                                                                                     | Healthy donors <sup>a</sup><br>(n = 17) | ITP Patients <sup>b</sup>             |                             |                               |
|-------------------------------------------------------------------------------------|-----------------------------------------|---------------------------------------|-----------------------------|-------------------------------|
|                                                                                     |                                         | Pre-treatment <sup>c</sup><br>(n = 5) | Post-treatment <sup>c</sup> |                               |
|                                                                                     |                                         |                                       | 1 week steroids<br>(n = 9)  | > 4 weeks steroids<br>(n = 7) |
| <sup>d</sup> Platelet count<br>(x 10 <sup>9</sup> /L)<br>(range)<br><sup>g</sup> P* | 247 ± 13<br>(137-360)                   | 25 ± 5<br>(2-35)                      | 121 ± 26<br>(24-268)        | 194 ± 41<br>(102-300)         |
|                                                                                     |                                         | ***                                   | ***                         | ns                            |
| Mean platelet vol<br>(fL)<br>P*                                                     | 8.45 ± 0.2                              | 9.6 ± 0.3                             | 8.6 ± 0.3                   | 8.4 ± 0.4                     |
|                                                                                     |                                         | *                                     | ns                          | ns                            |
| GPVI<br>(GeoMean)<br>P*                                                             | 274 ± 26                                | 86 ± 32                               | 159 ± 12                    | 158 ± 16                      |
|                                                                                     |                                         | **                                    | *                           | ns                            |
| <sup>e</sup> Plasma sGPVI<br>(ng/ml)<br>P*                                          | 19.9 ± 2.97                             | 40 ± 8                                | 50 ± 6                      | 71 ± 12                       |
|                                                                                     |                                         | **                                    | ***                         | ***                           |
| GPIbα<br>(GeoMean)<br>P*                                                            | 711 ± 37                                | 237 ± 91                              | 677 ± 33                    | 736 ± 35                      |
|                                                                                     |                                         | ***                                   | ns                          | ns                            |
| <sup>f</sup> GPIbα<br>Intact: Total<br>P*                                           | 0.922 ± 0.01                            | 0.48 ± 0.17                           | 0.90 ± 0.04                 | 0.92 ± 0.02                   |
|                                                                                     |                                         | ***                                   | ns                          | ns                            |

<sup>a</sup> Healthy Donors – aged 18-42 (59% female)<sup>b</sup> ITP Patients – aged 28-79 (67% female); P values compared to Healthy Donors

C:\WP\PortB\IDCC\DX\T44\68176\_3.DOC-7/25/2012

- 45 -

(ns, not significant). Platelet count < 40 when off treatment

<sup>c</sup> Earliest time point was 1 week, latest time point >4 weeks. Treatment with steroids (Prednisolone, Dapsone or in combination).

<sup>d</sup> Platelet count in platelet-rich plasma, reference range, 150-400 x 10<sup>9</sup>/L

<sup>e</sup> Measured by in-house ELISA. Reference values in healthy donors: mean 19 ± 4 ng/mL, range 11-24 ng/mL, n=10 (Al-Tamimi *et al.*, 2009 (*supra*))

<sup>f</sup> Ratio of intact GPIIb/IIIa to total amount of GPIIb/IIIa on circulating platelets measured with assay using antibodies described in Gardiner *et al.*, 2007 (*supra*).

<sup>g</sup> Difference from data obtained in healthy donors assessed by student t test. \*=P<0.1; \*\*=P<0.05; \*\*\*=P<0.001; ns = not significant

C:\NR\Patent\DOC\14486876\_1.DOC-7/25/2012

- 46 -

**BIBLIOGRAPHY**

- Al-Tamimi *et al.*, *Platelets* 20: 143-149, 2009
- Ausubel (Ed), "*Current Protocols in Molecular Biology*", 5<sup>th</sup> Edition, John Wiley & Sons, Inc, NY, Chapter 11, 2002
- Ausubel *et al.*, *Current Protocols in Molecular Biology* John Wiley & Sons Inc, in particular Section III of Chapter 11, 1994-1998
- Barbas *et al.*, *PNAS* 88: 7978-7982, 1991
- Barsam *et al.*, *Blood* 117(21): 5723-32, 2011
- Cautrecases, *J. Biol. Chem.* 245: 3059, 1970
- Clackson *et al.*, *Nature* 352: 624-628, 1991
- Coligan *et al.*, "*Current Protocols in Immunology*", John Wiley & Sons, Inc, 1991
- Gardiner *et al.*, *Blood* 111: 165-74, 2008a
- Gardiner *et al.*, *Journal of Thrombosis and Haemostasis* 6: 1175-1182, 2008b
- Gardiner *et al.*, *Journal of Thrombosis and Haemostasis* 5: 1530-1537, 2007
- Garrad *et al.*, *Bio/Technology* 9: 1373-1377, 1991
- Ge, *Nucleic Acids Res.* 28(2): e3, 2000
- Glockscuther *et al.* *Biochem.* 29: 1363-1367,
- Glorio-Paulet *et al.*, *J Agric Food Chem* 48 (5): 1678-1682, 2000
- Gram *et al.*, *PNAS* 89: 3576-3580, 1992
- Griffiths *et al.*, *EMBO J* 12: 725-734, 1993
- Harlow and Lane, "*Antibodies, A Laboratory Manual*", Cold Spring Harbor Publications, New York, 1988
- Hawkins *et al.*, *J Mol Biol* 226: 889-896, 1992
- Hoogenboom *et al.*, *Nuc Acid Res* 19: 4133-4137, 1991
- Huse *et al.*, *Science* 246: 1275-1281, 1989
- Jalkanen *et al.*, *J. Cell. Biol.* 101: 976-985, 1985
- Jalkanen *et al.*, *J. Cell. Biol.* 105: 3087-3096, 1987

