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# The p.Tyr157Cys variant in Protease Activated Receptor 4 reduces platelet functional responses and alters receptor trafficking

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**Running title:** The PAR4 Tyr157Cys variant.

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**ABSTRACT**

**Objective:** Protease activated receptor 4 (PAR4) is a key regulator of platelet reactivity and is encoded by *F2RL3* which has abundant rare missense variants. We aimed to provide proof of principle that rare *F2LR3* variants potentially impact on platelet reactivity and responsiveness to PAR1 antagonist drugs and to explore underlying molecular mechanisms.

**Approach and results:** We identified six rare *F2RL3* missense variants in 236 cardiac patients, of which the variant causing a tyrosine 157 to cysteine substitution (Y157C) was predicted computationally to impact most on PAR4 structure. Y157C platelets from three cases showed reduced responses to PAR4-activating peptide and to α-thrombin compared to controls, but no reduction in responses to PAR1 activating peptide. Pre-treatment with the PAR1 antagonist vorapaxar caused lower residual α-thrombin responses in Y157C platelets than in controls, indicating greater platelet inhibition. HEK293 cells transfected with a PAR4 Y157C expression construct had reduced PAR4 functional responses, unchanged total PAR4 expression but reduced surface expression. PAR4 Y157C was partially retained in the endoplasmic reticulum and displayed an expression pattern consistent with defective *N*-glycosylation. Mutagenesis of Y322, which is the putative hydrogen bond partner of Y157, also reduced PAR4 surface expression in HEK293 cells.

**Conclusions**: Reduced PAR4 responses associated with Y157C result from aberrant anterograde surface receptor trafficking, in part because of disrupted intra-molecular hydrogen bonding. Characterisation of PAR4 Y157C establishes that rare *F2RL3* variants have the potential to markedly alter platelet PAR4 reactivity particularly after exposure to therapeutic PAR1 antagonists.

**NON-STANDARD ABBREVIATIONS AND ACRONYMS**

| | |
|---|---|
| AFU | Arbitrary fluorescence units |
| BW | Ballesteros-Weinstein |
| ECL | Extracellular loop |
| EEA1 | Early endosomal antigen 1 |
| ER | Endoplasmic reticulum |
| GPCR | G protein-coupled receptor |
| ICL | Intracellular loop |
| IP$_3$ | Inositol 1,4,5 triphosphate |
| LTA | Light transmission aggregation |
| MA | Maximum aggregation |
| MFI | Median fluorescence intensity |
| PCR | Polymerase chain reaction |
| PAR1 | Protease activated receptor 1 |
| PAR4 | Protease activated receptor 4 |
| PRP | Platelet rich plasma |
| TM | Transmembrane |
| WP | Washed platelets |

**INTRODUCTION**

Protease activated receptors (PARs) are G protein-coupled receptors (GPCRs) that are critical mediators of haemostasis and inflammation in blood and vascular cells. [1] Human platelets express both PAR1 and PAR4, which mediate activation by the potent physiological agonist thrombin through stimulation of $G_{\alpha q}$ and phospholipase Cβ, leading to the mobilisation of cytoplasmic $Ca^{2+}$. [2,3] PAR1 has a greater affinity for thrombin and mediates rapid $Ca^{2+}$ mobilisation and platelet activation. By contrast, PAR4 activation requires higher concentrations of thrombin and leads to gradual and sustained $Ca^{2+}$ mobilisation, which may be necessary for stable platelet thrombus formation. [4,5] The importance of the PARs for platelet activation is illustrated by the efficacy of PAR1 and PAR4 antagonists as anti-thrombotic agents, [6,7] but also by observations that clinical use of the PAR1 antagonist vorapaxar increases the risk of intracranial bleeding. [8,9]

Platelet reactivity to activating agonists is a predictor of adverse cardiovascular events, particularly in patients receiving anti-platelet drugs for secondary prevention of arterial thrombosis. [10,11] Although platelet reactivity shows large variation across populations, it is a reproducible phenotype within individuals and has been estimated as 60-70% heritable. [12] Genome wide association studies have identified common variants in several genes that account for some of the inter-individual differences in platelet reactivity to epinephrine, collagen and ADP. [13,14] Smaller candidate gene studies have also identified associations between platelet reactivity to PAR agonists and common variants in *F2R* that encodes PAR1 [15,16] and in *F2RL3* that encodes PAR4. [17] Increased platelet PAR4 reactivity has been observed in African-American compared with European-American populations, suggesting a race-dependent genetic effect. [18] Part of this difference is accounted for by increased platelet levels of phosphatidylcholine transfer protein in African-American populations, which affects PAR4 reactivity indirectly. [18] However, there is also a contribution from the common *F2RL3* coding variant rs773902 that predicts an alanine 120 to threonine substitution (A120T) and is associated with increased PAR4 reactivity. [19] The rs773902 variant was observed at a frequency of 0.63 in an African-American population compared to 0.19 in European-Americans, and accounted for 48% of the overall population variance in PAR4 reactivity. [19]

In an analysis of population genetic databases, we recently showed that in addition to the common rs773902 variant, the *F2RL3* coding region also contains diverse rare genetic variants (global minor allele frequency <0.01), and that these occurred at a higher frequency than in other platelet GPCR genes. [20] *F2RL3* also contained a higher proportion of rare variants that were predicted computationally to be deleterious to receptor function compared to benign variants. [20] Together, these observations suggest that rare *F2RL3* variants may contribute to the population variance in PAR4 reactivity, alongside common variants such as rs773902. Since some rare *F2RL3* variants in population databases are predicted to have a large influence on the expression level or function of PAR4, [20] there may be a strong effect in individual patients on characteristics such as platelet haemostatic function or responsiveness to anti-thrombotic drugs that target the PAR pathway.

In order to provide proof of principle that rare *F2RL3* variants have the potential to impact on platelet PAR4 reactivity, we sequenced the *F2RL3* gene in a sample cohort of cardiac surgery patients at the Bristol Heart Institute and then evaluated the observed variants using computational prediction tools. The variant predicting a tyrosine 157 to cysteine (Y157C) substitution, which had the greatest predicted effect on PAR4 structure, was then analysed experimentally using platelets from PAR4 Y157C cases and transfected HEK293 cells.

**MATERIALS AND METHODS**
The study design and experimental methods are described in the online-only Data Supplement.

## RESULTS

**Characteristics of cases and *F2RL3* genotypes**
In a sample cohort of 236 cardiac surgery patients, (median age 69 years; 200 (85%) male; 227 (96%) Caucasian European), we identified seven different missense single nucleotide variants in the *F2RL3* coding region (Table 1). These included the common rs773902 (encoding PAR4 A120T), which has an allele frequency of 0.22 in Europeans in the Exome Aggregation Consortium Browser (ExAC) dataset [21] and occurred at a frequency of 0.14 in the study cohort. The remaining six variants were present as heterozygous alleles in a total of nine index cases and had MAFs of <0.01 in the ExAC dataset. [21]

The variants predicting alanine 82 to threonine, alanine 286 to threonine, proline 310 to leucine and valine 335 to leucine substitutions in PAR4 were classified as benign by meta-analysis of six different computational prediction tools using the PredictSNP consensus classifier [22] (Table 1 and Supplemental Table I). These substitutions were also predicted to have minimal impact on the structure of PAR4 determined from a homology model generated from the antagonist-bound PAR1 structure [23] (Supplemental Figure S1). The variant predicting a phenylalanine 296 to leucine (F296L) substitution was identified as deleterious to PAR4 by PredictSNP, although with a low likelihood score (Table 1 and Supplemental Table S1). In the PAR4 homology model, F296 projected centrally into the PAR4 transmembrane (TM) domain bundle, but was not predicted to contribute inter-TM domain hydrogen bonding (Supplemental Figure I). [23]

The remaining variant, that predicted tyrosine 157 to cysteine substitution (Y157C) was present in only two of the 57,476 *F2RL3* alleles reported in the ExAC database, and was classified as deleterious to PAR4 by PredictSNP with a high likelihood score (Table 1 and Supplemental Table S1). In the PAR4 homology model, Y157 projected centrally from TM domain 3 and was predicted to form a hydrogen bond with tyrosine 322 (Y322) in TM domain 7 (Figure 1A and B), thereby contributing to the PAR4 inter-TM domain contacts. Since this indicated that Y157C had a greater potential structural impact than the other variants observed in the study cohort, the index case harbouring Y157C and pedigree members were evaluated further.

