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Rare platelet G protein-coupled receptor variants:
What Can We Learn?

Header: (Platelet G protein-coupled receptor function)

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Summary

Platelet expressed G protein-coupled receptors (GPCRs) are critical regulators of platelet function. Pharmacological blockade of these receptors forms a powerful therapeutic tool in the treatment and prevention of arterial thrombosis associated with coronary atherosclerosis and ischaemic stroke. However, anti-thrombotic drug therapy is associated with high inter-patient variability in therapeutic response and adverse bleeding side-effects. In order to optimise the use of existing antiplatelet drugs and to develop new therapies more detailed knowledge is required relating to the molecular mechanisms that regulate GPCR and therefore platelet function. One approach has been to identify rare, function-disrupting mutations within key platelet proteins in patients with bleeding disorders. In this review we describe how an integrated functional genomics strategy has contributed important structure-function information about platelet GPCRs with specific emphasis upon purinergic (P2Y$_{12}$) and thromboxane (TP-$\alpha$) receptors. We also discuss the potential implications these findings have for pharmacotherapy and for understanding the molecular basis of mild bleeding disorders.

Abbreviations

GPCR, G Protein-Coupled Receptor; ECL, Extracellular loop; ICL, Intracellular loop; TMD, Transmembrane domain;

Introduction

Platelets are small anucleate cells derived from megakaryocytes in the bone marrow that circulate in the bloodstream and play a key role in haemostasis. Under physiological conditions platelets do not adhere to the vessel wall due to continual release of inhibitory prostaglandins and
nitric oxide which prevent platelet activation. When damage to the vasculature occurs, platelets bind to exposed collagen, become activated and release a number of stimulatory mediators which feedback in an autocrine manner to cause further platelet activation, recruitment of more platelets to the site of injury and the formation of a stable clot, preventing further blood loss (Kaplan et al., 2011).

Platelet activation is a tightly regulated process with both increased and decreased platelet reactivity resulting in significant sequelae. Decreased platelet activity is associated with an increased risk of bleeding. Conversely increased platelet activity, for example in response to atherosclerotic plaque rupture, can lead to the build-up of vessel-occluding thrombi, resulting in myocardial infarction or stroke. The majority of drugs that target platelets inhibit platelet activation. However a key challenge of pharmacotherapy in this area is to achieve sufficient inhibition of platelet activity to prevent thrombus formation whilst maintaining the haemostatic properties of platelets in order to avoid excessive bleeding. Although the main signalling pathways underlying platelet activation are now well defined (Stegner et al., 2011) further understanding of how platelet receptors are regulated, together with the identification of novel platelet proteins will aid the development of anti-platelet drugs with a better therapeutic index. Furthermore, with the development of rapid and detailed phenotypic and genotypic analyses there is also scope for tailoring therapy to the individual.

The aim of this review is to focus on rare, function-disrupting variants of platelet GPCRs and to discuss the significance of these findings in relation to GPCR structure / function and their potential implications for pharmacological therapy.

Platelet GPCRs and platelet activation pathways

There are a number of GPCRs present on the platelet cell surface including two purinergic receptors, P2Y1 and P2Y12, proteinase activated receptors (PAR) 1 and 4, thromboxane receptor A2 receptor (TP-α), serotonergic receptor 5HT2A, prostacyclin receptor (IP1), prostaglandin receptor E2 (EP3) and α2A adrenoceptor (Offermanns, 2006). There is also some evidence for the expression of other GPCRs in human platelets at the mRNA level although this is not backed by
conclusive evidence either through pharmacological or protein expression studies (Rowley et al., 2011). Of the GPCRs present on the platelet surface the key receptors involved in platelet activation are PAR\(_1\), PAR\(_4\), P2Y\(_1\), P2Y\(_{12}\) and TP-\(\alpha\). Of these the P2Y\(_{12}\) receptor is an established anti-thrombotic drug target whilst the PAR\(_1\) antagonist, Vorapaxar has recently been approved for use in a subset of patients with myocardial infarction and peripheral arterial disease (; Poole et al., 2014).

Platelet activation is a multi-step process, consisting of platelet adhesion, shape change, granule secretion and aggregation. These processes are mediated by a number of cell surface receptors, including integrins and ion channels as well as GPCRs. Activation of these platelet surface receptors triggers multiple signalling cascades that synergise to bring about a co-ordinated response to rapidly prevent excessive bleeding. The signalling events underlying platelet activation have been comprehensively reviewed elsewhere (Stegner et al., 2011) and are only very briefly outlined here focusing on the contribution of GPCRs to this process. Platelet adhesion is initiated by binding of sub-endothelial von Willebrand Factor (VWF) to platelet GPIb\(_\alpha\), which causes transient interaction between platelets and the vasculature. Firm adhesion is then mediated by the collagen receptors, \(\alpha2\beta1\) and GPVI, activation of which promotes platelet spreading and aggregation. Furthermore, thrombin generated at the site of vascular injury activates PAR receptors causing further shape change, aggregation, thromboxane A\(_2\) (TXA\(_2\)) generation and secretion of platelet granule cargo. Positive feedback loops are initiated following ADP release from dense granules and TXA\(_2\) generation which act via P2Y and TP\(\alpha\) receptors respectively, to potentiate platelet activation. These platelet activation pathways converge to promote activation of the integrin \(\alphaIIb\beta3\), which then is able to bind fibrinogen. Signalling via activated \(\alphaIIb\beta3\) is the final step of platelet activation, and results in cytoskeletal re-organisation and the formation of large, stable platelet aggregates, leading to the generation of stable thrombi (Watson et al., 2005; Rivera et al., 2009).