C:\NRPort\INDCCD\XT\4486876\_3.DOC-7/25/2012

- 47 -

- Köhler and Milstein, *Nature* 256: 495-497, 1975
- Kreber *et al.*, *J. Immunol. Methods*, 201(1): 35-55
- Lacobilli *et al.*, *Breast Cancer Research and Treatment* 11: 19-30, 1988
- Mu *et al.*, *Blood* 111: 4580-4587, 2008
- Neunert *et al.*, *Blood* 117: 4190-4120, 2011
- Plückthun *et al.*, In *Antibody engineering: A practical approach*, 203-252, 1996
- Reiter *et al.*, *Biochem.* 33: 5451-5459, 1994
- Reiter *et al.*, *Cancer Res.* 54: 2714-2718, 1994
- Reiter *et al.*, *J. Biol. Chem.* 269: 18327-18331, 1994
- Shen *et al.*, *Blood* 95: 903-910, 2000
- Webber *et al.*, *Mol. Immunol.* 32: 249-258, 1995
- Wild D, "*The Immunoassay Handbook*", Nature Publishing Group, 2001
- Winter and Milstein, *Nature* 349: 293, 1991

- 48 -

### WHAT IS CLAIMED IS:

1. An assay for assessing the proteolytic state of test platelets in a biological sample, the assay comprising detecting the level of intact platelet membrane receptor (PMR) (a) and detecting the level of total (cleaved and uncleaved) PMR (b) associated with platelets in the sample, and comparing a measure of the relationship between levels (a) and (b) ("test relationship") to a corresponding relationship measure derived from at least one reference biological sample ("reference relationship") selected from a sample obtained from one or more normal (e.g. healthy) reference subjects ("normal reference relationship") or from one or more reference samples having predetermined measured levels of intact and total PMR indicating that the reference subject has supernormal measured levels of cleaved PMR associated with platelets ("supernormal cleavage reference relationship"), wherein the similarity or difference between the test relationship and the reference relationship indicates the proteolytic state of platelets in the test sample.
2. The assay of claim 1 comprising (i) optionally contacting test platelets with an agent capable of permeabilizing platelets, (ii) contacting test platelets with a PMR antigen-binding molecule that binds to the ectodomain of PMR on the surface of platelets to provide a measure of the level of intact PMR; (iii) contacting test platelets with a PMR antigen-binding molecule that binds to the residual domain of PMR associated with platelets after ectodomain cleavage to provide a measure of the level of total (cleaved and uncleaved) PMR ("total"); (iv) comparing a measure of the relationship between the measured levels in (ii) and (iii) ("test relationship") to a corresponding relationship measure from at least one reference sample ("reference relationship") selected from a sample obtained from one or more normal (e.g. healthy) reference subjects ("normal reference relationship") or from one or more reference subjects having predetermined measured levels of intact and

C:\NR\Pub\1DCC\DX\T4486\T6\_3.DOC-7/25/2012

- 49 -

- total PMR indicating that the reference subject has supernormal measured levels of cleaved PMR associated with platelets ("supernormal cleavage reference relationship"), wherein the similarity or difference between the test and reference relationships indicates the proteolytic state of platelets in the test sample.
3. The assay of claim 1 or 2 wherein a similarity between the test relationship and the normal reference relationship or a difference between the test relationship and the supernormal cleavage reference relationship indicates the absence of supernormal levels of proteolytic cleavage in test platelets, and wherein a similarity between the test relationship and the supernormal cleavage reference relationship or a difference between the test relationship and the normal reference relationship indicate the presence of supernormal levels of proteolytic cleavage in test platelets.
  4. The assay of claim 1 or 2 or 3 wherein the test platelets are stored platelets, such as in stored blood or stored platelet concentrates.
  5. The assay of any one of claims 1 to 4 wherein the biological sample is whole blood.
  6. The assay of any one of claims 1 to 5 further comprising contacting platelets with an RNA-staining molecule and measuring the level of RNA in test platelets.
  7. The assay of any one of claims 1 to 6 wherein the PMR is GPIb $\alpha$ .
  8. The assay of claim 1 to 7 wherein the PMR is GPIb $\alpha$  and of the assay further comprises each step wherein the PMR is GPV and/or wherein the PMR is GPVI.
  9. The assay of any one of claims 1 to 8 for use in the diagnosis, prognosis, monitoring, treatment or management of conditions associated with

C:\NR\Perb\IDCCD\XT\4486876\_3.DOC-7/25/2012

- 50 -

thrombocytopenia or for use in the management of stored blood products.

