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**Evaluation of participants with suspected heritable platelet function disorders including recommendation and validation of a streamlined agonist panel**

Ban B Dawood[1], Gillian C Lowe[1], Marie Lordkipanidzé[1], Danai Bem[1], Martina E Daly[2], Mike Makris[2], Andrew Mumford[3], Jonathan T. Wilde[4] and Steve P Watson[1]

[1]Department of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham B15 2TT, UK

[2]Department of Cardiovascular Science, University of Sheffield, Sheffield, S10 2RX, UK

[3]Bristol Heart Institute, University of Bristol, Bristol Royal Infirmary, Bristol, BS2 8HW, UK

[4]Adult Haemophilia Centre, Queen Elizabeth Hospital, Birmingham, B15 2TH, UK


Author for correspondence: Dr Ban B Dawood, Centre for Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham B15 2TT, UK. e-mail: b.b.dawood@bham.ac.uk; tel: +44 121 4158680

Key words: aggregometry; bleeding; platelets; platelet function testing; secretion; ADP; thromboxane $A_2$

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**Abstract**

Light transmission aggregometry (LTA) is used worldwide for the investigation of heritable platelet function disorders (PFD), but interpretation of results is complicated by the feedback effects of ADP and thromboxane $A_2$ and by the overlap with the response of healthy volunteers. Over 5 years, we have performed lumi-aggregometry to nine platelet agonists in 111 unrelated research participants with suspected PFDs and in 70 healthy volunteers. Abnormal LTA or ATP secretion test results were identified in 58% of participants. In 84% of these, the patterns of response were consistent with defects in Gi receptor signalling, the thromboxane pathway and dense granule secretion. Participants with defects in signalling to Gq-coupled receptor agonists and to collagen were also identified. Targeted genotyping identified three participants with function disrupting mutations in the $P2Y_{12}$ ADP and thromboxane receptors. Our study illustrates that detailed phenotypic analysis using LTA and ATP secretion is a powerful tool for diagnosis of PFDs and enables subdivision at the level of platelet signaling pathways and in some cases to individual receptors. We further demonstrate that most PFD can be reliably diagnosed using a streamlined panel of key platelet agonists and specified concentrations suitable for testing in most clinical diagnostic laboratories.

**Introduction**

An adequate number of normally functioning platelets is essential in arresting haemorrhage from an injured blood vessel. Individuals with heritable platelet function disorders (PFDs) experience lifelong abnormal bleeding, typically at mucocutaneous sites or elsewhere after trauma or invasive procedures. This group of disorders is highly heterogeneous and can arise through defects in surface membrane receptors, signalling pathways, granule formation and secretion, cytoskeletal remodelling and expression of procoagulant activity[1,2]. Diagnosis of the severe PFDs, Glanzmann's thrombasthaenia and Bernhard-Soulier syndrome, or syndromic PFDs, such as Hermansky Pudlak syndrome [HPS]), is usually straightforward because of the characteristic clinical and laboratory features [3-6]. On the other hand, poor standardization of platelet function tests and the lack of consensus surrounding minimal diagnostic criteria have hampered diagnosis of the remaining PFDs.

Light transmission aggregometry (LTA) is a long established method for the diagnosis of PFDs. Responses to individual test agonists are typically classified as 'abnormal' if numerical parameters such as the level of maximal aggregation fall outside of the reference range of healthy donor controls or if there are qualitative differences between test and control aggregation curves [7,8]. However, most PFDs are associated with abnormal responses to multiple agonists and it is unclear whether it is the number of 'abnormal responses' [8] or the specific patterns of 'abnormal response' [9] is the more powerful in the diagnosis of PFDs. The additional diagnostic value of ATP secretion in diagnosis of PFDs is not known.

We have previously defined reference intervals for LTA and dense granule secretion to three concentration of nine platelet agonists in a cohort of 20 healthy volunteers[9]. We now present data from the UK Genotyping and Phenotyping of Platelets (GAPP) study [10] that includes extended data on 70 healthy donor controls that validates our previous reference intervals [9]. We also describe LTA and ATP secretion test results in a prospectively studied cohort of 111 patients with suspected PFDs registered at UK Haemophilia Comprehensive Care Centres. Detailed analysis of LTA and ATP secretion using several concentrations of the extended panel of nine agonists confirmed PFDs in approximately 60% of participants. Over 80% of this group exhibited defects in Gi receptor signaling, the thromboxane pathway or dense granule secretion. Further, a retrospective blinded analysis revealed that approximately 90% of PFDs can be identified with a restricted range of concentrations of six platelet agonists which we term a 'streamlined agonist panel'. This indicates that reliable diagnosis of PFDs

and definition of the major pathways is feasible in non-specialist clinical diagnostic laboratories using a combination of LTA and ATP secretion.

**Materials and Methods**

**Selection of Participants**

Participants with bleeding symptoms

Participants with suspected platelet function defects were referred from August 2006 to September 2011 from UK Comprehensive Care Haemophilia Centres and were invited to participate in this study if they satisfied all the following inclusion criteria: 1. Abnormal bleeding symptoms compatible with PFD (spontaneous mucocutaneous bleeding or abnormal bleeding at other sites following trauma or invasive procedures); 2. Results from coagulation factor tests all within local laboratory reference intervals (minimum panel of prothrombin time, activated thromboplastin time, Clauss fibrinogen activity, von Willebrand factor Ristocetin cofactor activity and activities of Factors VIII, IX and XI; and 3. Absence of demonstrable for acquired platelet dysfunction. Patient's with existing diagnoses of Glanzmann's thrombasthenia, Bernard-Soulier syndrome or Hermansky-Pudlak syndrome and those with platelet counts $< 100$ or $> 450 \times 10^9$ / litre were excluded. Laboratory testing was deferred in participants exposed within two weeks to drugs known to affect platelet function. Information on the age and sex of the participants is given in the results section.