GPCRs as targets for anti-platelet drugs

GPCRs remain the most widely and successfully targeted proteins for therapeutics and a number of anti-platelet drugs act, directly or indirectly at GPCRs expressed on the platelet cell surface. Alongside aspirin, which reduces thromboxane generation and hence reduces TP\(\alpha\) receptor stimulation, P2Y\(_{12}\) antagonists such as clopidogrel and prasugrel remain front-line therapy for a
variety of acute coronary syndromes. Interestingly, drugs that inhibit these ADP and thromboxane pathways have proved to be more successful antiplatelet drugs, with less adverse bleeding effects compared to integrin αIIbβ3 antagonists and PAR1 antagonists, which are associated with significant bleeding (Cox et al., 2000; Wiviott et al., 2011; Tricoci et al., 2012). This is likely due to the fact that ADP and thromboxane A2 are feedback mediators that contribute to activation via potentiation of other agonists. By contrast inhibition of PAR1 and αIIbβ3 integrin, which are activated by major platelet agonists, may cause a more significant inhibition of platelet activity and therefore an increased risk of bleeding.

Genetic variations associated with anti-platelet therapies targeting GPCRs.

A key issue associated with both aspirin and P2Y12 antagonists is a high degree of variability in inter-patient responsiveness to drug therapy and that a proportion of patients display “drug resistance”. For example, a third of patients taking clopidogrel are non-responsive as assessed by platelet function testing (Cattaneo, 2011b). The significant proportion of aspirin and P2Y12 receptor antagonist non-responders has led to considerable research into the underlying genetic causes of these variations. In the case of aspirin this has largely focused on cycloxygenase-1 (COX-1) polymorphisms, a number of which have been associated with reduced responsiveness to aspirin (Feher et al., 2009). Clopidogrel resistance meanwhile is largely associated with poor metabolism of the prodrug to the active form.

Clopidogrel is a pro-drug that requires metabolism by cytochrome P450 enzymes to form an active metabolite that subsequently binds irreversibly to the P2Y12 receptor. A number of studies have shown that clopidogrel does not effectively inhibit platelet activity in healthy volunteers and patients with acute coronary syndrome who have loss-of-function polymorphisms in CYP2C19 (Hulot et al., 2006; Giusti et al., 2007). Interestingly, loss-of-function variation in the cytochrome P450 2C19 gene (CYP2C19), caused by a single nucleotide polymorphism (rs4244285; c.681 G>A in NM_000769.1) within the coding region that creates a new splice site and thus a truncated and catalytically inactive protein, the CYP2C19*2 genotype, accounts for 12% of the variability in P2Y12 receptor inhibition seen with clopidogrel (Shuldiner et al., 2009). These variations in responsiveness led to the development of prasugrel, a structural analogue of clopidogrel. The hepatic metabolism of prasugrel, which produces the same active metabolite
that irreversibly antagonizes the P2Y\textsubscript{12} receptor, is less complex and therefore less susceptible to genetic variations in cytochrome P450 enzymes.

Responsiveness to anti-platelet agents is also affected by genetic variation in other key platelet genes, for example gain of function single nucleotide variations have been described in platelet integrins (GP1\(\alpha\) and \(\alpha\)II\(\beta\)3) (Cambria-Kiely et al., 2002). Therefore platelet reactivity in the general population is significantly influenced by genetic differences in a number of genes that reduce drug responsiveness and potentially contribute to an increased risk of adverse cardiovascular events.

Genetic Variation in Platelet GPCRs

In addition to exploring common genetic variations within the general population, an alternative and successful approach to understanding pathophysiological disease mechanisms is the study of patients with bleeding disorders. For example, the description of Glanzmann’s Thrombasthenia (Glanzmann, 1918) led to the identification of the key platelet integrin, \(\alpha\)II\(\beta\)3 (Nurden et al., 1974). Similarly, the P2Y\textsubscript{12} receptor was cloned in a study which described a patient with a bleeding history and a loss of function variation in the P2Y\textsubscript{12}R gene that encodes P2Y\textsubscript{12} (Hollopeter et al., 2001). These examples illustrate how the study of rare function disrupting variations affecting key platelet proteins can provide important information regarding platelet regulation and allow study of variant native proteins in vivo. This is especially important in platelets which are anucleate and therefore cannot be subjected to easy genetic approaches to change protein expression.