**Clinical characteristics and platelet aggregation responses**
The Y157C index case was a 66 year-old Caucasian male non-smoker (Y157C.1) who underwent elective coronary artery bypass grafting for symptomatic triple vessel disease. He had a history of upper gastrointestinal bleeding during previous aspirin treatment and intermittent bleeding from colonic diverticular disease, but no other bleeding symptoms. Y157C was also identified in two sons aged 39 and 36 years (Y157C.2 and Y157C.3; Supplemental Figure II) who had no history of cardiovascular disease or abnormal bleeding. Y157C.1, but not Y157C.2 or Y157C.3 harboured the heterozygous common variant rs773902 (PAR4 A120T). None of the study subjects were receiving aspirin or other drugs known to influence platelet function. There was no family history of abnormal bleeding.

Compared to controls with no rare *F2RL3* variants, platelets from the three Y157C cases all showed reduced maximum amplitude (MA) of LTA with 100 and 150 µM PAR4-activating peptide (PAR4-AP) and with 0.01 U/ml α-thrombin. Responses to PAR1-activating peptide (PAR1-AP) at concentrations in the range 1-10 µM were unchanged compared with controls. Shape change and lag phase of the aggregation responses to all the agonists were unchanged (Figure 2A and B). In order assess the impact of Y157C on platelet inhibition by vorapaxar, we first showed that pre-incubation of control platelets with 5 µM vorapaxar resulted in an approximately 90% reduction in LTA responses with 10 µM PAR1-AP (Supplemental Figure III), similar to that in healthy volunteers receiving therapeutic doses of vorapaxar. [24] At this concentration, LTA responses to 150 µM PAR4-AP were not reduced, indicating selective antagonism of PAR1. Pre-incubation of control platelets with 5 µM

vorapaxar caused an approximately 20% reduction in the LTA response with 0.1 U/mL α-thrombin, consistent with loss of the PAR1 component of the α-thrombin response (Figure 2C, 2D and Supplemental Figure S3). However with Y157C platelets, pre-incubation with vorapaxar almost completely abolished the LTA response to 0.1 U/mL α-thrombin (Figure 2D), indicating greater inhibition of platelet reactivity.

### Other platelet activation responses and PAR4 expression

Compared with controls, platelets from all three Y157C cases showed reduced cytoplasmic $Ca^{2+}$ mobilisation with PAR4-AP at concentrations of 50-1000 µM (Figure 3A). No significant changes in $Ca^{2+}$ mobilisation were observed in Y157C platelets compared to controls in response to 1-100 µM PAR1-AP or to 0.5-5 U/ml α-thrombin (Figure 3B and C and Supplemental Figure IV).  Integrin $\alpha_{IIb}\beta3$ activation and α-granule release with 150 µM PAR4-AP were both reduced in Y157C platelets compared with controls but responses with 5 µM PAR1-AP were unchanged (Figure 3D).

In Western blots of lysates from Y157C platelets and from controls, PAR4 and PAR1 migrated as bands of approximately 40 and 70 kDa respectively (Figure 4A).  When expressed relative to the corresponding tubulin bands, the densities of the PAR4 and PAR1 bands were unchanged in Y157C platelets compared with controls (Figure 4B), indicating that the reduced PAR4 reactivity was not the result of diminished PAR4 synthesis or abnormal catabolism.

### PAR4 Y157C expression and function in HEK293 cells

In order to investigate further the mechanism of the reduced PAR4 reactivity suggested in the platelet experiments, we next studied HEK293 cells transfected with Y157C variant (PAR4 Y157C) or non-mutagenised (PAR4 WT) expression constructs with a CFP tag is expressed at the PAR4 carboxyl-terminus. Total expression of the PAR4-CFP fusion protein, determined from the CFP fluorescence, was unchanged in PAR4 Y157C cells compared to PAR4 WT cells (Figure 5A), similar to the findings in Y157C platelets. However, we additionally found that surface PAR4 expression, assessed by measuring binding of an antibody recognising the PAR4 extracellular domain, was reduced in PAR4 Y157C cells compared to PAR4 WT cells (Figure 5B).

Stimulation of HEK293 cells transfected with empty vector with PAR4-AP did not increase the cytoplasmic $Ca^{2+}$ concentration. However, stimulation of PAR4 WT cells caused significant $Ca^{2+}$ responses with PAR4-AP at concentrations of 10 µM-1 mM (Figure 5C), similar to the range previously shown to increase cytoplasmic $Ca^{2+}$ in control platelets. This confirmed that PAR4 WT expressed as a CFP fusion protein retained functional activity. Compared to the PAR4 WT cells, the PAR4 Y157C cells showed reduced cytoplasmic $Ca^{2+}$ mobilisation with 50-500 µM PAR4-AP (Figure 5C) and less accumulation of inositol phosphate (IP3) with 10-100 µM PAR4-AP (Figure 5D). This confirmed that the defect in surface expression of PAR4 Y157C in the HEK293 cells was associated with reduced PAR4 reactivity.

### Trafficking of PAR4 Y157C in HEK293 cells

In order to investigate the defect in surface expression further, the PAR4-CFP fusion proteins were visualised in both the WT and Y157C cells by immunofluorescence confocal microscopy.  In the WT cells, PAR4-CFP was localised at the cell membrane, but also to discrete structures within the cytoplasm. By contrast, in the Y157C cells, PAR4-CFP was diffusely localised to the peri-nuclear region, with minimal surface expression (top panels of Figure 6A and B). In order to further define the abnormal distribution of PAR4 Y157C, the HEK293 cells were also visualised after incubating with antibodies recognising early endosome antigen 1 (EEA1), a marker of the endosomes that mediate surface receptor internalisation, and calnexin, an endoplasmic reticulum (ER) exit marker. Whilst in the WT cells, PAR4-CFP co-localised with EEA1-positive compartments, in the Y157C cells, there

was less co-localisation (Figure 6A and 6C). By contrast, there was greater co-localization with calnexin in the Y157C cells than in the WT cells (Figure 6B and 6C), suggesting that PAR4 Y157C undergoes abnormal anterograde trafficking between the ER and cell membrane.

In Western blots of lysates from both the WT and Y157C cells, the CFP fusion proteins migrated as prominent bands of approximately 55 kDa, comparable with the predicted molecular weight of PAR4-CFP. In the WT cell lysates, PAR4-CFP also migrated as a series of broad bands between 60 and 80 kDa, consistent with post-translationally modified forms. [25] Levels of these modified forms were reduced in the Y157C cell lysates, similar to a control cell line expressing PAR4 in which the consensus *N*-linked glycosylation site in the amino-terminal region of PAR4 had been removed by mutagenesis (PAR4 N56A; Figure 6D). PAR-CFP expression in the N56A cells was also absent at the cell membrane (Figure 6F). These data are consistent with receptor retention in the ER and further confirm the inability of PAR4 Y157C to be efficiently transported to the plasma membrane.

In the PAR4 homology model, Y157 was predicted to form a hydrogen bond with Y322 in TM domain 7, which is essential for the structural integrity of related class A GPCRs. We tested the effect of disruption of this bond using HEK293 cells transfected with a PAR4 Y322A expression, in which the Y157-Y322 hydrogen bond was disrupted by mutagenesis of Y322. Similar to PAR4 Y157C, Western blots of lysates from the Y322A cells showed reduced high molecular weight PAR4-CFP forms (Figure 6E). There was also reduced cell surface expression in the Y322A cells compared to the WT cells, and cytoplasmic retention in a similar distribution to the Y157C cells (Figure 6F). Together, this suggests that PAR4 Y322A also undergoes defective anterograde trafficking towards the cell membrane and highlights the importance of the Y157-Y322 hydrogen bond for this process.