Healthy controls

Healthy donor volunteers aged 18 years or older were also included in this study. Controls were considered healthy if they did not have a history of bleeding symptoms, did not require long-term medical therapy and had refrained from drugs known to influence platelet function in the previous 2 weeks.

Ethics

This study was approved by the National Research Ethics Service Committee West Midlands – Edgbaston (REC reference: 06/MRE07/36) and participants and controls gave written informed consent in accordance with the Declaration of Helsinki.

**Platelet agonists and other reagents**

ADP, adrenaline and U46619 were purchased from Sigma (Poole, UK). Arachidonic acid (sodium salt) was from Cayman Chemical (Cambridge Bioscience Ltd., UK). Horm collagen was purchased from Nycomed Austria (Linz, Austria). PAR-1 receptor specific peptide SFLLRN was purchased from Alta Bioscience Laboratory (University of Birmingham, UK) and the PAR-4 receptor specific peptide AYPGKF and collagen related peptide (CRP) were purchased from Dr Richard Farndale (Cambridge University, UK). Luciferin-Luciferase

reagent (Chrono-Lume®) was purchased from Chrono-log Corporation (Havertown, PA, USA). AR-C67085 was a gift from AstraZeneca. Rhodocytin was a gift from Dr Johannes Eble, Frankfurt University Hospital, Germany.

**Platelet preparation and measurement of aggregation and secretion**

Blood from participants and a simultaneous healthy volunteer control were taken into sodium citrate (3.8%) and transported at ambient temperature (~20 $^o$C) to the laboratory. In the majority of cases, blood was taken in the adjacent clinic but in some instances, samples from participants and healthy volunteers were transported over distances of up to 100 miles by courier to Birmingham, UK. We have demonstrated previously that the results in platelet rich plasma (PRP) from samples that have been transported in this way are indistinguishable to those from locally collected samples provided that the PRP is prepared at the site of testing[9].

PRP was prepared by centrifugation at 200$g$ for 10 min in a spin out rotor. Platelet aggregation and ATP secretion were measured in PRP using a dual Chronolog lumiaggregometer (Model 460 VS, Havertown, PA, USA). Autologous platelet-poor plasma (PPP) was used to set the aggregation scale prior to each study according to the manufacturer's instruction. All experimentation was performed within six hours of preparation of the PRP as previously published [9]. Priority was given to time sensitive agonists such as ADP and adrenaline which were always used first within four hours of blood collection [9,11]. No adjustment was made to the platelet count as there was no significant difference in the aggregation curves to platelet agonists within the normal range of platelet count by comparison of the response to three platelet agonists in healthy volunteers at the two extreme ends of the normal range (Supplementary Figure 1). This is consistent with previous reports [12-15].

Platelets from the participants and healthy volunteers were routinely exposed to ADP (3, 10, 30 and 100µM), adrenaline (10, 30 and 100µM), collagen (0.3, 1 and 3µg/ml), collagen-related peptide (CRP: 1, 3 and 10µg/ml), PAR1 (10, 30 and 100µM) and PAR4 (100, 250 and 500µM) peptides, arachidonic acid (0.5, 1 and 1.5 mM), U46619 (1 and 3 mM) and ristocetin (1, 1.25, 1.5 and 2 mg/ml). The responses to ADP and arachidonic acid were the first to be investigated as these are the major two feedback agonists and a significant number of participants were found to have defects in Gi-signalling or in the TxA$_2$ pathway [16]. This information was used to guide subsequent testing, notably in cases where the platelet volume was limiting.

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### *P2RY12* analysis

The *P2RY12* coding sequence was amplified from genomic DNA and directly sequenced as described previously [17].

### Analysis of data

Aggregation responses at each agonist concentration were classified as *abnormal* by reference to a bank of local healthy volunteers [9]. ATP secretion was calculated by addition of a known concentration of ATP (4nmol) and normalised to a platelet count of $1 \times 10^8$ platelets /ml (Supplementary Figure 2). Results are shown as mean + s.d. Statistical analysis was performed using a one tailed Student's t-test and Cohen's kappa statistics.

### Results

The 111 index cases were comprised of 81 female and 30 males. There were 70 healthy volunteers. The mean age and standard deviation of the participants with a clinically diagnosed PFD was 41±16 years and 31±19 years for female and male participants, respectively. A full distribution of ages of participants is shown in Supplementary Figure 3.

LTA and ATP secretion were determined to nine platelet agonists (up to three concentrations) on each participant alongside a control (see methods). The response of each participant was evaluated by a minimum of two experts in platelet function testing and compared to the control on the day and to a reference range that originally consisted of 20 controls but which had built up to 70 controls by the end of the study. A retrospective analysis was performed to verify the original diagnoses and a small number of adjustments made. This approach revealed that 64/111 (58%) participants (43 female and 21 male) had a clear defect in platelet aggregation or ATP secretion to several concentrations of platelet agonists.