As part of the Genotyping and Phenotyping of Platelets consortium (GAPP) we have developed an approach for the rapid identification and characterization of rare genetic variations causing defects within platelet proteins, including GPCRs (Watson et al., 2010; Watson et al., 2013). This study has recruited a cohort of patients who attend UK Haemophilia Centres with symptoms of mild, lifelong bleeding but who have no demonstrable defect in platelet number or coagulation factors. For each study subject platelets were isolated from blood samples and were analysed by light transmission aggregation and secretion assays using a wide panel of agonists at different concentrations. Test results were compared to reference intervals for each agonist concentration determined by measurement of platelet aggregation and secretion in a cohort of healthy volunteer
controls without bleeding symptoms (Dawood et al., 2012). The aim of this detailed analysis is to identify the defective signalling pathway responsible for a loss of platelet function thus allowing targeted sequencing of a subset of genes that form the signalling pathway in which loss-of-function variants may underlie the platelet dysfunction. This approach has recently been validated following the description of a novel change in the HPS4 gene in a patient with reduced hair, eye and skin pigmentation; bleeding phenotype and platelet dysfunction (Jones et al., 2012).

If a nucleotide variation is identified that predicts loss of protein function then the structure-function of the variant protein is studied in detail in heterologous cell systems and by further analysis of platelets from the study subject. This approach has been highly successful in identifying loss of function variants in a number of GPCRs including the P2Y₁₂ and TP-α receptors (detailed in Tables 1 and 2) and has provided significant information regarding structure-function relationships of these important anti-thrombotic drug targets. The findings from these and other studies are discussed in further detail below.

Rare Variants of the Thromboxane Receptor

Thromboxane receptor deficiency (MIM #614009) is inherited in an autosomal recessive or dominant manner and has been identified in several individuals from different kindreds who display mild mucocutaneous bleeding symptoms (Kamae et al., 2011). To date, one quantitative defect causing reduced TP-α receptor expression and four qualitative defects caused by TP receptor amino acid substitutions have been reported. These naturally occurring variants are listed in table 1 and the findings of these studies summarized below.

A nucleotide variation which caused loss of TP-α receptor expression was first described in a patient with a history of mucocutaneous bleeding (Kamae et al., 2011). Sequence analysis of TBXA2R in the patient and her father revealed that these individuals were heterozygous for a single nucleotide duplication at c.167 (c.167dupG in NM_001060.5) resulting in a frame shift from amino acid 58. Corresponding cell lines studies showed that this caused significantly reduced receptor expression.

The first qualitative defect in the TP-α receptor caused by a missense nucleotide variation in the TBX2R gene was reported in 1994 by Hirata et al., (Hirata et al., 1994). This resulted from an
Arg60Leu amino acid substitution at the start of the 1st intracellular loop (Figure 1) and occurred in a patient with a history of post-surgical bleeding (Hirata et al., 1994) but has also since been described in another kindred with a history of mild bleeding (Fuse et al., 1996; Higuchi et al., 1999). The platelets from these affected individuals show absent or reduced aggregation to the synthetic thromboxane analogue U46619. In patients homozygous for the Arg60Leu amino acid substitution, the defect in aggregation was also accompanied by a reduction in TXA2-induced IP3 generation and Ca2+ mobilization. Interestingly, heterozygous Arg60Leu patients whose platelets also showed reduced aggregation to TP-α receptor agonists, showed apparently normal calcium mobilization, suggesting a possible additional pro-aggregatory effect of TP-α receptor activation that is independent of calcium signalling. Initial expression studies in Chinese Hamster Cells (CHOs) showed that co-expression of both the wild-type (WT) receptor with the variant Arg60Leu TP-α receptor was associated with a reduction in Ca2+ mobilization although the signalling ability of the variant receptor alone was not evaluated (Hirata et al., 1994). These conflicting data in cell lines and human platelets merit further investigation. More recent studies have shown that when expressed alone the Arg60Leu TP-α receptor variant has dramatically attenuated receptor responses when compared to WT despite comparable ligand binding affinities and receptor surface expression (Chakraborty et al., 2013). Molecular modelling indicates that Arg 60 interacts via hydrogen bonds with Met 126 and Arg 130 in transmembrane domain 3 and that this interaction is lost when the Arg is substituted for Leu (Chakraborty et al., 2013). Importantly Arg 130 is part of the D/ERY motif (Figure 1) that is highly conserved amongst GPCRs (Rovati et al., 2007) and is critical for receptor activation. Therefore it is likely that the variant Arg60Leu TP-α receptor is unable to undergo the required conformational changes required to promote efficient G protein coupling. This recent modelling data correlate with previous mutagenesis studies of the ERY motif within TP-α which confirmed the involvement of Arg 130 in receptor coupling to Gq (Capra et al., 2004). Mutation of the Arg 130 to Val resulted in a loss in inositol phosphate accumulation in response to U46619, a consequence of defective PLC activation via Gq.