## DISCUSSION

We have demonstrated that in a small cohort of 236 cardiac patients, nine cases harboured six different rare missense variants in the PAR4 gene *F2RL3*. This observation is consistent with our previous analysis of the 1000 genomes and Exome Sequencing Project population databases, which suggested that *F2RL3* was genetically diverse and that when considered together, rare *F2RL3* variants may be abundant at population level. [20]

Amongst the rare variants identified in the study cohort, sequenced based computational analysis using the PredictSNP consensus classifier identified two as potentially impactful to PAR4 reactivity. These included F296L, which is predicted to disrupt the conserved FxxCW/FxP motif in the PAR4 TM domain 6 that mediates activation of related class A GPCRs. [26] Substitution of PAR4 F296 with valine (F296V), is a common variant in African-Africans (MAF 0.06) [21] and has been shown previously to influence PAR4 reactivity,[19] suggesting that F296L may also have a functional impact. A second variant Y157C, was predicted to disrupt a hydrogen bond between TM domains 3 and 7 in the PAR4 homology model. Inter-TM domain hydrogen bonds have been proposed as essential for class A GPCR folding, [23, 27] suggesting high impact effect. We acknowledge that computational analysis and structural homology modelling may be insensitive to some impactful variants, as illustrated by the failure of these methods to identify the A120T as a change of function variant. [19] However, we concluded that Y157C was likely to have a greater effect on PAR4 reactivity than the other variants in the study cohort, and select this variant for further analysis in this proof-of-concept study.

Consistent with this prediction, we found that platelets from the Y157C index case and from the two Y157C pedigree members showed reduced responses to PAR4-AP when compared with controls in multiple functional assays. By contrast, responses to PAR1-AP were

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unchanged. Since both PAR4 and PAR1 signal predominantly through $G_{\alpha q}$ in platelets, [2, 3] this is most consistent with a defect at PAR4 receptor level. Y157C platelets also showed reduced functional responses to α-thrombin, in keeping with the requirement for both PAR4 and PAR1 activation for the full platelet response to this agonist. [2] The preservation of PAR1-AP responses in the PAR4 Y157C platelets is less consistent with previous observations in transfected cell models that PAR1-PAR4 heterodimer formation is necessary for full PAR1 expression and function [28]. It is not possible to resolve from these functional data whether the heterozygous Y157C substitution in the cases disrupts PAR4 homodimer formation [29], which could contribute to the loss of PAR4 responsiveness phenotype.

The common coding PAR4 A120T variant (rs773902), has previously been associated with gain of PAR4 receptor function. [19] Since the index case Y157C.1 was heterozygous for A120T, this may have modified the phenotype of platelets from this case. However, despite the presence of A120T, platelets from Y157C.1 showed reduced reactivity when compared to the control group of which 80% were homozygous for the reduced function A120 allele. Moreover, there were no detectible differences in the PAR4 reactivity of platelets from Y157C.1 compared to the other Y157C cases who did not harbour A120T. Other potential modifiers of the PAR4 reactivity include non-coding *F2RL3* variants, [17] and other regulators such as miRNA [18] which potentially affect PAR4 expression level. These were also unlikely to have impacted on the reduced PAR4 reactivity in the Y157C platelets since there was no reduction in total PAR4 expression compared to controls. Together, these observations suggest that Y157C has a larger impact on platelet PAR4 reactivity compared to other potential modifiers. We acknowledge that statistical proof of an association between Y157C and reduced PAR4 reactivity is hampered by the small number of affected cases, similar to other rare variant studies. However, we have also analysed Y157C using HEK293 cells, which do not express endogenous PAR4 and are an established PAR expression model. [19, 25] Our demonstration of reduced PAR4 reactivity in HEK293 cells expressing PAR4 Y157C provides strong evidence that Y157C is causally associated with the platelet phenotype and that this effect is not dependent on other modifiers.

It is significant that Y157C was associated with gastrointestinal bleeding only during aspirin treatment in the index case Y157C.1, and that the pedigree members with Y157C had no abnormal bleeding. This suggests that heterozygosity for Y157C alone is insufficient to disrupt clinical haemostasis. However, in the presence of the PAR1 antagonist vorapaxar *ex vivo*, we found a greater inhibitory effect on the platelet thrombin response in the Y157C cases than in controls. This finding is consistent with impairment of both PAR1 and PAR4 thrombin activation pathways in the Y157C platelets. Vorapaxar is currently approved by the US Food and Drug Administration for the secondary prevention of cardiovascular disease in selected patients, [30] but was associated with abnormal bleeding in the pivotal clinical trials. [8, 9] Our findings raise the possibility that loss-of-function *F2RL3* variants could be potential pharmacogenetic markers of increased anti-thrombotic effect and of bleeding during anti-platelet treatment, particularly with PAR1 antagonists and possibly with emerging drugs that target PAR4. [6, 31] However, this requires further experimental confirmation.

Using HEK293 cells, we studied the expression and function of PAR4 Y157C with a CFP tag appended to the carboxyl-terminus that has previously been shown not to disrupt cellular localisation or PAR4 functional responses. [25, 28] Although there may have been a small reduction in total receptor expression in this overexpression model, we showed that the Y157C substitution resulted in much more marked reduction of surface expression of PAR4, thereby reducing the availability of receptor to respond extracellular agonists. Consistent with this, PAR4 Y157C showed greater co-localisation than PAR4 WT with the ER exit-marker calnexin, but less co-localisation with EEA1, which is a marker of endosomes that mediate GPCR internalisation after surface membrane expression. [32] Compared to PAR4 WT, there were also less high molecular weight forms of PAR4, similar to mutagenized PAR4 N56A, which is not glycosylated because of the absence of a consensus *N-*

glycosylation site. [25] Correct anterograde trafficking of cargo through the ER and Golgi is usually required for full *N*-glycosylation. [33] Therefore, the simplest explanation for these observations is that aberrant folding of PAR4 caused by the Y157C substitution leads to failure of trafficking beyond the ER, thereby reducing *N*-glycosylation and impairing cell surface expression.

There are several potential explanations of how the Y157C substitution could alter PAR4 folding sufficiently to disrupt anterograde trafficking. One possibly is that folding is disrupted by non-native disulphide bond formation by the new cysteine at position 157. This cannot be excluded from our data, although there were no candidate binding partners for cysteine 157 in the homology model of the correctly folded receptor. A more plausible explanation is that abnormal receptor folding is caused by loss of the predicted hydrogen bond between Y157 in TM domain 3 and Y322 in TM domain 7. These binding partners are conserved in class A GPCRs and contribute to the network of inter-TM domain interactions which maintain receptor stability in both inactive and active states. [27] In support of this, we showed that disruption of this hydrogen bond by mutagenesis of the cognate Y157 binding partner Y322, resulted in accumulation of PAR4 in the cytoplasm and reduced high molecular forms, similar to PAR4 Y157C. It is noteworthy that mutagenesis of the homologous Y353 residue in PAR1 also resulted in abnormal surface receptor expression. [23]

We conclude that reduced PAR4 reactivity in platelets from the study cases is most likely a direct effect of the rare Y157C substitution in *F2RL3.* At least in HEK293 cells, this is mostly a consequence of reduced cell surface PAR4 expression arising from defective anterograde trafficking, most likely caused by loss of the Y157-Y322 hydrogen bond and abnormal receptor folding. It is noteworthy that mutagenesis of residues that are homologous to Y157 in other class A GPCRs, such as the P2Y purinoreceptor 1 [34] and adenosine receptor A1, [35] reduced receptor ligand binding, but did not affect surface expression. Therefore, although reduced surface expression provides an adequate explanation for loss of PAR4 reactivity in the Y157C cells, an additional functional defect in any correctly trafficked PAR4 Y157C cannot be excluded from our data. It is a limitation of our study that in the limited number of blood samples available from the Y157C cases, we were unable to reliably resolve whether platelet surface PAR4 expression was diminished.

To our knowledge, Y157C is the first naturally occurring rare PAR4 variant to have undergone mechanistic characterisation. The association between Y157C and reduced platelet PAR4 reactivity establishes the principle that rare *F2RL3* variants potentially contribute to inter-individual differences in platelet function and responses to therapeutic PAR antagonists.

