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The Protease-Activated Receptor 4 Ala120Thr Variant Alters Platelet Responsiveness to Low-Dose Thrombin, Protease-Activated Receptor 4 Desensitization and Is Blocked by Noncompetitive $P2Y_{12}$ Inhibition

Short Title: Functional Consequences of the PAR4-A120T Variant

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Essentials

1. The rs773902 SNP results in differences in platelet protease-activated receptor (PAR4) function
2. The functional consequences of rs773902 were analyzed in human platelets and stroke patients
3. rs773902 affects thrombin-induced platelet function, PAR4 desensitization, stroke association
4. Enhanced PAR4 Thr120 effects on platelet function are blocked by ticagrelor

Summary

Background: *F2RL3* encodes protease-activated receptor 4 (PAR4) and harbors an A/G SNP (rs773902) with racially dimorphic allelic frequencies. This SNP mediates an alanine to threonine substitution at residue 120 that alters platelet PAR4 activation by the artificial PAR4-activation peptide, AYPGKF.

Objectives: We determined the functional effect of rs773902 on stimulation by a physiological agonist, thrombin, and on antiplatelet antagonist activity.

Methods: Healthy human donors were screened and genotyped for rs773902. Platelet function was assessed in response to thrombin without and with antiplatelet antagonists. The association of rs773902 alleles with stroke was assessed in the Stroke Genetics Network study.

Results: Compared to rs773902 GG donors, platelets from rs773902 AA donors had increased aggregation to subnanomolar concentrations of thrombin, increased granule secretion and decreased sensitivity to PAR4 desensitization. In the presence of PAR1 blockade, this genotype effect was abolished by higher concentrations of or longer exposure to thrombin. We were unable to detect a genotype effect on thrombin-induced PAR4 cleavage, dimerization, and lipid raft localization; however, rs773902 AA platelets required 3-fold higher PAR4-AP for receptor desensitization. Ticagrelor, but not vorapaxar, abolished the PAR4 variant effect on thrombin-induced platelet aggregation. A significant association of modest effect was detected between the rs773902 A allele and stroke.

Conclusion: The *F2RL3* rs773902 SNP alters platelet reactivity to thrombin; the allelic effect requires $P2Y_{12}$, and is not affected by gender. Ticagrelor blocks the enhanced reactivity of rs773902 A platelets. PAR4 encoded by the rs773902 A allele is relatively resistant to desensitization and may contribute to stroke risk.

Keywords
Blood Platelets; Genetic Variation; Receptors, Thrombin; Stroke; Thrombin

Introduction

Human platelets express two thrombin receptors, protease activated receptor (PAR) 1 and PAR4 [1, 2]. Comparatively less research has been performed on PAR4, perhaps because it was shown early that PAR1 had a higher affinity for thrombin [3-5]. However, work from the Kuliopolos laboratory in 2000 demonstrated platelet PAR1 mediates a rapid but transient platelet calcium signaling response to thrombin, while PAR4 mediates a slower, sustained rise producing the majority of the calcium response [6]. PAR4 also plays a more important role in thrombin generation than PAR1 [7]. Compared to platelets from white individuals, platelets from black individuals are hyper-reactive when stimulated with the AYPGKF activation peptide (AP) that specifically stimulates PAR4 [8-10]. Genome-wide approaches identified a G/A single nucleotide polymorphism (SNP) in PAR4, rs773902, that accounted for approximately 50% of the racial variation in PAR4 activity [9]. The G/A SNP of rs773902 results in either an alanine (Ala) or threonine (Thr), respectively, at position 120 in the second transmembrane domain, with the threonine-containing variant being more common in Blacks than Whites (63% vs. 19% allelic frequency, respectively [9]). The reasons for differences in signaling and kinetics between PAR1 and PAR4 and between PAR4 Ala120 and PAR4 Thr120 are incompletely understood, in part due to a lack of suitable reagents and a murine model. Thus, the naturally-occurring and common functional PAR4 Ala120Thr genetic variant provides a unique opportunity to gain a deeper understanding of PAR biology and PAR1-PAR4 relationships.