The remaining TBXA2R gene rare variants causing amino acid substitutions in the TP-α receptor protein sequence (Table 1) have been described using the GAPP approach outlined above. In 2010 Mumford et al., identified a patient with a history of bruising and prolonged epistaxes since
infancy (Mumford et al., 2010). Platelet aggregation and secretion were reduced in response to arachidonic acid and U46619, whereas responses to other agonists were within normal range. Sequencing of the TXA2R gene showed that the patient was heterozygous for a c.190G>A variation predicting an Asp304Asn substitution in the 7th transmembrane domain of the receptor (Figure 1). Ligand binding studies in platelets revealed a 50% reduction in maximal binding to the variant Asp304Asn TP-α receptor compared to WT, without a change in binding affinity. Further studies in CHO cells also showed that the variant Asp304Asn TP-α receptor had a significantly impaired ability to bind radioligand despite expression at the cell surface being comparable to WT. These observations suggested that the reduction in TXA2-mediated platelet activation in the patient may be due to impaired ligand binding. Interestingly, this Asp304Asn substitution occurred in the highly conserved NPXXY motif (Figure 1), where the Asn at position 1 is substituted for Asp in 21% of class A GPCRs (Mirzadegan et al., 2003). High resolution structural studies suggest that this motif, which is located near the cytoplasmic end of TMD7, interacts with a network of water molecules to weakly stabilize the inactive state of the receptor and also allows rapid conformational changes to occur for activation (Rosenbaum et al., 2009).

The observation that an Asn residue at position 1 of the NPXXY motif (P 7.49 as identified by Ballesteros-Weinstein numbering) might suggest that the substitution of Asp to Asn observed in this study would not be function-disrupting. However, data from two separate mutagenesis studies suggest that the Asp and Asn are not interchangeable. In the human gonadotrophin-releasing hormone receptor (GnRH) an Asp318Asn mutation impairs G protein coupling (Zhou et al., 1994). In addition Johnson and co-workers showed that there were differences in binding to ADP-ribosylation factor (ARF) isoforms between GPCRs that possess the NPXXY versus the DPXXY motif (Johnson et al., 2006). It is unclear why the Asp304Asn substitution causes a decrease in ligand binding since the majority of studies of variations within the NPXXY motif have found differences in receptor activation. The GnRH Asp318Asn substitution showed no alterations in ligand binding (Zhou et al., 1994), which suggests that this effect may be specific for the thromboxane receptor, although there is some evidence that residue 7.49 (Ballesteros-Weinstein numbering) interacts via hydrogen bonding with the ligand binding pocket (Li et al., 2004; Pardo et al., 2007).
More recently, two further function-disrupting TBXA2R gene variations have been identified, both predicting amino acid substitutions within the TMD1 (Figure1) (Mumford et al., 2013; Nisar et al., 2014). Interestingly, both these variations reduce TP-α receptor expression at the cell surface, suggesting an important role for TMD1 in the regulation of anterograde receptor traffic. The Trp29Cys substitution was identified in a patient who displayed abnormal post-surgical bleeding and whose platelets showed reduced aggregation and secretion in response to arachidonic acid and U46619. Ligand binding studies in both patient platelets and in HEK293 cells expressing the variant receptor showed a reduction in Bmax and Kd, indicating a reduction in receptor surface expression and ligand binding affinity. Further studies showed no change in total receptor expression, but a significant reduction in cell surface expression which was accompanied by a reduced ability to signal via Gq.

The Trp29 residue (1.37 Ballesteros-Weinstein numbering), alongside other residues within TMD1 has previously been shown to be important for the formation of heterodimers between TP-α and the alternative receptor isoform TP-β (Fanelli et al., 2011). Interestingly, this study showed that a variant TP-α receptor in which a number of key residues within TMD1 (including Trp29 in combination with Ile25, Cys35, Val36, Leu39, Leu43, Leu44 and Ser47) were replaced with alanine, resulted in reductions in receptor signaling and ligand binding that were comparable to the Trp29Cys variant reported by Mumford and colleagues. Taken together these findings suggest that Trp29 of the TP-α receptor is a key residue contributing to surface expression seen with the TMD1 mutant studied by Fanelli and co-workers, possibly by reducing the ability of TP-α to form functional dimers at the cell membrane. Whilst expression of TP-β in platelets at the protein level is not clear, the first 343 residues are shared between the two isoforms, therefore it is likely the same residues are involved in homodimer formation. Although the ability of the Trp29Cys variant receptor to dimerize was not directly studied in the Mumford et al study (Mumford et al., 2012) subsequent work has shown that the variant Trp29Cys TP-α receptor does not interact with the WT TP-α at the cell surface (unpublished observations).