### Major subgroups of PFDs

Comparing the pattern of response in the 64 participants with an identified defect in LTA or ATP secretion revealed that 54/64 (84%) could be assigned to three major diagnostic groups, namely with defects in Gi signaling, thromboxane $A_2$ pathway and granule secretion. The distribution of defects is in shown in Table 1 and the characteristic features of each subgroup are described below:

**(i) Gi-like defect**: 21 (32.8%) of the 64 index cases were diagnosed with a Gi-like defect. This subgroup exhibited a defect in aggregation and secretion to the two Gi-coupled

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heterotrimeric receptors agonists, ADP and adrenaline. A key diagnostic feature was a transient aggregation to ADP (10 μM), a reduced or absent primary wave with no secondary wave of aggregation to adrenaline, and absence of ATP secretion to both agonists. Similar numbers of participants had a reduced primary wave or absent primary response to adrenaline [16]. In comparison, high concentrations of ADP and adrenaline induced sustained aggregation in the 70 healthy volunteers. Representative traces to ADP and adrenaline are shown in Figure 1.

Reduced aggregation and secretion to low and intermediate concentrations of other platelets agonists, most notably collagen, was also observed in this group, consistent with impairment of the known feedback role of ADP. At higher agonist concentrations, sustained aggregation and a normal level of ATP secretion was usually seen. A robust response to 1mM arachidonic acid (Figure 1) helps to distinguish this group of participants from those with a defect in the $TxA_2$ pathway as described below.

**(ii) Thromboxane $A_2$ pathway defect**: 14 (21.9%) of the 64 index cases were diagnosed with a thromboxane pathway defect. This was characterized by a marked and selective defect in aggregation and secretion to arachidonic acid (1mM) as illustrated by the representative trace in Figure 2. In three of the fourteen participants with this defect, a reduced response to the $TxA_2$-mimetic U46619 was also seen indicating a defect at the level of the thromboxane receptor or its downstream signalling cascade. A normal aggregation response to U46619 is evidence for a defect in conversion of arachidonic acid to $TxA_2$ as the response to U46619 is insensitive to cyclooxygenase blockade [9,18]. Aggregation and secretion to low concentrations of other platelet agonists was also reduced in this group, consistent with the positive feedback role of $TxA_2$. For most agonists, full recovery was seen at higher concentrations (not shown), with the exceptions of ADP and adrenaline which exhibited a *slowly* decaying aggregation response and severely reduced or absent secondary wave, respectively (see Figure 2).

**(iii) Dense granule-secretion defect:** 19 (30%) of the 64 index cases were diagnosed with a dense granule secretion defect which could be due either to a defect in storage or secretion. The level of secretion was dependent on the platelet count which was normalized to the response observed to PAR-1 (Figure 3A) or PAR-4 peptides as outlined in the methods. Participants were considered to have a significant defect in secretion when the response fell outside of the normal range determined in controls. A decrease in ATP secretion was observed to all agonists even though several, including PAR-1, PAR-4 and CRP, induced maximal, sustained aggregation at high concentrations. There was however a reduction in

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aggregation to low concentrations of most platelet agonists, consistent with impairment of the feedback role of ADP and $TxA_2$. The response to a low concentration of collagen was notably markedly impaired as illustrated in the representative trace in Figure 3A. One agonist which showed a minimal change in its dose response curves for aggregation was ADP; this is consistent with our previous conclusion that secretion plays a minimal role in aggregation to the nucleotide [9].

It is noteworthy that the PAR-1 peptide (100μM) elicited a limited degree of secretion of ATP in all index cases diagnosed with a secretion disorder (Figure 3B), with the exception of the index case (and also other related family members) with an HPS-8 mutation (homozygous)[19]. Secretion to the PAR-1 peptide (100μM) in participants diagnosed with a Gi or thromboxane pathway defect, as well as one participant with a homozygous $P2Y_{12}$ receptor mutation (see below) and two with a heterozygote $TxA_2$ receptor defect (see below and refer to [18]) fell within the normal range (Figure 3B). These results demonstrate that the vast majority of participants diagnosed with a secretion disorder elicit a limited degree of dense granule secretion and that the maximal level of secretion to a high concentration of a PAR-1 peptide is unaffected by mutations in the $P2Y_{12}$ ADP or $TxA_2$ receptors.

**Other, less common subgroups of PFDs**

The comparison of the LTA and ATP secretion results in the remaining 10/64 (16%) of participants, along with additional tests, revealed several further subgroups as shown in Table 1, namely defects in the GPVI signaling pathway, Gq receptor signaling and in the ADP and $TxA_2$ receptors (confirmed by gene sequencing). In two other participants, the pattern of defects could not assign to any of these groups and is described as complex to indicate the presence of defects in multiple pathways. These further subgroups are described below:

**$P2Y_{12}$ ADP receptor mutation:** The index case was a 31 year old Asian female with a life long history of severe bruising and prolonged bleeding from cuts who had required a blood transfusion following a caesarean section. Her parents were first cousins, although neither had a history of bleeding. Her brother also had a lifelong history of prolonged bleeding from cuts but was not available for investigation. Her full blood count and basal coagulation tests were within the normal range. The participant's platelets exhibited a weak, transient aggregation and absence of secretion to a high concentration of ADP (100μM) that was preceded by shape change (Figure 4A). The response was not altered in the presence of the $P2Y_{12}$ receptor antagonist, AR-C67085, while addition of the antagonist reduced the response to ADP in the healthy volunteer to that of the participant (Figure 4A). The participant's

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platelets exhibited a biphasic aggregation to adrenaline and a mild defect in aggregation to low concentrations of other platelet agonists, most notably collagen (consistent with the feedback role of ADP in supporting activation), which normalized at higher concentrations (Figure 4A). The level of ATP secretion by maximal concentrations of the PAR1- peptide was within the normal range indicating that the participant did not have a secretion defect (Figure 4B). ADP had no effect on cAMP formation by $PGE_1$, whereas adrenaline induced a marked decrease in the second messenger (supplementary Figure 4).