## DISCLOSURES
The authors declare no conflicts of interest

**REFERENCES**

1.	Coughlin SR. Protease-activated receptors in hemostasis, thrombosis and vascular biology. *J Thromb Haemost*. 2005; 3:1800-14.
2.	Kahn ML, Nakanishi-Matsui M, Shapiro MJ, Ishihara H, Coughlin SR. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. *J Clin Invest*. 1999;103:879-887.
3.	Gieseler F, Ungefroren H, Settmacher U, Hollenberg MD, Kaufmann R. Proteinase-activated receptors (PARs) - focus on receptor-receptor-interactions and their physiological and pathophysiological impact. *Cell Commun Signal*. 2013;11:86.
4.	Shapiro MJ, Weiss EJ, Faruqi TR, Coughlin SR. Protease-activated receptors 1 and 4 are shut off with distinct kinetics after activation by thrombin. *J Biol Chem*. 2000;275:25216-21.
5.	Covic L, Gresser AL, Kuliopulos A. Biphasic kinetics of activation and signaling for PAR1 and PAR4 thrombin receptors in platelets. *Biochemistry*. 2000;39:5458-5467.
6.	Young SE, Duvernay MT, Schulte ML, Lindsley CW, Hamm HE. Synthesis of indole derived protease-activated receptor 4 antagonists and characterization in human platelets. *PLoS One*. 2013;8:e65528.
7.	Covic L, Misra M, Badar J, Singh C and Kuliopulos A. Pepducin-based intervention of thrombin-receptor signaling and systemic platelet activation. *Nat Med*. 2002;8:1161-5.
8.	Tricoci P, Huang Z, Held C, *et al*. Thrombin-receptor antagonist vorapaxar in acute coronary syndromes. *N Engl J Med*. 2012;366:20-33.
9.	Morrow DA, Braunwald E, Bonaca MP *et al*.  Vorapaxar in the secondary prevention of atherothrombotic events. *N Engl J Med*. 2012;366:1404-1413.
10.	Breet NJ, van Werkum JW, Bouman HJ, Kelder JC, Ruven HJ, Bal ET, Deneer VH, Harmsze AM, van der Heyden JA, Rensing BJ, Suttorp MJ, Hackeng CM, ten Berg JM. Comparison of platelet function tests in predicting clinical outcome in patients undergoing coronary stent implantation. *JAMA*. 2010;303:754-762.
11.	O'Donnell CJ, Larson MG, Feng D, Sutherland PA, Lindpaintner K, Myers RH, D'Agostino RA, Levy D, Tofler GH, Framingham Heart S. Genetic and environmental contributions to platelet aggregation: the Framingham heart study. *Circulation*. 2001;103:3051-3056.
12.	Bray PF, Mathias RA, Faraday N, Yanek LR, Fallin MD, Herrera-Galeano JE, Wilson AF, Becker LC, Becker DM. Heritability of platelet function in families with premature coronary artery disease. *J Thromb Haemost*. 2007;5:1617-1623.
13.	Qayyum R, Becker LC, Becker DM, Faraday N, Yanek LR, Leal SM, Shaw C, Mathias R, Suktitipat B and Bray PF. Genome-wide association study of platelet aggregation in African Americans. *BMC Genet*. 2015;16:58.
14.	Johnson AD, Yanek LR, Chen MH, Faraday N, Larson MG, Tofler G, Lin SJ, Kraja AT, Province MA, Yang Q, Becker DM, O'Donnell CJ, Becker LC. Genome-wide meta-analyses identifies seven loci associated with platelet aggregation in response to agonists. *Nat Genet*. 2010;42:608-613.
15.	Smith SM, Judge HM, Peters G, Armstrong M, Dupont A, Gaussem P, Storey RF. PAR-1 genotype influences platelet aggregation and procoagulant responses in patients with coronary artery disease prior to and during clopidogrel therapy. *Platelets*. 2005;16:340-345.
16.	Dupont A, Fontana P, Bachelot-Loza C, Reny JL, Bieche I, Desvard F, Aiach M, Gaussem P. An intronic polymorphism in the PAR-1 gene is associated with platelet receptor density and the response to SFLLRN. *Blood*. 2003;101:1833-1840.
17.	Muehlschlegel JD, Perry TE, Liu KY, Fox AA, Smith SA, Lichtner P, Collard CD, Shernan SK, Hartwig JH, Body SC, Hoffmeister KM. Polymorphism in the protease-activated receptor-4 gene region associates with platelet activation and perioperative myocardial injury. *Am J Hematol*. 2012;87:161-166.
18.	Edelstein LC, Simon LM, Montoya RT, Holinstat M, Chen ES, Bergeron A, Kong X, Nagalla S, Mohandas N, Cohen DE, Dong JF, Shaw C, Bray PF. Racial differences in human platelet PAR4 reactivity reflect expression of PCTP and miR-376c. *Nat Med*. 2013;19:1609-1616.

19.     Edelstein LC, Simon LM, Lindsay CR, Kong X, Teruel-Montoya R, Tourdot BE, Chen ES, Ma L, Coughlin S, Nieman M, Holinstat M, Shaw CA, Bray PF. Common variants in the human platelet PAR4 thrombin receptor alter platelet function and differ by race. *Blood*. 2014;124:3450-3458.

20.     Jones ML, Norman JE, Morgan NV, Mundell SJ, Lordkipanidze M, Lowe GC, Daly ME, Simpson MA, Drake S, Watson SP, Mumford AD. Diversity and impact of rare variants in genes encoding the platelet G protein-coupled receptors. *Thromb Haemost*. 2015;113:826-837.

21.     Exome Aggregation Consortium (ExAC) C, MA- URL: http://exac.broadinstitute.org.

22.     Bendl J, Stourac J, Salanda O, Pavelka A, Wieben ED, Zendulka J, Brezovsky J, Damborsky J. PredictSNP: robust and accurate consensus classifier for prediction of disease-related mutations. *PLoS Comput Biol*. 2014;10:e1003440.

23.     Zhang C, Srinivasan Y, Arlow DH, Fung JJ, Palmer D, Zheng Y, Green HF, Pandey A, Dror RO, Shaw DE, Weis WI, Coughlin SR, Kobilka BK. High-resolution crystal structure of human protease-activated receptor 1. *Nature*. 2012;492:387-392.

24.     Kosoglou T, Reyderman L, Tiessen RG, van Vliet AA, Fales RR, Keller R, Yang B, Cutler DL. Pharmacodynamics and pharmacokinetics of the novel PAR-1 antagonist vorapaxar (formerly SCH 530348) in healthy subjects. *Eur J Clin Pharmacol*. 2012;68:249-258.

25.     Cunningham MR, McIntosh KA, Pediani JD, Robben J, Cooke AE, Nilsson M, Gould GW, Mundell S, Milligan G, Plevin R. Novel role for proteinase-activated receptor 2 (PAR2) in membrane trafficking of proteinase-activated receptor 4 (PAR4). *J Biol Chem*. 2012;287:16656-16669.

26.     Rasmussen SG, Choi HJ, Fung JJ *et al.* Structure of a nanobody-stabilized active state of the beta(2) adrenoceptor. *Nature*. 2011;469:175-180.

27.     Venkatakrishnan AJ, Deupi X, Lebon G, Tate CG, Schertler GF, Babu MM. Molecular signatures of G-protein-coupled receptors. *Nature*. 2013;494:185-194.

28.     Arachiche A, Mumaw MM, de la Fuente M, Nieman MT. Protease-activated receptor 1 (PAR1) and PAR4 heterodimers are required for PAR1-enhanced cleavage of PAR4 by alpha-thrombin. *J Biol Chem*. 2013;288:32553-32562.

29.     de la Fuente M, Noble DN, Verma S, Nieman MT. Mapping human protease-activated receptor 4 (PAR4) homodimer interface to transmembrane helix 4. *J Biol Chem*. 2012;287:10414-10423.

30.     Baker NC, Lipinski MJ, Lhermusier T, Waksman R. Overview of the 2014 Food and Drug Administration Cardiovascular and Renal Drugs Advisory Committee meeting about vorapaxar. *Circulation*. 2014;130:1287-1294.

31.     Wu CC, Teng CM. Comparison of the effects of PAR1 antagonists, PAR4 antagonists, and their combinations on thrombin-induced human platelet activation. *Eur J Pharmacol*. 2006;546:142-147.

32.     Holleman J, Marchese A. The ubiquitin ligase deltex-3l regulates endosomal sorting of the G protein-coupled receptor CXCR4. *Mol Biol Cell*. 2014;25:1892-1904.