10. The assay of any one of claims 1 to 10 wherein the antigen binding molecule that binds to the ectodomain of PMR on the surface of platelets is labelled with a first fluorescent or other detectable molecule, wherein the antigen binding molecule that binds to the residual domain of PMR associated with platelets after receptor cleavage is labelled with a second fluorescent or other detectable molecule, and wherein test platelets are subjected to flow cytometry to provide a measure of the level of intact PMR or proportion of platelets with intact PMR ("intact") and a measure of the level of total (cleaved and uncleaved) PMR or the proportion of platelets with cleaved and uncleaved PMR ("total").
11. The assay of claim 10 wherein the first and second fluorescent or other detectable molecules are different.
12. The assay of claim 9 or 11 further comprising contacting platelets with an RNA-staining fluorescent molecule and measuring the level of RNA in test platelets.
13. The assay of any one of claims 1 or 12 wherein the test and reference relationships are compared for two or more PMR.
14. A kit for assessing the proteolytic state of test platelets in a biological sample the kit comprising: (i) a PMR antigen-binding molecule that binds to the shed ectodomain of PMR, wherein the antigen-binding molecule is either immobilized (bound) to one or more test portions and/or contained within a conjugate portion; and/or (ii) a PMR antigen-binding molecule that binds to the residual domain of PMR associated with platelets after ectodomain cleavage, wherein the antigen-binding molecule is either immobilized (bound) to one or more test portions and/or

C:\NRPort\B\CCDXT\4486876\_3.DOC-7/23/2012

- 51 -

- contained within a conjugate portion and (iii) instructions for using the device for assessing the proteolytic status of platelets.
15. A kit of claim 14 for use in the diagnosis, prognosis, monitoring, treatment or management of conditions associated with thrombocytopenia.
  16. The kit of claim 14 for use in the management of stored blood products.
  17. The kit of any one of claims 14 to 16 wherein the PMR is GPIb $\alpha$ .
  18. The kit of claim 14 to 17 wherein the PMR receptor is GPIb $\alpha$  and wherein the kit further comprises antigen-binding molecules that bind to the shed ectodomain and residual domain of GPVI and/or GPV.
  19. An assay for differentially treating a population with respect to thrombocytopenia comprising (i) detecting the level of intact PMR (a or ta) and detecting the level of total (cleaved or uncleaved) PMR (b or tb) associated with platelets in a test biological sample, and (ii) comparing a measure of the relationship between levels (a) and (b) (the "test" relationship) to a control, wherein subjects in the population are stratified with respect to the proteolytic state of their platelets determined using the test relationship, and wherein subjects receive different treatment regimes and/or monitoring regimes based upon the proteolytic state of the platelets determined using the test relationship.
  20. The assay of claim 19 wherein the PMR is GPIb $\alpha$ .