A further variant of the TP-α receptor, Asn42Ser, has also been described (Nisar et al., 2014). Like the Trp29Cys variant, this substitution also occurs within TMD1 and results in reduced surface receptor expression. This patient had a history of menorrhagia, excessive post-operative bleeding and easy bruising, a phenotype indicative of a platelet-function disorder. The Asn42
residue (1.5 Ballesteros-Weinstein numbering) is the most conserved residue in class A GPCRs and is involved in hydrogen bonding with Gly51, Ala299 and Asp83 in bovine rhodopsin (Smith, 2010), all of which are conserved in the human TP-α receptor. Cell line studies show that the Asn42Ser variant TP-α receptor is retained intracellularly, likely in a trans Golgi network (TGN)/ER compartment. Therefore it is likely that interactions with the Asn42 residue are required for correct processing and transport of this receptor to the cell surface. Another notable feature of the protein sequence of the TP-α receptor is the potential presence of an arginine-based ER retention motif (RxR; Figure 1) within intracellular loop 3 (ICL3) of the receptor. These motifs regulate anterograde traffic of proteins including GPCRs (Michelsen et al., 2005; Cunningham et al., 2012). The predominately intracellular localisation of this Asn42Ser variant and the ability of this site to interact with other amino acids through hydrogen bonding mean that this substitution has the potential to impact conformational rearrangement of the receptor. Such rearrangement may expose motifs such as the RxR motif present in ICL3, which may otherwise be masked in native WT form. The consequence of unmasking through conformational rearrangement may result in impaired export from the ER to the plasma membrane, as observed for the Asn42Ser variant. This remains to be explored in more detail.

The description of the Asn42Ser mutation takes the number of TP receptor variants identified in patients with abnormal bleeding to a total of 5, indicating that these remain rare contributors to bleeding risk even in selected populations. However, study and characterization of these mutants have provided key insights into TP receptor structure/function and in particular highlighted the role of TMD1 in the regulation of TP cell surface expression.

Rare Variants of the P2Y₁₂ Receptor

P2Y₁₂ receptor deficiency (MIM #609821) has been identified as an autosomal dominant or recessive disorder characterized by mild to moderate mucocutaneous bleeding and excessive bleeding in response to trauma or after surgery (Cattaneo et al., 2003). The first description of genetic variation in P2RY12 occurred in the report describing the cloning of the gene and was a heterozygous dinucleotide deletion within the coding region (c.717_718delCA) (Hollopeter et al., 2001) in a previously described patient (Nurden et al., 1995) which resulted in a significant loss of P2RY12 expression. Platelets from patients who are heterozygous for a variation that
causes loss of P2Y₁₂ receptor expression (see Table 2) display reduced and reversible aggregation to ADP and reduced aggregation to sub-maximal concentrations of other agonists. Platelet secretion is also reduced due to the positive feedback role of P2Y₁₂ in amplification. Therefore the phenotype is similar to the effects seen in patients with a primary secretion defect. A number of further patients have now been described with P2Y₁₂ receptor deficiency, which have been the subject of several comprehensive reviews (Cattaneo, 2011a; Cattaneo, 2011c) and are summarized in Table 2.

The first P2Y₁₂ receptor defect that caused an alteration in receptor function (as opposed to absent expression) was reported by Cattaneo and colleagues who described an individual who was compound heterozygous for two amino acid substitutions (Arg256Gln and Arg265Trp) in the 6th transmembrane domain and the ECL3 of the P2Y₁₂ receptor respectively (Cattaneo et al., 2003) (Figure 2). The patient had a lifelong history of easy bruising and excessive post-surgical bleeding. Platelet studies showed reduced and reversible aggregation to high concentrations of ADP, whilst shape change remained intact. Signalling studies showed a reduced ability to inhibit PGE1-induced cAMP generation despite normal ligand binding. Further analysis was also carried out on the two children of the index case, both of whom were heterozygous only for the Arg265Trp substitution. Interestingly, these showed a reduction in aggregation and signaling at low concentrations of ADP but at higher ADP concentrations there was no difference compared to healthy controls. Cell line studies, in which the variant Arg256Gln and Arg265Trp P2Y₁₂ receptors were individually expressed in CHO cells, revealed that both amino acid substitutions caused a similar reduction in receptor activation. In subsequent studies in cell line models, the Arg256 residue has also been shown to be important for ligand interactions and antagonist recognition (Hoffmann et al., 2008; Hoffmann et al., 2009; Mao et al., 2010; Chen et al., 2011; Schmidt et al., 2013). The study by Mao and co-workers showed that both the variant Arg256Gln and Arg265Trp P2Y₁₂ receptor expressed in CHO cells were significantly more sensitive to blockade by the P2Y₁₂ antagonist AR-C69931MX than the WT receptor. Furthermore, the recent description of the P2Y₁₂ crystal structure also showed that Arg256, alongside other residues forms the ligand binding pocket and is involved in both antagonist and agonist binding (Zhang et al., 2014a; Zhang et al., 2014b). The discovery of genetic variants...
that result in alterations in sensitivity to receptor blockade is clearly advantageous to aiding rational drug design.