33.     Moremen KW, Tiemeyer M, Nairn AV. Vertebrate protein glycosylation: diversity, synthesis and function. *Nat Rev Mol Cell Biol*. 2012;13:448-462.

34.     Moro S, Guo D, Camaioni E, Boyer JL, Harden TK, Jacobson KA. Human P2Y1 receptor: molecular modeling and site-directed mutagenesis as tools to identify agonist and antagonist recognition sites. *J Med Chem*. 1998;41:1456-1466.

35.     Rivkees SA, Barbhaiya H, IJerman AP. Identification of the adenine binding site of the human A1 adenosine receptor. *J Biol Chem*. 1999;274:3617-3621.

**SINGIFICANCE OF THIS RESEARCH**

Protease activated rceptor 4 (PAR4) mediates platelet activation by thrombin, and is a potential target for anti-thrombotic drugs. Rare varaints in the PAR4 gene (*F2RL3*) are a

potential source inter-individual differences in platelet reactivity, yet the impact of these is unknown. Here we provide proof-of-principle that rare *F2RL3* varaints have a high potential impact on platelet reactivity by characterising a variant predicting a tyrosine 157 to cysteine substitution (Y157C) in PAR4. We show that heterozygous Y157C platelets have reduced PAR4 activation responses and greater levels of inhibition with the therapeutic PAR1 antagonist vorapaxar compared to controls. In cell models the Y157C substitution causes reduced PAR4 activation responses by altering anterograde trafficking of receptor to the surface membrane. This first detailed characterisation of a high impact PAR4 variant highlights that rare genetic variants could be potential risk factors for bleeding, particularly during anti-thrombotic therapies that target the PAR pathway.

## FIGURE LEGENDS

### Figure 1. Structural interactions of PAR4 Y157
**(A)** In a PAR4 homology model based on the antagonist-bound PAR1 structure, Y157 projects centrally from transmembrane (TM) domain 3 into the TM domain bundle. **(B)** A higher resolution image showing the close proximity and likely formation of a hydrogen bond between the side chains of Y157 and Y322 in TM7. TM=transmembrane domain; ECL1-extracellular loop 1. Superscript indicates Ballesteros-Weinstein numbering.

### Figure 2. Light transmission aggregation responses in Y157C platelets.
**(A)** Representative light transmission aggregation (LTA) responses of platelets from Y157.1 and a control in response to 150 µM PAR4-AP, 0.01 U/mL α-thrombin or 5 µM PAR1-AP. **(B)** Maximum amplitude (MA) of LTA responses to PAR4-AP, α-thrombin and PAR1-AP. **(C)** Representative LTA responses of platelets from Y157C.1 and control, pre-incubated with vehicle (left panel) or with 5 µM vorapaxar (right panel) before stimulation with 0.1 U/mL α-thrombin. **(D)** Residual platelet aggregation in response to 0.1 U/mL α-thrombin after pre-incubation of control or Y157C platelets with 5 µM vorapaxar. Data are expressed as a percentage of the amplitude of LTA responses in the absence of vorapaxar. The data in B and D are means ± SEM (* $p \leq 0.05$; ** $p \leq 0.005$; ***$p \leq 0.0005$; ns=not significant; two way ANOVA with Bonferroni *post hoc* test (B) or Student's t test (F)) and represent measurements from at least three experiments in all the Y157C cases and from a minimum of six controls.

### Figure 3. Calcium response, integrin α$_{IIb}$β3 activation and α-granule release in Y157C platelets
**(A-C)** Calcium mobilisation responses to PAR4-AP, PAR1-AP and α-thrombin in the three Y157C cases and from controls (minimum n=7). Data are presented as the fold increase in fluorescence emission following stimulation of the Fluo4-AM loaded platelets expressed relative to the fluorescence emission before stimulation. **(D)** Integrin α$_{IIb}$β3 activation indicated by PAC1 binding, and α-granule release indicated by P-selectin exposure, in response to either 150 µM PAR4-AP (P4-AP) or 5 µM PAR1-AP (P1-AP). Data are expressed as the median fluorescence intensity (MFI) of binding of the detecting antibody quantified in arbitrary units. Data are means ± SEM from at least four independent experiments at each agonist concentration; * $p \leq 0.05$; ** $p \leq 0.005$; ***$p \leq 0.0005$; ns=not significant; two way ANOVA with Bonferroni *post hoc* test.

### Figure 4. PAR4 expression in Y157C platelets.
**(A)** Representative Western blot of platelet lysates from Y157C.1 and a control probed with anti-PAR4, anti-PAR1 or anti-tubulin antibodies. **(B)** Total platelet PAR4 and PAR1 expression measured by densitometry of the principal 70 and 40 KDa immunoreactive bands for PAR4 and PAR1 respectively. The data are expressed as relative density units (RDU) after normalisation to the tubulin band within each lane. The expression data are from three

experiments using Y157C platelets and are presented as means ± SEM; *=p≤0.05, ns=non-significant; Student's t test.
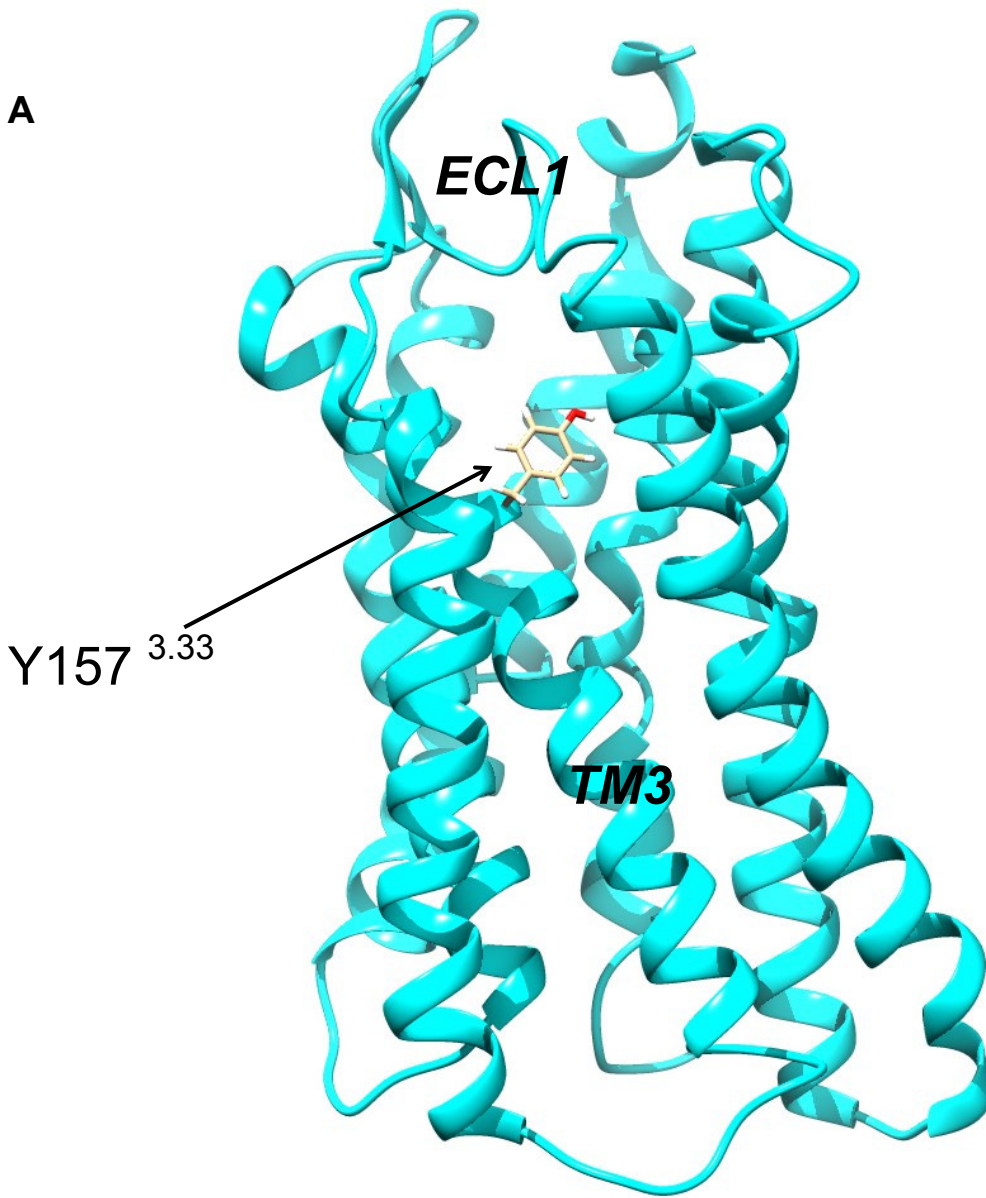
**Figure 5. PAR4 expression and function in Y157C cells.**
**(A)** Total PAR4 expression in HEK293 cells transfected with the PAR4 Y157C or PAR4 WT CFP-fusion constructs or with empty vector, determined by measuring CFP fluorescence. Data are expressed as median fluorescence intensity (MFI) measurements from six experiments. **(B)** Surface PAR4 expression determined by measuring binding of an antibody recognising the PAR4 extracellular domain to non-permeabilised HEK293 cells. Data are expressed as the MFI measurements from six experiments in cells transfected with expression constructs for PAR4 WT (WT) or PAR4 Y157C (Y157C). In all experiments there was a total of 10 µg of construct per culture plate. **(C)** Calcium mobilisation responses to 10-1000 µM PAR4-AP in Fura2-AM loaded HEK293 cells. Data are expressed as the increase in $Ca^{2+}$ concentration at 100 s after stimulation from three to five experiments. **(D)** Fold increase in [$^3$H] inositol phosphate (IP) accumulation in the presence of lithium chloride to prevent IP breakdown, after stimulation with10-100 µM PAR4-AP.  Data are expressed as fold increase in [$^3$H] (IP) in a minimum of three experiments. Data are means ± SEM; * p≤0.05; ** p≤0.005; ***p≤0.0005; ns=not significant; Student's t test (A and B), two way ANOVA with Bonferroni *post hoc* test (C and D).