Although platelet activation with the PAR4-AP is dependent on the rs773902 polymorphism, it is important to characterize the thrombin response for multiple reasons. First, thrombin is the major *in vivo* physiologic agonist for PAR4 and AYPGKF is an artificial sequence that does not occur *in vivo* [11]; furthermore, PAR signaling can differ between thrombin and PAR activation peptides [12]. PAR-APs are used at micromolar or milimolar concentrations and

do not allow assessment of relevant thrombin concentrations for PAR activation. Secondly, because thrombin functions as a platelet agonist through both platelet PAR4 and PAR1 [13], and because PAR4 has been shown to heterodimerize with PAR1 [13], it is critical to characterize thrombin-induced PAR4 signaling in human platelets expressing both PAR4 and PAR1. Lastly, acute coronary syndrome patients who are homozygous A for rs773902 exhibited less bleeding in a clinical trial of the PAR1 blocker vorapaxar [14], but a direct effect of the PAR4 variant on platelets inhibited with vorapaxar has not been studied.

The goals of the current work were to assess the effects of the PAR4 variant on stimulation by the physiological agonist thrombin, the actions of common anti-platelet agents, and the risk of *in vivo* thrombotic events. We now report the PAR4 Ala120Thr variant regulates low-dose thrombin-induced platelet aggregation and that the Thr120 isoform is relatively resistant to desensitization. In addition, we report an effect of the rs773902 genotype on risk for stroke.

Methods

Subject Recruitment and Genotyping

Healthy human donors between ages 21-60 of self-identified race from the greater Philadelphia area were screened and genotyped for rs773902 (Fig. S1) in *F2RL3* using the TaqMan SNP Genotyping Assay (Life Technologies, Carlsbad, CA). Because the rs773902 A allele is less common in the general population but more common in blacks than whites, we recruited more blacks than whites (total donors screened were 109 blacks and 75 whites). Exclusion criteria are shown in Fig. 1. Of the 184 screened donors, 17 blacks and 16 whites met inclusion criteria and were available for recall for *ex vivo* platelet studies for the duration of the study.

Platelet Aggregation, Activation and Desensitization

Washed platelets were prepared and adjusted to 2 x 10^8 platelets/mL in Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 0.485 mM NaH_2PO_4, 12 mM $NaHCO_3$, 25 mM HEPES, 1mM $MgCl_2$, 2mM $CaCl_2$, 0.1% glucose, 0.35% bovine serum albumin, pH = 7.35) as previously described [8]. Platelet aggregation was measured with a PAP-8E Aggregometer using 0.5 mg/mL arachidonic acid (Bio/Data Corporation, Horsham, PA), α-thrombin (1 nM = 0.1 U/mL; Enzyme Research Laboratories, South Bend, IN), PAR1-AP (SFLLRN), and PAR4-AP (AYPGKF) (GL Biochem Ltd, Shanghai, China). ATP release was measured from washed platelets with a Chronolog Model 700 aggregometer (Chrono-Log, Havertown PA) as per

manufacturer's instructions. P-selectin expression and calcium mobilization were measured by flow cytometry as previously described [10, 16].

Desensitization was performed as previously described [17-19], where washed platelets were incubated with 100-300 μM PAR4-AP or 10 μM PAR1-AP in the presence of 0.5 μM PGI_2 for 30 minutes, followed by aggregometry with PAR4-AP or PAR1-AP.

PAR4 Variant Studies in Heterologous Cell Lines

PAR4 Cleavage Quantification. COS7 cells from the ATCC (Manassas, VA) were transfected with pCMV-3FLAG-PAR4-Ala120 or PCMV-3FLAG-PAR4-Thr120 expression vectors validated by sequencing using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were harvested 24 hours later, incubated with thrombin for 5, 10 or 20 minutes, and analyzed with a monoclonal Anti-FLAG M2-FITC antibody (Sigma-Aldrich, St. Louis, MO) by flow cytometry.