This profile is indicative of a defect in the $P2Y_{12}$ ADP receptor. In confirmation of this, genomic DNA sequencing revealed that the participant was homozygous for a single base deletion at nucleotide position 36 of the *P2RY12* gene. This mutation is predicted to cause a frameshift leading to introduction of a premature stop codon (c.36delG, p.Gly12fs) resulting in a failure to express the $P2Y_{12}$ receptor.

**$TxA_2$ receptor mutations:** We have recently described a participant with a function-disrupting heterozygous mutation in the $TxA_2$ receptor[18]. The defining features of this participant were a marked defect in aggregation and secretion to low concentrations of arachidonic acid and U46619. We have since identified a second participant who is heterozygous for a distinct mutation in the $TxA_2$ receptor which alters receptor trafficking (Nisar and Mundell in preparation). In both participants, the defect in aggregation and secretion is similar to that in participants with defective arachidonic acid metabolism described above, with the significant difference being the reduction in response to U46619.

**GPVI pathway defect:** We have identified four index cases with a GPVI-like defect on the basis of a selective reduction in aggregation and secretion to the GPVI-specific agonist CRP, whereas the response to ADP, arachidonic acid and PAR1- and PAR4- specific peptides, were within the normal range (see Figure 5 and not shown). Aggregation and secretion to low concentrations of collagen were also reduced, although recovery was seen at higher concentrations as illustrated by the representative traces in Figure 5. The weaker effect on collagen relative to CRP can be accounted for by the presence of a second collagen receptor on the platelet surface, integrin, $\alpha2\beta1$, and by the marked dependency of the response of collagen on the feedback actions of ADP and $TxA_2$. The snake venom toxin rhodocytin activates platelets through the C-type lectin receptor CLEC-2 which signals through a closely related signaling cascade to that of GPVI[20]. The demonstration of an impairment in aggregation to rhodocytin as illustrated in Figure 5 is indicative of a signalling defect downstream of GPVI or CLEC-2.

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**Gq-like defect**: One male index case, and his sister, from a non-consanguineous relationship, were observed to have a partial defect in aggregation and secretion to intermediate concentrations of agonists that signal through the heterotrimeric G proteins Gq and $G_{13}$, namely PAR1- and PAR4- specific peptides and the $TxA_2$ analogue U46619 (Supplementary Figure 5). In contrast, the responses to ADP and CRP were only marginally inhibited arguing against a general defect in platelet activation. This is further supported by the similar nature of the response to the phorbol ester PMA in the participant's platelets (Supplementary Figure 5). Thus, the defect is putatively at the level of Gq or $G_{13}$ or their downstream signalling proteins.

## Streamlined agonist panel testing

We next considered whether a reduced panel of LTA and ATP secretion tests would be sufficient to enable reliable diagnosis of PFD. Following review of the above results, we developed a streamlined panel of agonists and concentrations based on their ability to diagnose and discriminate the above subgroups of PFDs (Table 2). We then undertook a comparison of the diagnosis of a PFD using this streamlined panel with the original agonist panel. These studies took into account the extent and time course of aggregation (noting whether it was transient or delayed) and the level of secretion of ATP, as the extent of maximal aggregation alone does not distinguish between the major three platelet function defect groups from controls (Figure 6).

Two independent experts were blinded to previous diagnoses and reviewed data that would have been created using the streamlined agonist panel in 94 cases which were suitable for full analysis. Inter observer variation was minimal with an agreement of approximately 90% (kappa statistic 0.829, p value for significance <0.001). A mutually acceptable consensus was subsequently reached for the 10% of cases with a difference of opinion. The diagnoses from this process were then compared to the previous historical diagnoses using the expanded agonist list (Table 3). There was a significant level of agreement with a kappa statistic of 0.721 (p value for significance < 0.001). In addition, the sensitivity (87%), specificity (86%), negative predictive value (84%) and positive predictive value (88%) provided further verification of the streamlined agonist panel as shown in Table 3.

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**Discussion**

The most widely used test for platelet function is LTA which monitors the increase in light transmission through a suspension of platelets as aggregation proceeds. Among the advantages of this test is its relative simplicity, the ability to monitor responses to individual agonists over time and (in our experience) its reproducibility at the level of each donor. Furthermore, it can be combined with real-time monitoring of ATP secretion in a lumi-aggregometer by addition of the Luciferin-Luciferase reagent. The drawbacks of this method include the time taken to perform the assays and the fact that many investigators consider it to be operator-dependent and to give inconsistent results. The interpretation of aggregation traces is also complex due to the feedback effects of redundant platelet activation pathways, although this applies to all tests of platelet function. Several of these limitations can be minimised by standardisation of the aggregation and secretion assays as performed in this study. A similar standardisation of LTA was also used in a recent study of 229 participants to detect participants with platelet dysfunction [8].