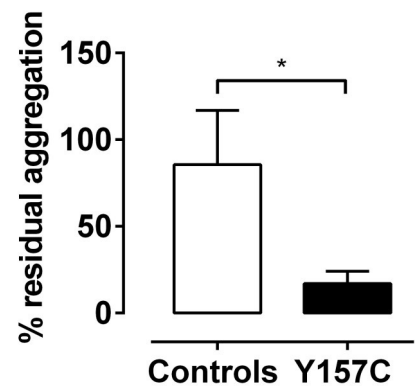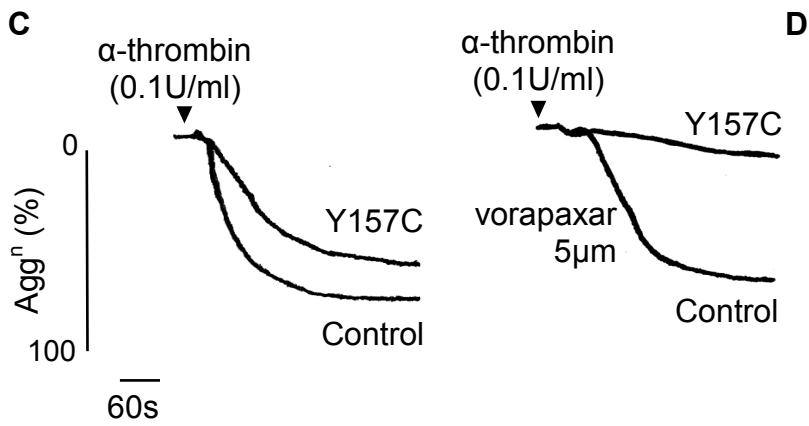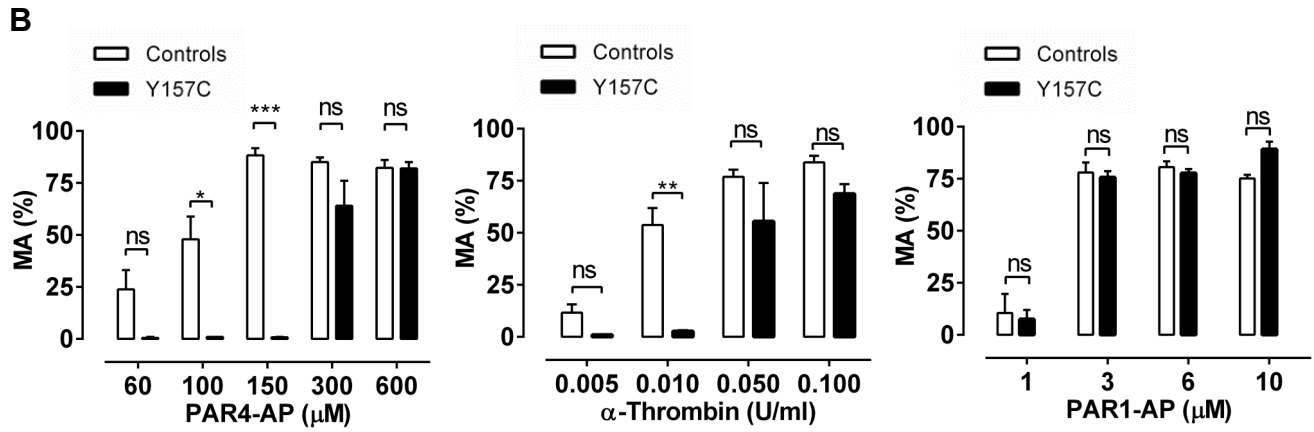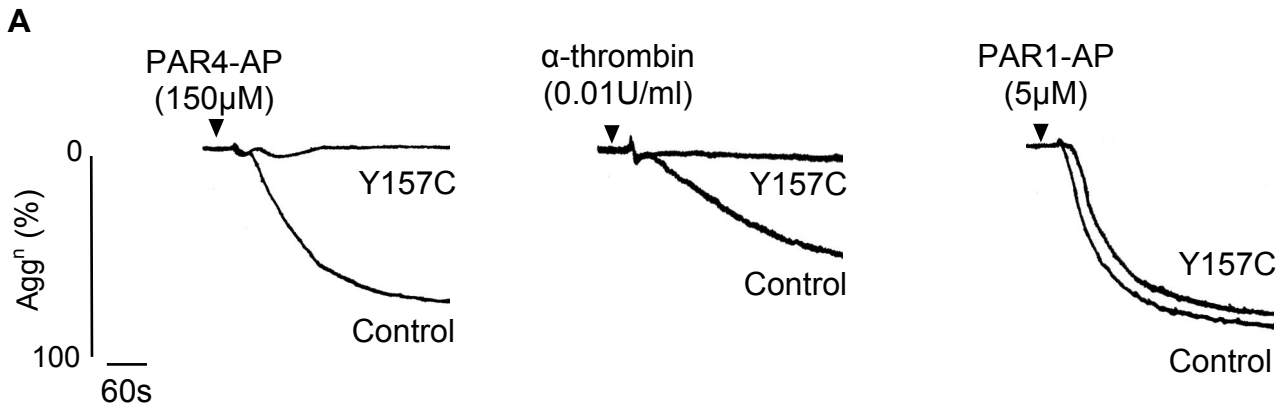**Figure 6. PAR4 Y157C, N56A and Y322A in HEK293 cells**
**(A** and **B)** Representative immunofluorescence confocal microscopy images of HEK293 cells transfected with the PAR4 Y157C or PAR4 WT CFP-fusion expression constructs. CFP is represented as green and the endoplasmic reticulum marker calnexin and early endosome antigen 1 (EEA1) as red. Areas of co-localisation are indicated by white arrows. (**C**) Co-localisation of PAR4-CFP with EEA1 or calnexin quantified by Velocity imaging software and expressed as Manders' overlap co-efficient.  Data are means ± SEM from 20 cells; *p≤ 0.05; one-way ANOVA with Dunnett's post-test. (**D** and **E)** Representative Western blots of lysates from HEK293 cells transfected with wild type (WT), Y157C, N56A or Y322A PAR4 (P4) -CFP expression constructs probed with anti-CFP or anti-tubulin antibodies. (**F**) Representative immunofluorescence confocal microscopy images of PAR4 WT, PAR4 N56A and PAR4 Y322A cells. The scale bars represent 10 µm.