A similar platelet phenotype was observed by Remijn and co-workers who reported a Pro258Thr substitution in the P2Y₁₂ receptor in a patient with a history of epistaxis, easy bruising and post-traumatic bleeding (Remijn et al., 2007). This patient’s platelets also showed reduced and reversible aggregation in response to high ADP concentrations, however the exact mechanism by which this the Pro258Thr change within the 3rd extracellular loop (Figure 2) affects P2Y₁₂ receptor function is not known.

Two further amino acid substitutions in P2Y₁₂ were identified in a cohort of patients who had been previously been recruited to the European Molecular and Clinical Markers for the Diagnosis and Management of Type 1 von Willebrand Disease (MCMMDM-1VWD) study. Type 1 von Willebrand Disease is caused by a quantitative reduction in the plasma concentration of von Willebrand factor (VWF) which is required for platelet adhesion. The bleeding symptoms associated with Type 1 von Willebrand Disease (VWD) are indistinguishable from those seen in patients with mild platelet function disorders and show significant inter-individual variation, even between members of the same family. Data from the MCMMDM-1VWD study have confirmed the contribution of genetic loci outside of the VWF locus to the pathogenesis of bleeding in type 1 VWD (Goodeve et al., 2007). This raises the possibility that loss-of-function variations in genes that affect platelet function may contribute to the severity of bleeding in some individuals with type I VWD.

In order to test this hypothesis, P2Y₁₂R from the MCMMDM-1VWD index cases and geographically matched healthy controls were sequenced as part of the GAPP study. The first of the two variant receptors identified was a Lys174Glu substitution in the 2nd extracellular loop of the P2Y₁₂ receptor (Daly et al., 2009). Platelets from the patient and two related individuals showed reduced aggregation and secretion to ADP. Ligand binding studies revealed a significant reduction in the ability of the variant P2Y₁₂ Lys174Glu receptor to bind radioligand compared to WT receptor, whilst total and surface expression were not affected. The 174 lysine residue is adjacent to a cysteine residue (Cys175; Figure 2) which has previously been shown to be involved in ligand binding (Costanzi et al., 2004; Savi et al., 2006). Interestingly, the P2Y₁₂
crystal structure suggests that Lys174 does not directly interact with the agonist, but instead stabilizes the agonist-bound conformation via the formation of a salt bridge with Glu273 in the TMD7 (Zhang et al., 2014a).

A further patient from the MCMMD-1VWD cohort was found to be heterozygous for a Pro341Ala substitution in the extreme C terminus of the P2Y<sub>12</sub> receptor. Platelet studies on the mother of the patient, who did not have VWD but was heterozygous for the Pro341Ala P2Y<sub>12</sub> receptor substitution, revealed a reduced ability to signal via Gi at low ADP concentrations and a reduction in maximal P2Y<sub>12</sub> ligand binding (Nisar et al., 2011). Although there was no effect on aggregation to ADP or other agonists, further studies revealed that the variant receptor was unable to correctly recycle back to the surface following receptor stimulation and internalization. Studies in cells transfected with the variant Pro341Ala P2Y<sub>12</sub> receptor revealed that the receptor was retained intracellularly in the TGN and rab-7 positive compartments and was not able to recycle and resensitize following agonist exposure (Nisar et al., 2011; Cunningham et al., 2013). The description of the Pro341Ala substitution was particularly interesting because of its location within the PDZ ligand of the P2Y<sub>12</sub> receptor (Figure 2). This PDZ ligand consists of a short amino acid sequence that is found at the extreme C terminus of a large number of proteins including more than 30 GPCRs (Marchese et al., 2008). GPCR PDZ ligands can bind to a number of PDZ domain-containing proteins and these interactions regulate various aspects of receptor regulation including stabilization, signaling and trafficking (Weinman et al., 2006). Indeed, the P2Y<sub>12</sub> receptor has been shown to interact basally with the NHERF1 in a PDZ-dependent manner (Nisar et al., 2012). Despite a vast number of cell line studies which have highlighted the importance of PDZ interactions, this was the first report of a naturally occurring mutation that demonstrated the in vivo importance of a GPCR PDZ ligand.

More recently, the in vivo importance of the P2Y<sub>12</sub> DRY motif was also demonstrated by the description of a naturally occurring variant Arg122Cys P2Y<sub>12</sub> receptor identified in a patient who had a lifelong history of spontaneous bleeding, and haemorrhage upon surgical challenge (Patel et al., 2014). Importantly this patient was homozygous for the Arg122Cys substitution in the P2Y<sub>12</sub> receptor and displayed significantly reduced platelet aggregation in response to ADP as a result of reduced receptor expression at the platelet cell surface. Further analysis in cell lines
suggested that this was due to enhanced agonist-independent receptor internalization and accumulation in lysosomes. The DRY motif (Figure 2) is a highly conserved region found in almost all GPCRs and is known to play a critical role in regulating receptor conformational states. The recent resolution of the P2Y\textsubscript{12} crystal structure shows that P2Y\textsubscript{12} is atypical for a class A GPCR due to the absence of interactions between the DRY motif and helix VI, which may explain high constitutive receptor activity (Zhang et al., 2014b). Data from the R122C study suggest that disruption of this motif further prevents stabilization of the P2Y\textsubscript{12} receptor in an inactive conformation in the absence of agonist, resulting in increased constitutive internalization (Patel et al., 2014). Interestingly, platelet phenotyping of this patient along with related family members also showed a reduction in PAR1 peptide-induced platelet activation (Patel et al., 2014). Subsequent sequencing analysis of the F2R gene which encodes the PAR\textsubscript{1} receptor found that patient was also homozygous for the PAR\textsubscript{1} rs168753 single nucleotide polymorphism (SNP) which has previously been shown to result in reduced PAR1 expression in platelets (Dupont et al., 2003; Smith et al., 2005). This is the first description of two GPCRs being affected by loss of function mutations that combine to reduce platelet activity. Similar to VWD cohort patients described above, this demonstrates the multi-factorial nature of mild bleeding disorders.