Bioluminescence Resonance Energy Transfer (BRET)

HEK293 cells (1×10^5) were transfected with donor plasmid HA-PAR1-Luc or HA-P2Y12-Luc and increasing amounts (0-1 μg) of acceptor plasmid (PAR4-120T-GFP or PAR4-120A-GFP), stimulated with 10 nM α-thrombin for 10 min followed by 5 μM luciferase substrate (Coelenterazine 400a, Biotium Inc., Hayward, CA). Emission was detected using a Perkin Elmer Victor 3 plate reader equipped with the appropriate BRET2 filter set. BRET signal was calculated by the ratio of emission at 515 nm to emission at 410 nm minus the BRET in the absence of GFP, as previously described [20-22].

Platelet Lipid Raft Fractionation

Platelet Lipid Raft Fractionation was performed as described [23]. Untreated or stimulated (1 nM thrombin) washed platelets were lysed, fractionated on a 5%-30% sucrose gradient and centrifuged at 200,000 x *g* for 18 hours. Twelve equal fractions were collected, blotted on a nitrocellulose membrane and immunoblotted for GM1 with Cholera Toxin B subunit (CTxB, Sigma Aldrich, St. Louis, MO) or anti-PAR4 14H6 [24].

Antagonist Studies

Washed platelets were incubated with various concentrations of the PAR1 inhibitor vorapaxar (Axon Medchem, Reston, VA), the PAR4 inhibitor BMS-986120 (Cayman Chemical, Ann Arbor, MI), the $P2Y_{12}$ inhibitors Ticagrelor (Selleckchem, Houston, TX) or 2-

methylthioadenosine 5'-monophosphate triethylammonium salt hydrate (MeSAMP) (Sigma Aldrich, St. Louis, MO), or the cyclooxygenase inhibitor aspirin (ASA) (Sigma-Aldrich, St. Louis, MO), and maximal aggregation or calcium mobilization measured.

Stroke Association Study

Genotype and phenotype data were downloaded from the Stroke Genetics Network (SiGN) study, containing over 11,000 individuals from NCBI's repository via the National Center for Biotechnology Information (NCBI) database of Genotypes and Phenotypes (dbGAP) [25]. SiGN is a large international collaboration designed to detect genetic variants that predispose to subtypes of ischemic stroke. Stroke status was defined using the Trial of Org 10172 in Acute Stroke Treatment (TOAST) scale [26]. Rs773902 genotypes were extracted using PLINK v1.07 [27]. Since the control population was exclusively white ($N_{control}$ = 832) and the prevalence of the PAR4 variant differs by race [9], only data from the self-identified white cases (N_{cases} = 7338) were used to avoid confounding with race. Among these cases, 6255 had rs773902 genotype information (Table S1). Each TOAST subgroup of SiGN was analyzed for overall stroke risk. We employed multivariate logistic regression models for stroke outcome and included both categorical and continuous variables as potential contributors to stroke risk. The rs773902 A allele was considered the risk allele in a dominant model (heterozygous AG or homozygous AA), recessive model (homozygous AA) and an additive model (A allele copy number) after controlling for the stroke risk contributions of age, sex, hypertension, smoking, and diabetes status. We also examined models that included an interaction effect between diabetes and genotype for the impact on stroke risk because of prior reports indicating a relationship between diabetes and PAR4 [28] and based on our finding of an interaction between genotype and diabetes status.

Results

Platelet Aggregation to Thrombin is Enhanced with the rs773902 AA Genotype

Healthy donors were recruited, genotyped and recalled for *ex vivo* platelet studies. Prior work with 154 donors [9] and additional preliminary studies showed heterozygotes have an intermediate phenotype for platelet functional assays, so platelet function comparisons were limited to subjects homozygous for rs773902 AA or GG, encoding Thr/Thr and Ala/Ala, respectively (Fig. 1). As expected, there were more self-identified blacks with the rs773902 AA

genotype. There were no significant differences in age, gender, BMI, or hematologic parameters between the two study groups (Table 1).