- 1/7 -

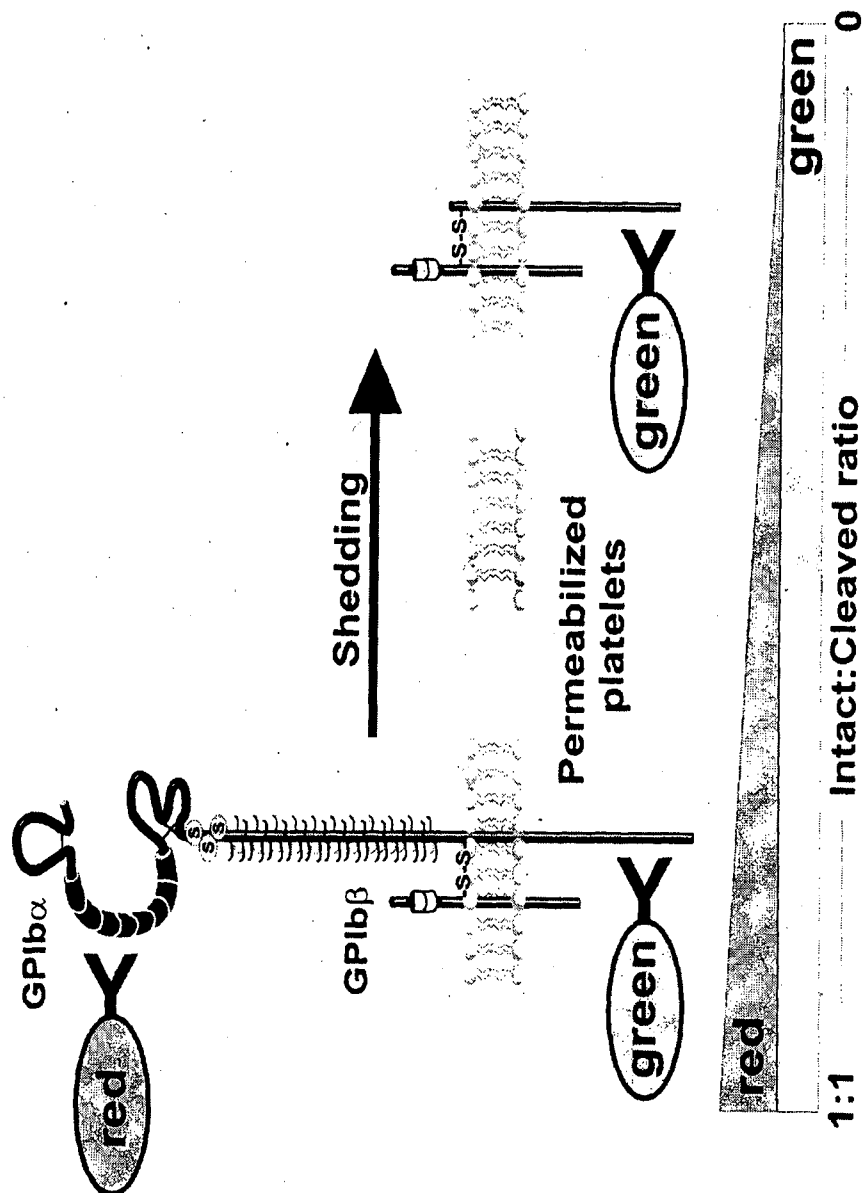


FIGURE 1

- 2/7 -

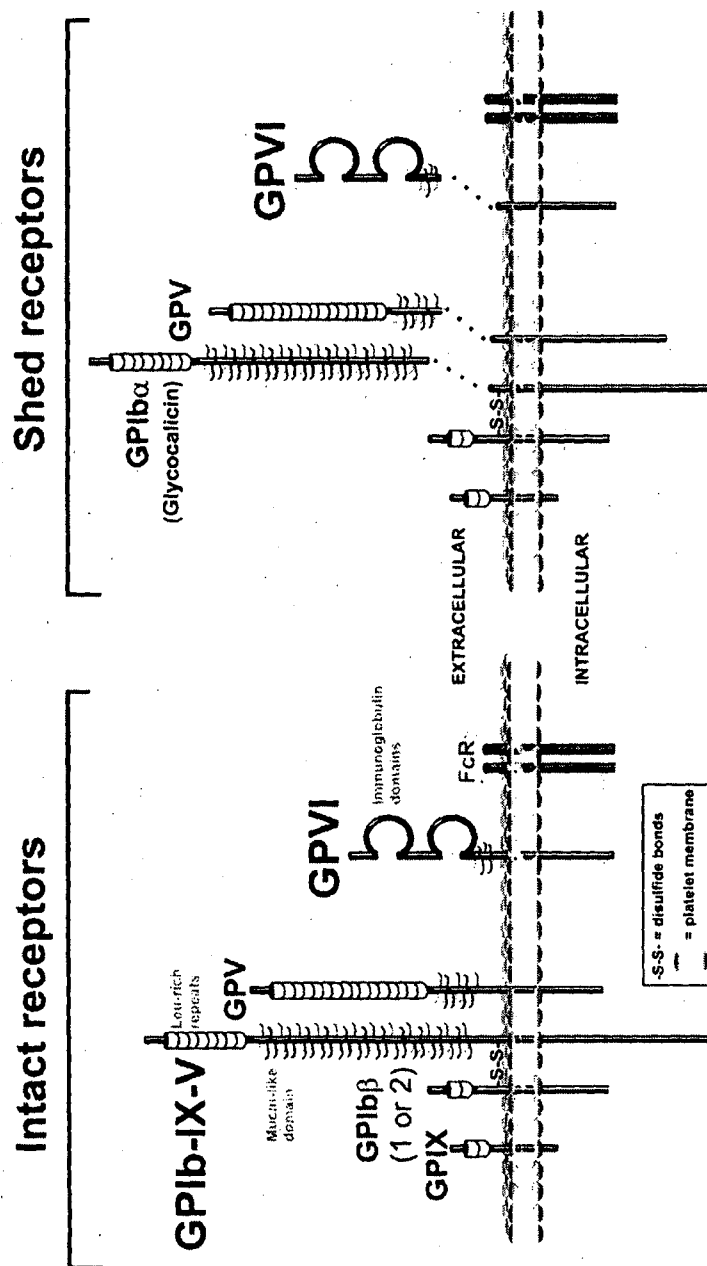


FIGURE 2

- 3/7 -

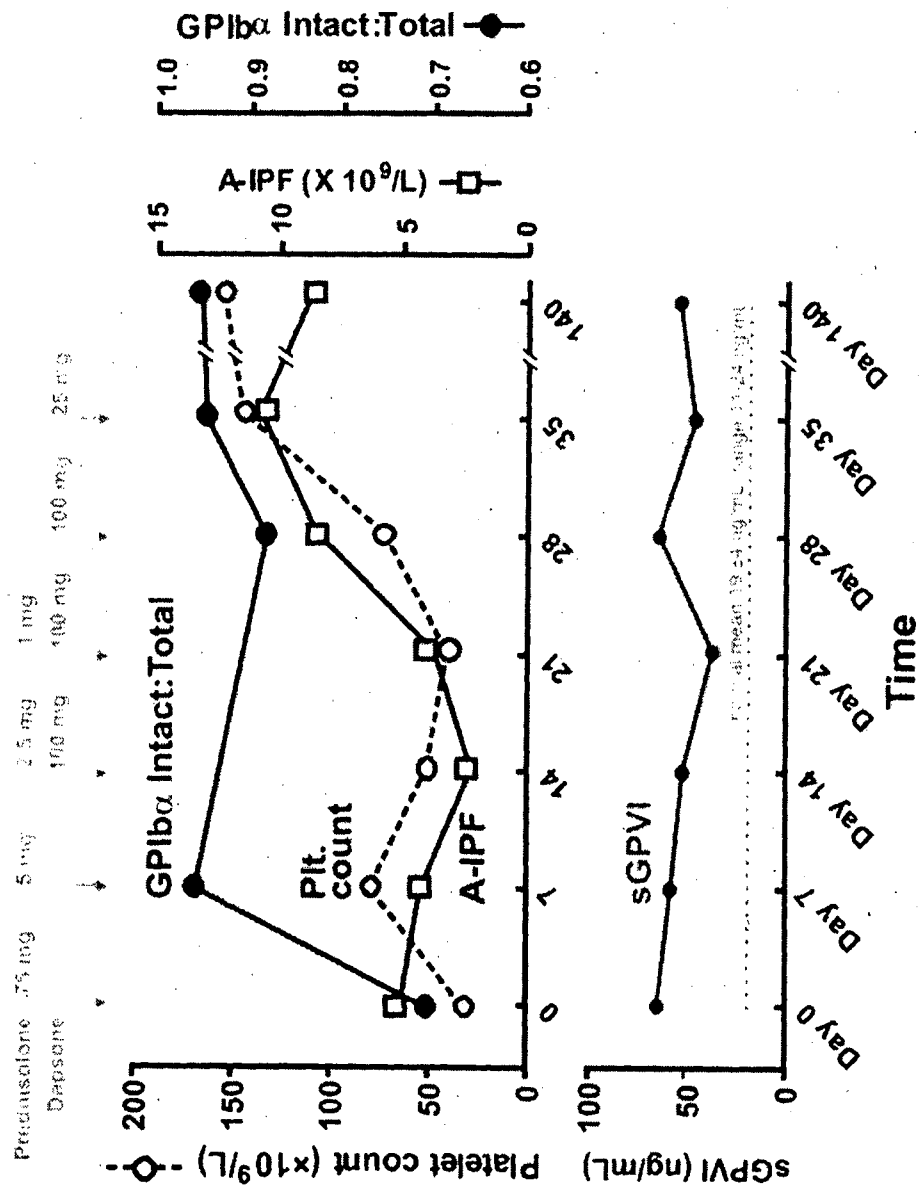


FIGURE 3

- 4/7 -

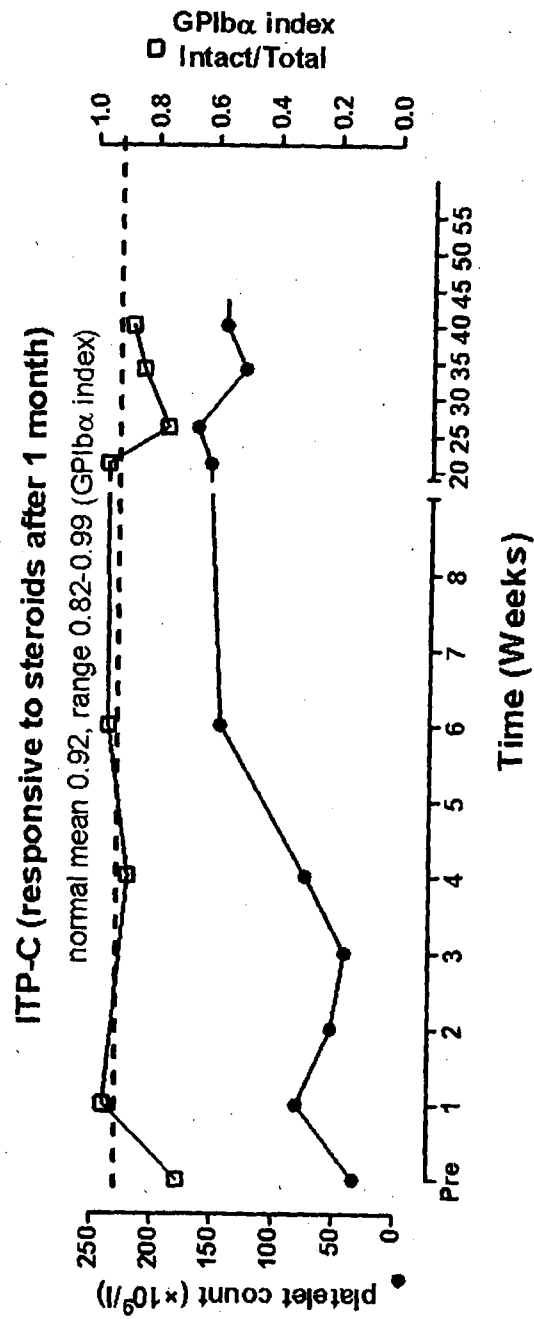


FIGURE 4

- 5/7 -

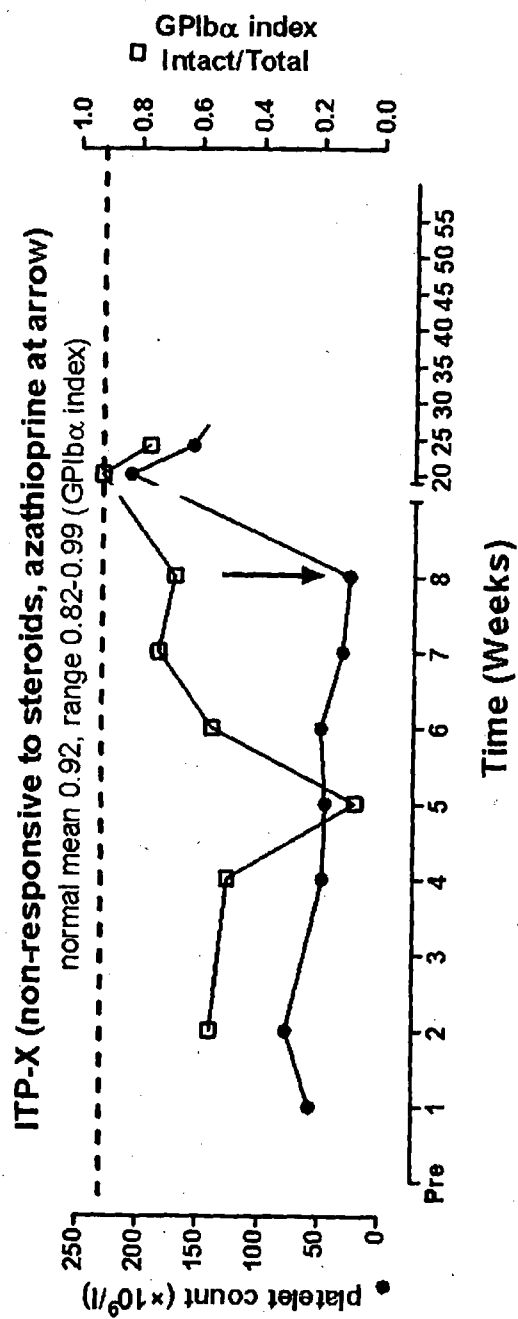


FIGURE 5

- 6/7 -

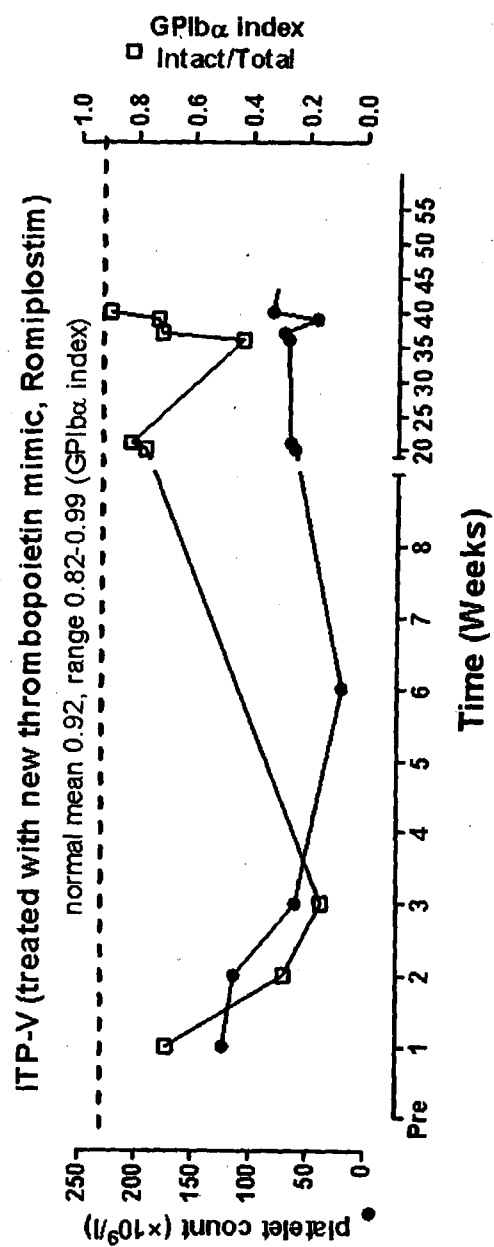


FIGURE 6

- 7/7 -

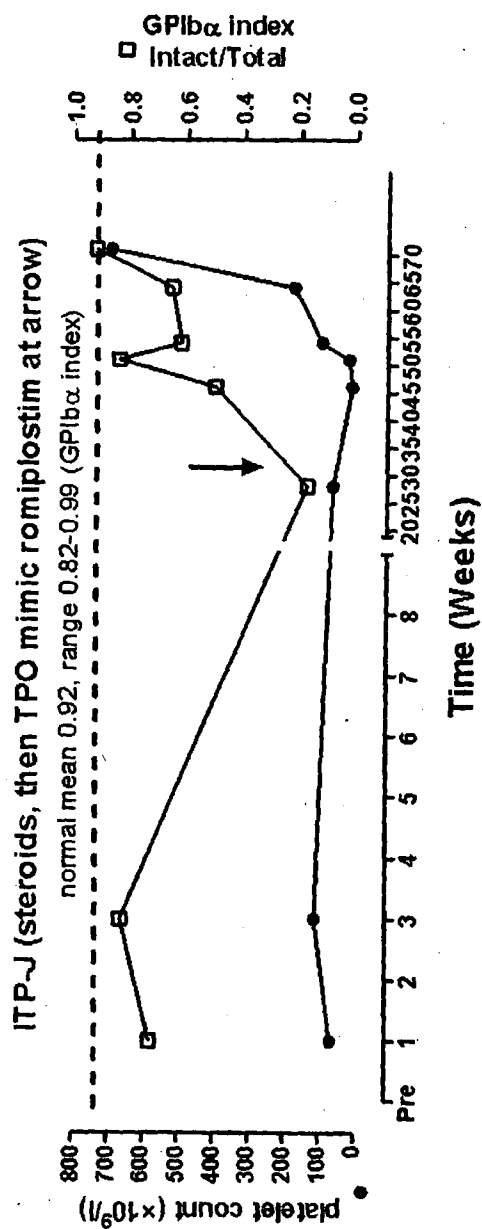


FIGURE 7

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2012/000883

## A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/532 (2006.01) G01N 33/533 (2006.01) G01N 33/534 (2006.01)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, CAPLUS, EMBASE, WPI, EPODOC: platelet, thrombocyte, glycoprotein 1b alpha, glycoprotein V, glycoprotein VI, receptor, shedding, cleavage, release, proteolysis, detect, assay, diagnose, prognosis, assess, thrombocytopenia, disease, and like terms.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|-----------|------------------------------------------------------------------------------------|-----------------------|
|           | Documents are listed in the continuation of Box C                                  |                       |

☒ Further documents are listed in the continuation of Box C☒ See patent family annex

|                                                                                                                                                                         |                                                                                                                                                                                                                                                  |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| * Special categories of cited documents:                                                                                                                                |                                                                                                                                                                                                                                                  |