Defining the platelet phenotype through the analysis of aggregation and secretion to nine platelet agonists, alongside other functional tests, provides important information on the defective pathway(s) and in some cases the defective protein allowing targeted genetic analysis. It is not practical for hospital laboratories to follow this procedure however because of the time taken for the analysis and the relatively low numbers of participants that are referred to each centre. Nevertheless, a limited analysis of platelet aggregation using one or two concentrations of the 'standard' platelet agonists (ADP, adrenaline, AA, collagen and ristocetin) is extremely valuable in aiding diagnosis [8].

In the present study, we have validated the use of a streamlined panel of six platelet agonists (see Table 3) by comparing the diagnoses made with the extended agonist panel. This streamlined agonist panel could be used in nearly all clinical testing centres and would serve to guide further subtyping of the PFD by a specialist laboratory or a clinical research study through functional investigations and targeted genotyping or analysis of whole exome sequencing data. The functional tests could include an expanded range of platelet agonists and more specialised tests, such as measurement of second messengers (cAMP and $Ca^{2+}$ elevation), $TxA_2$ formation, shear-based assays of platelet adhesion and aggregation, and flow cytometric measurements of α-granule secretion and glycoprotein receptor levels. The streamlined agonist list would also identify nearly all of the 40% of patients in whom such tests would appear to have limited value.

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The majority of participants with an identified defect in platelet function (~80%) were assigned to one of three groups characterized by defects in Gi-signalling, thromboxane formation and dense granule secretion. This serves to emphasise the importance of these three pathways in supporting platelet activation during haemostasis. In the vast majority of these cases, the causative gene mutation(s) are not known and the value of the subgrouping is to enable further functional studies and targeted gene sequencing / gene interrogation.

The relatively small number of participants with defects in the Gq-pathway is perhaps surprising given the importance of this pathway in platelet activation. This may however reflect the fact that several platelet receptors signal through Gq-mediated pathways, namely PAR1, PAR4, $P2Y_{12}$ and $TxA_2$ receptors, thereby reducing the impact of a partial defect. Alternatively, a mutation in this pathway could cause a more severe phenotype in other tissues that could be lethal in utero. The small number of participants with defects in the GPVI pathway is consistent with the relatively mild nature of bleeding associated with defects in the collagen receptor [21].

There are several possible explanations for the failure to identify a defect in platelet function in almost 40% of participants suspected of having a platelet disorder. These include the likely possibility that they do not have a defect in platelet activation pathways but an abnormality in another component of the haemostatic response such as increased fibrinolysis or an impairment of vascular integrity. Alternatively, it is possible that they have a defect that has not been detected in the platelet function tests that have been performed such as an enhanced activity of an inhibitory pathway. The limited repertoire of platelet function tests used in this study may also have contributed to the failure to diagnose a platelet defect. However, in work that is not reported in this study, we have performed additional platelet tests in many of the participants that have been investigated in this study including measurement of platelet aggregation on collagen at intermediate rate of shear $(1,000 \text{ s}^{-1})$, and investigation of the inhibitory action of prostacyclin and sodium nitroprusside, which elevate cAMP and cGMP, respectively, and clot retraction. No defect was observed in any of these responses in patients for whom aggregation and secretion fell within the normal range

The phenotyping of platelets has been used to direct genotyping and has led to the identification of participants with a homozygous defect in the $P2Y_{12}$ ADP receptor gene (this study) and two heterozygous mutations in the $TxA_2$ receptor, one of which has been previously reported [18]. The homozygous $P2Y_{12}$ ADP receptor gene mutation reported in this study is the 10th mutation to be reported in the nucleotide receptor [10]. A third heterozygous

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mutation in the $TxA_2$ receptor has also recently been reported [22]. The only other reported mutation in the thromboxane receptor, Arg60Leu [23], may be a rare SNP as there is no clear defect in function of the receptor in cell lines studies [24,25]. We have not identified participants with mutations in the PAR1 and PAR4 thrombin receptors or the collagen receptor GPVI. There are no reported participants with mutations in the PAR1 and PAR4 receptors, possibly because they are incompatible with life, and only two patients with mutations in GPVI have been reported[26,27]. The small number of participants with mutations in platelet surface receptors suggests that receptor mutations account form a relatively small proportion of mutations that give rise to bleeding symptoms, with the majority being in intracellular signalling pathways or functional processes such as secretion or thromboxane formation. Identifying the mutations that give rise to these function disorders requires a more high throughput approach offered by for example whole exome sequencing or a targeted second generation sequencing array [28].

The present study represents a comprehensive investigation of participants with a clinically suspected platelet disorder alongside healthy volunteers. The participants have been grouped on the basis of the identified disorder, with the majority having defects in one of the major feedback pathways of platelet activation, namely Gi signalling, the thromboxane pathway and dense granule secretion. A handful of these participants have defects at the level of the ADP and thromboxane receptor but the majority have mutations downstream of receptor activation necessitating the use of second generation sequencing and targeted interrogation based on the functional results to establish the causative mutation(s). A streamlined platelet agonist list that could be performed in most clinical testing centres and a sequence of interpretation has been developed based on these results (Table 3). Importantly, this interpretation takes into account the pattern but not the magnitude of the defect in aggregation, and whether secretion is also defective.

**Authorship Contributions**

BBD designed and performed the research, collected, analysed and interpreted data and wrote the first draft of the manuscript. SPW designed the study, interpreted data, and revised the draft and final manuscript. JTW was involved in participants' recruitment, interpreting data, contributed to and revised the manuscript. MED analysed *P2RY12* gene and revised the manuscript. MM and AM were involved in participants' recruitment and revision of the manuscript. DB performed a specialized platelet test. GL and ML have led the research governance of the GAPP study since October 2010, revised the manuscript and contributed to the analysis of the results. The authors have no conflict of interest to declare.