Genetic variants of other platelet GPCRs

To date there have been no reported function-disrupting rare variants for the P2Y\textsubscript{1}, PAR\textsubscript{1} and PAR\textsubscript{4} receptors, although common population variations in the genes encoding the PAR\textsubscript{1} and P2Y\textsubscript{1} receptors have been associated with weak alterations in platelet activity. For example, the rs168753 variation in F2R, that encodes the PAR\textsubscript{1} receptor, is associated with reduced platelet activity. The P2Y\textsubscript{1} SNP (1622 G/G genotype) has been associated with a reduced anti-platelet effect of aspirin (Lordkipanidze et al., 2011). The GAPP consortium has found a single missense PAR\textsubscript{1} rare variant and one missense PAR\textsubscript{4} variant (Stockley et al., in submission) although no change in receptor function has yet been detected. The lack of rare mutations in these receptors (that are widely expressed and have key non-platelet functions) suggests that substitutions that significantly alter function of these receptors may be incompatible with life. An inherited defect
in the alpha 2A adrenoceptor that is associated with reduced receptor aggregation to epinephrine has been described (Rao et al., 1988) however the causative gene defect is unknown.

There are also a number of inhibitory GPCRs expressed on the platelet surface, such as the prostanoid receptors, (EP2 and IP) and the adenosine 2A receptor (A2A). These receptors couple to Gs, raise intracellular cAMP levels and inhibit platelet activation via Protein Kinase A. Although ADORA2A variants have been associated with a variety of diseases (Deckert et al., 1996; Hohoff et al., 2007), loss-of-function changes in inhibitory receptors are unlikely to contribute to an increased bleeding risk. However cAMP is a vital regulatory pathway in platelets and loss-of-function mutations in genes involved in this pathway could contribute to increased platelet reactivity and therefore increased risk of adverse cardiovascular events.

Implications of Receptor Mutations for Mild Bleeding

Whilst these studies have provided useful information regarding receptor function, it is important to note that the presence of a heterozygous variation in a GPCR gene does not always correlate with a clinical bleeding phenotype. For example, in the previously reported kindreds with TP-α caused by Asp304Asn and Trp29Cys substitutions, the index cases presented with mild bleeding. However, in both kindreds, there were other first degree relatives who were also heterozygous for these loss-of-function substitutions, but were asymptomatic (Mumford et al., 2010; Mumford et al., 2012). Similarly, patients with P2Y12 receptor deficiency display bleeding symptoms of variable severity that does not correlate well with genotype, or the presence of homozygous versus heterozygous changes. These findings highlight the multifactorial nature of mild bleeding symptoms and suggest that there may be further unidentified defects in the index cases of these kindreds that contribute to the bleeding diathesis. For example the index case with the Pro341Ala P2Y12 receptor substitution also had VWD type 1 disease and a more severe bleeding history than his mother who had an identical platelet P2Y12 receptor defect but did not have VWD (Nisar et al., 2011). The requirement for a closed high-pressure cardiovascular system for life necessitates multiple pathways for haemostasis with a high degree of redundancy. Thus, whilst studies using a detailed phenotyping approach combined with targeted genotyping (Dawood et al., 2012), such as described above can be used to diagnose platelet function disorders, wider speculation on the causes of bleeding should be avoided in the absence of whole
Discussion and Future Perspectives

Variation in GPCR genes results in the disruption of receptor function in a wide variety of human genetic diseases, including mild platelet bleeding disorders. The description and characterization of a number of rare thromboxane and P2Y_{12} receptor variants has provided key insights into the physiological significance of GPCR subdomains. Although there have been a number of studies that have undertaken mutation analysis of these receptors, these naturally occurring variants provide the opportunity to study the effect of changes such as amino acid substitutions in endogenously expressed receptors in human platelets. Platelets have a clear advantage over other cell types as they can be taken repeatedly and with relative ease from patients and family members. Whilst murine knock-out models have been important for delineating roles of individual surface receptors in platelet activation and in vivo thrombosis, more subtle study of receptor function at the molecular level in platelets is associated with a number of technical challenges. For example there is an inherent lack of receptor specific antibodies able to detect receptor expression. Furthermore, there are often difficulties in translating findings in cell expression studies into platelets because of differences in cell machinery, membrane trafficking and receptor expression levels.