| "A" document defining the general state of the art which is not considered to be of particular relevance                                                                | "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention                                              |
| "E" earlier application or patent but published on or after the international filing date                                                                               | "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone                                                                     |
| "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "O" document referring to an oral disclosure, use, exhibition or other means                                                                                            | "&" document member of the same patent family                                                                                                                                                                                                    |
| "P" document published prior to the international filing date but later than the priority date claimed                                                                  |                                                                                                                                                                                                                                                  |

Date of the actual completion of the international search  
2 October 2012Date of mailing of the international search report  
02 October 2012

Name and mailing address of the ISA/AU

AUSTRALIAN PATENT OFFICE  
PO BOX 200, WODEN ACT 2606, AUSTRALIA  
Email address: pct@ipaustalia.gov.au  
Facsimile No.: +61 2 6283 7999

Authorised officer

Jason MacKenzie  
AUSTRALIAN PATENT OFFICE  
(ISO 9001 Quality Certified Service)  
Telephone No. 0262837934

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2012/000883

## Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:
  - a. (means)
    - ☐ on paper
    - ☐ in electronic form
  - b. (time)
    - ☐ in the international application as filed
    - ☐ together with the international application in electronic form
    - ☐ subsequently to this Authority for the purposes of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

A sequence listing was filed in both paper and electronic forms, but was not used for the purposes of the search or opinion.