**Conflict of interest disclosure**

None

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**Table legends**

**Table 1: Classification of participants with mild platelet-based bleeding defects.**

Participants who exhibited a defect in platelet function were subdivided on the basis of their major platelet phenotype as described in the text.

**Table 2: A streamlined panel of agonists with interpretative notes for diagnosing platelet function defects.**

Different concentrations of agonists with the percentage of maximal aggregation ± s.d. are shown. ATP secretion from dense granules should be measured for the following agonist concentration: ADP (30 µM), adrenaline (30 µM), arachidonic acid (1mM), PAR-1 specific peptide (100 µM) and collagen 3µg/ml.

**Table 3: A comparison between the expanded agonist panel and a streamlined agonist panel in diagnosing platelet funtion defect.**

The data shows a comparison between diagnoses of platelet function defect using the expanded agonist panel and a streamlined agonist panel. The sensitivity (87%), specificity (86%), negative predictive value (84%) and positive predictive value (88%). Kappa statistic of 0.721 (p<0.001).

**Figure legends**

**Figure 1: Aggregation to ADP and adrenaline in two participants diagnosed with a Gi-like defect.**

Aggregation in two participants (p) diagnosed with a Gi-like defect is shown. The participant in (A) shows a partial primary wave response to adrenaline while for a second participant shown in (B) the primary wave is absent. c: control (healthy volunteer). Note that the biphasic aggregation to ADP in Figure 1B would eventually decline. The patterns of aggregation are representative of other participants diagnosed with a Gi-like defect.

**Figure 2: Aggregation in a participant diagnosed with a TxA$_2$ pathway defect.**

Aggregation in a participant (p) diagnosed with a TxA$_2$ pathway defect. The TxA$_2$ pathway defect also results in the abolition of response to arachidonic acid (1 mM) and impairment in response to other agonists, including ADP and adrenaline, but not to U46619 indicating a defect in arachidonic acid metabolism. c: control (healthy volunteer). The pattern of aggregation is representative of other participants diagnosed with a defect in arachidonic metabolism..

**Figure 3: Aggregation and ATP secretion in a participant diagnosed with a dense granule defect.**

(A) Aggregation in a participant diagnosed with a defect in dense granule secretion on the basis of a significantly reduced level of secretion to high concentrations of PAR-1 specific peptide and other platelet agonists including PAR-4 specific peptid and CRP relative to a panel of controls. c: control (healthy volunteer). The pattern of aggregation is representative of other participants diagnosed with a secretion disorder. (B) ATP secretion was measured alongside aggregation in a Born-lumiaggregometer in platelet-rich plasma using Chronolume reagent for detection of ATP. The degree of ATP secretion (following normalisation to platelet count – see Supplementary Figure 2) to PAR1-specific peptide (100 μM) in healthy volunteers and participants diagnosed with defective dense granule secretion is shown. Participants identified with mutations in the P2Y$_{12}$ (present study) and TxA$_2$ receptors [10] and HPS-8 [19] are identified by the presence of the square bracket.

**Figure 4: Aggregation and secretion in a participant with a homozygous P2Y$_{12}$ mutation that prevents receptor expression.**

Aggregation and secretion in a participant (p) with a homozygous mutation in P2Y$_{12}$ which introduce an frame-shift early in the coding sequence (amino acid no. 12; see text). Responses are shown alongside a control (c). The PRP platelet count in the control and participant were 4.1 x $10^8$/ml and 3.9 x $10^8$/ml, respectively.

**Figure 5: Aggregation and secretion in a participant with a GPVI-like defect.**

Aggregation in a participant (p) diagnosed with a GPVI-like defect on the basis of a reduced response to CRP and to rhodocytin. A similar pattern of aggregation was observed in other participants diagnosed with a GPVI-like defect. c: control.

**Figure 6: Maximal aggregation response in participants with platelet function defects and healthy volunteers**

The percentage of maximal aggregation was measured in a Born-lumiaggregometer in platelet rich plasma in response to the shown concentrations of the following agonists: ADP, adrenaline, arachidonic acid, collagen, PAR-1 specific peptide and ristocetin . The results are shown as % increase in light transmission relative to platelet-poor plasma.

**Table 1**

| Type of platelet defect | Number of participants | Percentage of participants |
|---|---|---|
| Membrane Gi signaling | 21 | 32.8 % |
| TxA$_2$ pathway | 14 | 21.9 % |
| GPVI | 4 | 6.2 % |
| Gq | 1 | 1.6 % |
| Dense granule | 19 | 29.7 % |
| Complex | 5 | 7.8 % |
| Total | 64 | 100 % |

**Table 2**

| agonist | concentration | % of maximal aggregation mean ± s.d. | expected | Abnormal pattern and further testing |
|---|---|---|---|---|
| *ADP | 10µM | 59.8 ± 12.5 | maximal, sustained aggregation and secretion | reduced or transient aggregation and absent secretion: use 30µM |
| *adrenaline | 10µM | 70.3 ± 13.7 | biphasic aggregation with secretion coincident with second phase | Reduced or absent primary wave and absent secretion: use 30µM |
| arachidonic acid | 1mM | 71.0 ± 8.5 | maximal, sustained aggregation and secretion | Absent or delayed / reduced aggregation and secretion: use U46619 (3 $\mu$M) |
| *PAR-1 receptor specific peptide (SFLLRN) | 100µM | 73.8 ± 11.1 | maximal, sustained aggregation and marked secretion; | Tranisent aggregation and reduced secretion: use PAR-4 receptor specific peptide (AYPGKF; 500uM). |
| collagen | 1µg/ml | 50.4 ± 22.5 | sustained aggregation and secretion | Reversible aggregation and absent secretion: use 3µg/ml and collagen related peptide (CRP; 3 µg/ml) or convulxin |
| ristocetin | 1.5mg/ml | 70.8± 16.1 | maximal sustained aggregation (often biphasic) and secretion | Reduced or absent aggregation and secretion |