**A**



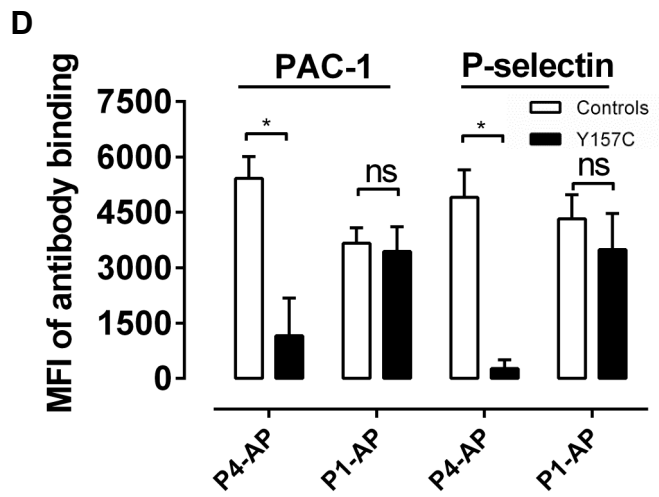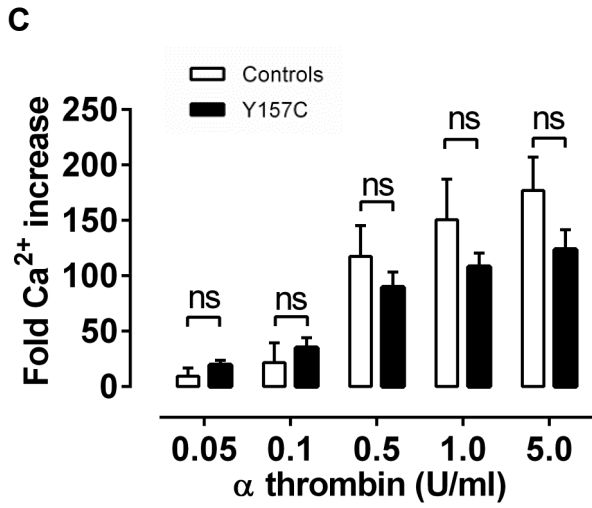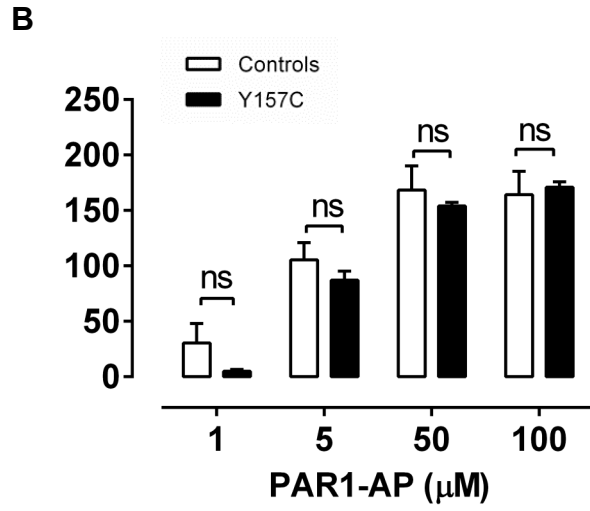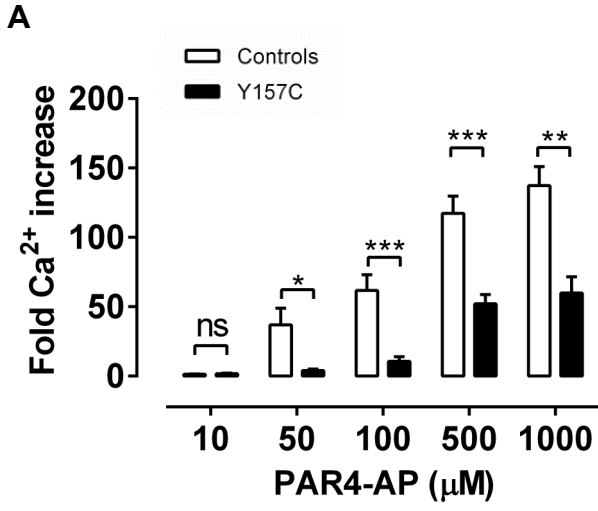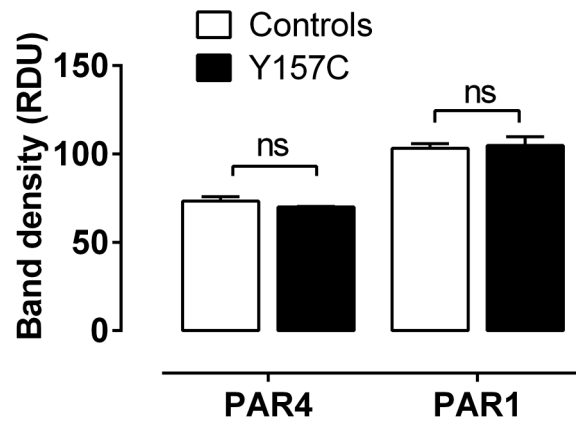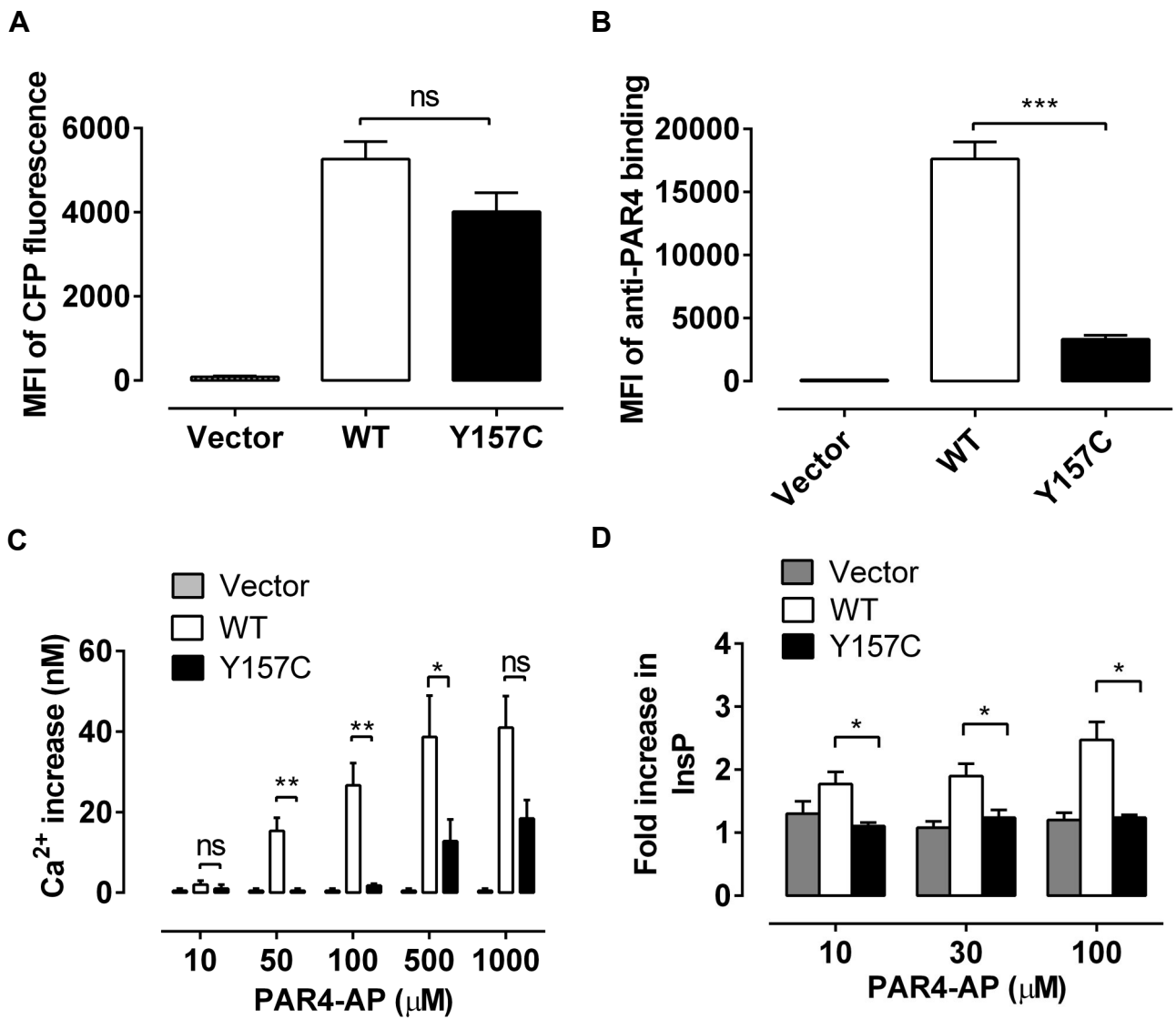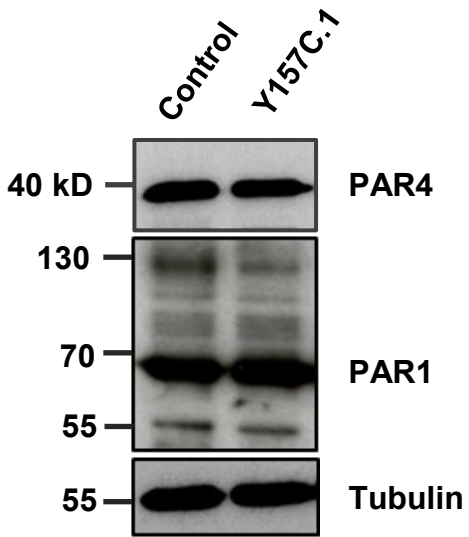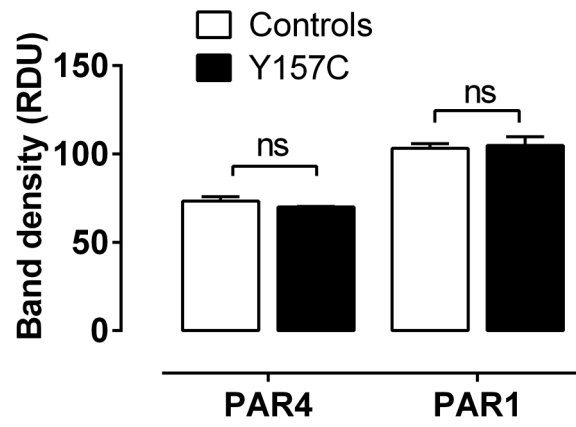| | Control | Y157C.1 | |
|---|---|---|---|
| 40 kD — | | | PAR4 |
| 130 — | | | |
| 70 — | | | PAR1 |
| 55 — | | | |
| 55 — | | | Tubulin |
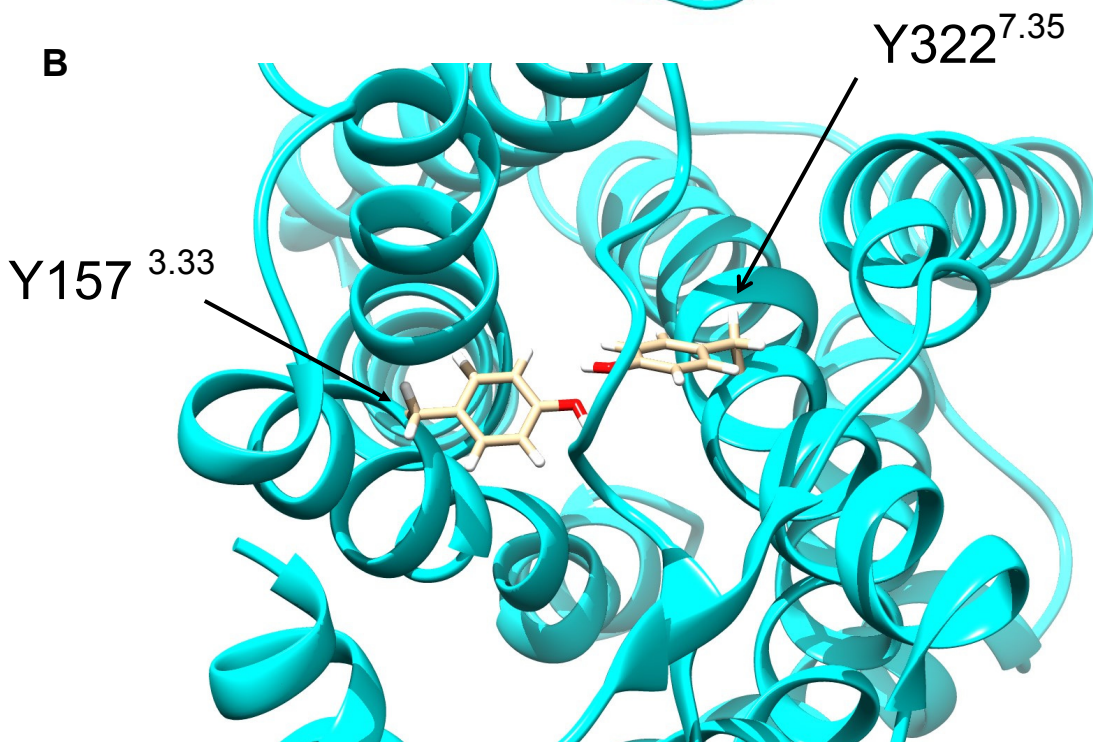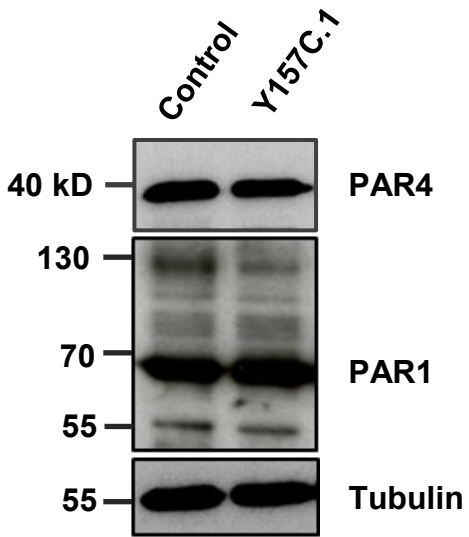
**B**