| INTERNATIONAL SEARCH REPORT                           |                                                                                                                                                                                                                                                                                                                                                          | International application No. |
|-------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------|
| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |                                                                                                                                                                                                                                                                                                                                                          | PCT/AU2012/000883             |
| Category*                                             | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                                                       | Relevant to claim No.         |
| X                                                     | Qiao JL, Shen Y, Gardiner EE, Andrews RK. Proteolysis of platelet receptors in humans and other species. Biol Chem. 2010 Aug;391(8):893-900. Review.<br>See the whole document, particularly the abstract, Shedding in vivo on pages 895-97, and Summary on pages 897-98.                                                                                | 1-20                          |
| X                                                     | Mo X, et al. Transmembrane and trans-subunit regulation of ectodomain shedding of platelet glycoprotein Ibalpha. J Biol Chem. 2010 Oct 15;285(42):32096-104. Epub 2010 Aug 17.<br>See the whole document, particularly the abstract, Figures 1-2 and supporting text, Flow Cytometry and measurement of glyco-calicin on page 32098, and the Discussion. | 1-20                          |
| X                                                     | US 2005/0019743 A1 (Wagner et al) 27 January 2005<br>See the whole document, particularly the abstract, paragraphs [0004], [1101 - 0015], examples 6-9, claims 1-22 and the supporting description.                                                                                                                                                      | 1-20                          |