**Table 3**

| | (expanded agonist panel) positive | (expanded agonist panel) negative | |
|---|---|---|---|
| (streamlined agonist panel) positive | 45 | 6 | **Positive predictive value 88%** |
| (streamlined agonist panel) negative | 7 | 36 | **Negative predictive value 84%** |
| | **Sensitivity ( 87% )** | **Specificity ( 86% )** | |

**Figure 1**
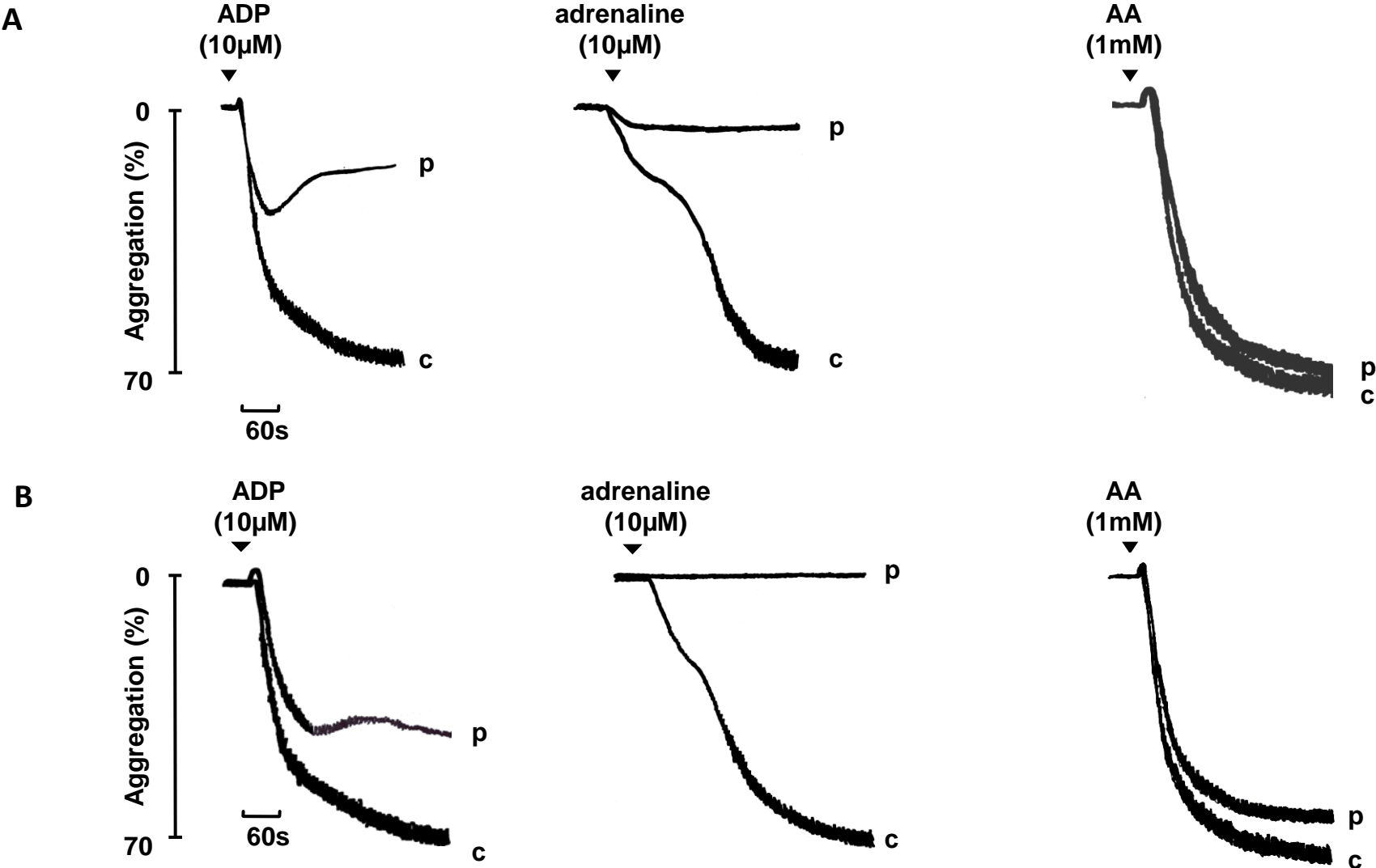
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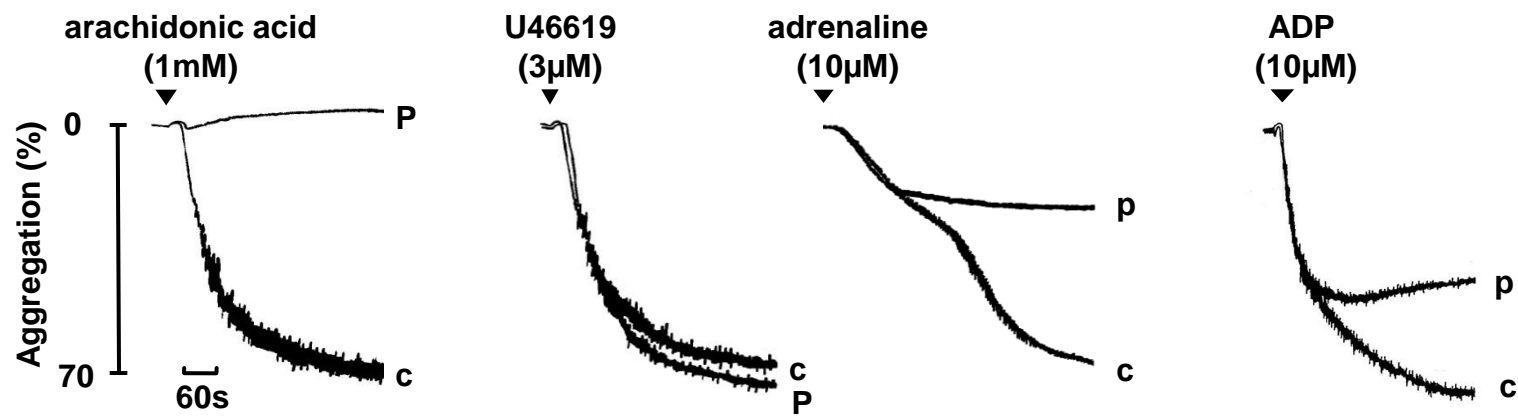
**Figure 3**
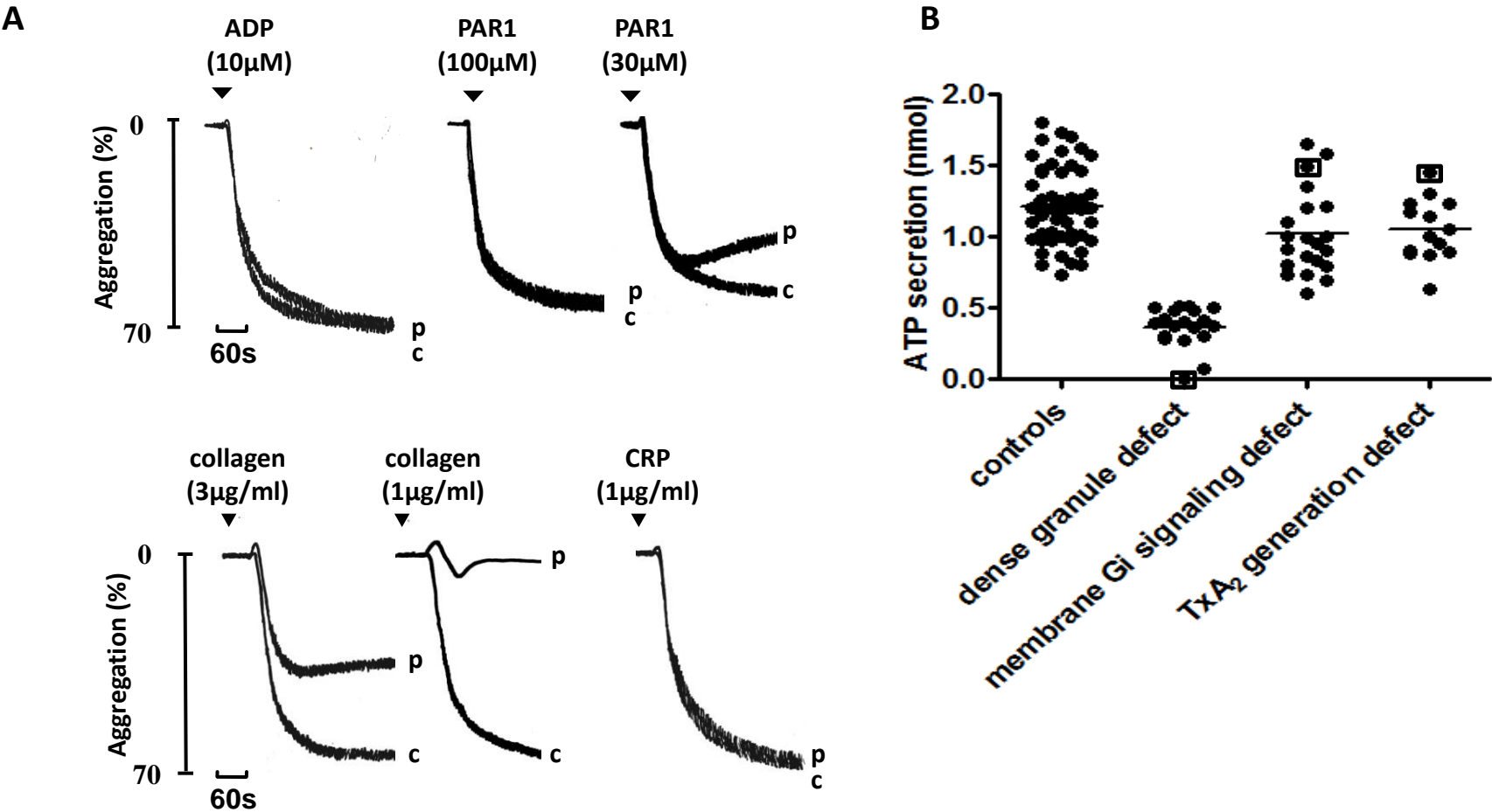
**Figure 4**

**Figure 5**

**Figure 6**