Importantly the rare receptor variants described above validate findings found in the vast number of cell expression and modeling studies which have previously assigned structure-function relationships to particular receptor motifs. For example, rare receptor variants that disrupt the NPXXXXY, PDZ and DRY motifs (figures 1 & 2) have all been shown to affect endogenous receptor function in platelets. The description of these rare variants that affect ligand binding, surface expression, G protein coupling and intracellular trafficking highlights the critical importance of each of these processes upon GPCR function, and for the P2Y_{12} and TP-\alpha receptors, maps these functions to specific domains and residues. This is significant because these receptors remain at the forefront of anti-platelet drug therapy. The descriptions of variant receptors such as the Arg256Gln/Arg265Trp P2Y_{12} receptor that affect antagonist sensitivity may aid targeted rational drug design in this area. Recent data from phase III clinical trials of
PAR1 antagonists (Capodanno et al., 2012; Tricoci et al., 2012) confirm the need for more subtle approaches to combat the bleeding associated with anti-platelet therapy. Greater insight into how platelet GPCRs are regulated may also provide novel mechanisms to prevent excessive platelet activation whilst preserving haemostasis.

Genome-wide analysis has shown that variants of platelet receptors, including ADRA2 and P2Y12R, are associated with increased platelet reactivity in the normal population (Jones et al., 2009; Johnson et al., 2010), a finding which has confirmed previous candidate gene studies (Kunicki et al., 2012). Recent advances in second generation sequencing technologies mean that large numbers of individuals can be rapidly tested for genetic changes in their whole exome or through pre-selected candidate genes (Jones et al., 2012). However, it is clear that an integrated approach involving detailed phenotyping and cell validation studies are required in order to truly dissect out and then demonstrate meaningful changes in platelet activity. The study of patients with mild bleeding has been highly successful in identifying a number of rare GPCR mutants that affect receptor function. It is hoped that the extension of this candidate gene-led approach to whole exome analysis will provide further information regarding known platelets genes and to identify novel regulators of platelet function.

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Author Contributions

SPN, MLJ and SJM wrote the manuscript. MRC and ADM reviewed and commented on the manuscript.

Conflict of Interest
The authors declare no conflict of interest.

References


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Figure 1
Thromboxane (TP-α) receptor snake plot.
Sites of naturally-occurring variants found in patients with a bleeding history are highlighted in green. Key amino-acid regulatory motifs are highlighted in yellow (specifically RXR ER retention motif; D/NPXXY motif, E/DRY motif).

Figure 2
P2Y_{12} Receptor Snake Plot
Sites of naturally-occurring variants found in patients with a bleeding history are highlighted in green. Key amino-acid regulatory motifs are highlighted in yellow (specifically E/DRY motif and type 1 PDZ ligand).

Table 1.
Variants of TBXA2R. The numbering used to described coding region variants relates to the RefSeq transcript NM_001060.5. ICL: intracellular loop. TMD: transmembrane domain.

Table 2.
Variants of P2RY12.
<table>
<thead>
<tr>
<th>Description</th>
<th>Variation in coding DNA</th>
<th>Inheritance</th>
<th>Region</th>
<th>Defect</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Receptor deficiency</td>
<td>c.167dupG</td>
<td>heterozygous</td>
<td></td>
<td>reduced receptor expression</td>
<td>Kamae et al., 2011</td>
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<tr>
<td>R60L</td>
<td>c.179G&gt;T</td>
<td>homozygous or heterozygous</td>
<td>ICL1</td>
<td>reduced receptor coupling to Gq</td>
<td>Hirata et al., 1994 &amp; Higuchi et al., 1999</td>
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<td>D304N</td>
<td>c.190G&gt;A</td>
<td>heterozygous</td>
<td>TMD7</td>
<td>reduced ligand binding</td>
<td>Mumford et al., 2010</td>
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<td>W29C</td>
<td>c.87G&gt;C</td>
<td>heterozygous</td>
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<tr>
<td>N42S</td>
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<tr>
<td>Description</td>
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<td>Inheritance</td>
<td>Region</td>
<td>Defect</td>
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<td>Receptor deficiency</td>
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<td>haploinsufficiency: only 1 variant P2RY12 allele detected</td>
<td>no protein</td>
<td>no signaling via P2Y12</td>
<td>Fontana et al., 2009</td>
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<tr>
<td>Partial receptor deficiency</td>
<td>none detected</td>
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<td>reduced receptor function</td>
<td>Fontana et al., 2009</td>
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<td>R256Q &amp; R265W</td>
<td>c.767G&gt;A; 793C&gt;T</td>
<td>compound heterozygous</td>
<td>TMD6; ECL3</td>
<td>reduced signaling via Gi</td>
<td>Cattaneo et al., 2003</td>
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<td>P258T</td>
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