| X                                                     | Al-Tamimi M, et al. Coagulation-induced shedding of platelet glycoprotein VI mediated by factor Xa. Blood. 2011 Apr 7;117(14):3912-20. Epub 2011 Jan 20.<br>See the whole document, particularly the abstract, measuring sGPVI on pages 3913-4, Table 1 and the supporting text, Figure 6A and B and supporting text, and Conclusions.                   | 1-20                          |
| X                                                     | US 7645592 B2 (Takizawa et al) 12 January 2010<br>See the whole document, particularly the abstract, Column 7 lines 9-30, Examples 12-13, claims 15-21 and the supporting description.                                                                                                                                                                   | 1-20                          |
| P,X                                                   | Gardiner EE, Al-Tamimi M, Andrews RK, Berndt MC. Platelet receptor shedding. Methods Mol Biol. 2012;788:321-39. Review.<br>See the whole document, particularly the Introduction, Table 1, Figure 1, part 10. Shedding in Human Disease on pages 332, and Conclusions.                                                                                   | 1-20                          |
| P,X                                                   | Qiao J, et al. Analysis of Platelet Receptor Expression in ITP. Blood (ASH Annual Meeting Abstracts) 2011 118: Abstract 3289.<br>See the whole abstract.                                                                                                                                                                                                 | 1-20                          |
| P,X                                                   | Stivala S, et al. Analysis of the Mechanisms of Increased Platelet Count Induced by Dietary alpha-Linolenic Acid Indicates Reduced Cleavage of GPIb and Reduced p38 Phosphorylation. Blood (ASH Annual Meeting Abstracts) 2011 118: Abstract 2198.<br>See the whole abstract.                                                                            | 1-20                          |