**A**



**B**

| Position | Predicted substitution | Domain (BW) | Hom cases (%) | Het cases (%) | refSNP | Minor allele frequency$^*$ | Computational annotation $^\dagger$ | Structural homology modelling |
|----------|------------------------|-------------|---------------|---------------|--------|----------------------------|-------------------------------------|-------------------------------|
| 19:16889707 | Ala82Thr | AT/TM 1 (1.37) | 0 | 1 | rs139190744 | $<10^{-3}$ | Neutral | Neutral |
| 19:16889821 | Ala120Thr | TM2 (2.48) | 9 | 50 | rs773902 | 0.22 | Neutral | Neutral |
| 19:16889933 | Tyr157Cys | TM3 (3.33) | 0 | 1 | - | $<10^{-4}$ | Deleterious | Deleterious |
| 19:16890319 | Ala286Thr | TM6 (6.39) | 0 | 1 | - | $<10^{-4}$ | Neutral | Neutral |
| 19:16890349 | Phe296Leu | TM6 (6.48) | 0 | 1 | rs2227346 | $<10^{-3}$ | Deleterious | Uncertain |
| 19:16800392 | Pro310Leu | ECL3 | 0 | 4 | rs2227376 | $<10^{-2}$ | Neutral | Neutral |
| 19:16890466 | Val335Leu | TM7 (7.48) | 0 | 1 | rs1339232968 | $<10^{-3}$ | Neutral | Neutral |

**Table 1. Missense single nucleotide variations in the *F2RL3* coding region in 236 cardiac surgery index cases.** Variants are expressed relative to ENST00000248076 and are annotated to PAR4 topological regions defined in UNIPROT dataset Q96R10 and by using Ballesteros-Wienstein (BW) generic G protein receptor nomenclature. AT-amino-terminal region; TM-transmembrane domain; ECL- extracellular loop. *Global minor allele frequency determined from the Exome Aggregation Consortium Browser [23]. $^\dagger$Computational prediction determined from the PredictSNP consensus classifier [21].