| INTERNATIONAL SEARCH REPORT                                                                                                                                                                                                                                           |                  | International application No. |                  |
|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------|-------------------------------|------------------|
| Information on patent family members                                                                                                                                                                                                                                  |                  | PCT/AU2012/000883             |                  |
| This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information. |                  |                               |                  |
| Patent Document/s Cited in Search Report                                                                                                                                                                                                                              |                  | Patent Family Member/s        |                  |
| Publication Number                                                                                                                                                                                                                                                    | Publication Date | Publication Number            | Publication Date |
| US 2005/0019743 A1                                                                                                                                                                                                                                                    | 27 Jan 2005      | AU 2002305333 A1              | 18 Nov 2002      |
|                                                                                                                                                                                                                                                                       |                  | EP 1399466 A2                 | 24 Mar 2004      |
|                                                                                                                                                                                                                                                                       |                  | JP 2004535389 A               | 25 Nov 2004      |
|                                                                                                                                                                                                                                                                       |                  | US 2002165166 A1              | 07 Nov 2002      |
|                                                                                                                                                                                                                                                                       |                  | US 6861504 B2                 | 01 Mar 2005      |
|                                                                                                                                                                                                                                                                       |                  | US 2004248813 A1              | 09 Dec 2004      |
|                                                                                                                                                                                                                                                                       |                  | US 7271152 B2                 | 18 Sep 2007      |
|                                                                                                                                                                                                                                                                       |                  | US 2004048803 A1              | 11 Mar 2004      |
|                                                                                                                                                                                                                                                                       |                  | US 2004072750 A1              | 15 Apr 2004      |
|                                                                                                                                                                                                                                                                       |                  | US 2005019743 A1              | 27 Jan 2005      |
|                                                                                                                                                                                                                                                                       |                  | WO 02089730 A2                | 14 Nov 2002      |
|                                                                                                                                                                                                                                                                       |                  | WO 2004105837 A2              | 09 Dec 2004      |
| US 7645592 B2                                                                                                                                                                                                                                                         | 12 Jan 2010      | AR 048638 A1                  | 10 May 2006      |
|                                                                                                                                                                                                                                                                       |                  | AU 2005243193 A1              | 24 Nov 2005      |
|                                                                                                                                                                                                                                                                       |                  | AU 2005243193 B2              | 27 Jan 2011      |
|                                                                                                                                                                                                                                                                       |                  | CA 2565063 A1                 | 24 Nov 2005      |
|                                                                                                                                                                                                                                                                       |                  | CA 2567394 A1                 | 16 Apr 2008      |
|                                                                                                                                                                                                                                                                       |                  | CN 1964990 A                  | 16 May 2007      |
|                                                                                                                                                                                                                                                                       |                  | EP 1745076 A2                 | 24 Jan 2007      |
|                                                                                                                                                                                                                                                                       |                  | EP 1745076 B1                 | 13 Jun 2012      |
|                                                                                                                                                                                                                                                                       |                  | JP 2008507476 A               | 13 Mar 2008      |
|                                                                                                                                                                                                                                                                       |                  | KR 20070010038 A              | 19 Jan 2007      |
|                                                                                                                                                                                                                                                                       |                  | RU 2006140407 A               | 27 May 2008      |
|                                                                                                                                                                                                                                                                       |                  | US 2007202108 A1              | 30 Aug 2007      |
|                                                                                                                                                                                                                                                                       |                  | US 7611707 B2                 | 03 Nov 2009      |
|                                                                                                                                                                                                                                                                       |                  | US 2007207155 A1              | 06 Sep 2007      |
|                                                                                                                                                                                                                                                                       |                  | US 7645592 B2                 | 12 Jan 2010      |
|                                                                                                                                                                                                                                                                       |                  | WO 2005111083 A2              | 24 Nov 2005      |

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