Thrombin dose response curves demonstrated that platelet aggregation initiates at ~0.3 nM thrombin and that platelets from subjects with the rs773902 AA genotype had a leftward-shifted dose response curve relative to subjects with the rs773902 GG genotype (p=0.022, Fig. 2A and 2B, solid lines). The concentration of thrombin required to produce 50% maximal aggregation (EC50) was significantly lower for platelets from rs773902 AA homozygotes than rs773902 GG homozygotes (0.41 nM ±0.02 vs. 0.49 nM ±0.02 respectively, p<0.001). Because there were more females in the GG genotype group, additional analyses were performed that showed no gender effect on thrombin response (Fig. 2C). In addition, the genotype effect on the thrombin dose response curves did not vary by race (Fig. 2D and 2E). The number of platelet surface PARs did not differ by genotype (Fig. S2). When PAR-APs were used, an even greater effect of genotype was observed (Fig. S3), presumably because thrombin-induced activation of PAR1 shifts the agonist dose response curve further leftward for rs773902 GG homozygous platelets and a PAR1-mediated shift does not occur with PAR4-AP. Thus, compared to rs773902 GG homozygous platelets, rs773902 AA homozygous platelets demonstrate enhanced platelet aggregation to thrombin regardless of gender or race.

PAR4-PAR1 Interaction in Low Concentration Thrombin-Induced Platelet Aggregation

Because PAR4 has a lower affinity for thrombin than does PAR1 [3, 5, 29], it has generally been thought that PAR4 activation requires a substantially higher thrombin concentration [4]. But the data in Figure 2 using human platelets with PAR4 variants imply an effect of PAR4 at low thrombin concentrations. Consequently, we tested the contribution of PAR1 and PAR4 to thrombin stimulation following inhibition with vorapaxar and BMS-986120, respectively. We found that 400 nM BMS-986120 fully inhibited PAR4-AP aggregation in both rs773902 A (Fig. S4A) and G genotypes (data not shown). Neither BMS-986120 nor vorapaxar had an effect on activation of the non-targeted receptor by peptides (Fig. S4B and S4C).

Inhibition of PAR4 with the PAR4-specific BMS-986120 compound shifted the thrombin dose response curve of both genotypes to the right (Fig. 2B, dotted lines, p<0.0001). In the absence of PAR4 signaling, PAR1-induced platelet aggregation begins at ~0.5 nM thrombin and the EC50 increases to ~0.7 nM. These data indicate PAR4 contributes to thrombin-induced platelet aggregation at low thrombin concentrations in the range of ~0.4 nM to ~0.8 nM (i.e., the difference between the solid and dotted lines in Fig. 2B). There was no significant difference between the two genotype curves for the PAR1-mediated thrombin dose-response.

Inhibition of PAR1 with vorapaxar caused an even greater right shift in the thrombin dose response curves (Fig. 2B, dashed lines, $p<0.0001$). In the absence of PAR1 signaling, PAR4-induced platelet aggregation begins at ~0.8 nM thrombin and the average EC50 increases to ~1.0 nM. Although there was no statistically significant difference between vorapaxar-treated thrombin dose-response curves, there is a suggestion that the genotype effect may persist in the 0.8 nM-1.0 nM thrombin range. Lastly, the data in Fig. 2 also suggest that *maximal* platelet aggregation may be greater for PAR4 alone than PAR1 alone (~72% vs. ~57%).

Because GPIbα can also bind thrombin and activate PAR1 [30], we considered whether GPIbα was contributing to the low dose thrombin response. SZ2, a GPIbα specific monoclonal antibody that blocks thrombin binding [31, 32], had no effect on rs773902 AA or GG aggregation at 0.45 nM thrombin (Fig. S5), indicating that GPIbα does not contribute to the low dose thrombin response. Collectively, these data indicate that PAR4 contributes to platelet aggregation at low thrombin concentrations.

The Kinetic Effect of rs773902 on Platelet Aggregation to Thrombin

It has recently been shown that Gq and G13 activation is increased by purified PAR4 Thr120 protein compared to purified PAR4-Ala120 protein, peaking at about 2 minutes; thereafter the genotype effect is lost [33]. Therefore, we determined if there were genotype effects on time-dependent platelet aggregation. In the absence of PAR inhibitors, the genotype difference persisted for 15 min at 0.4 and 0.5 nM thrombin but was lost at 0.6 nM thrombin (Fig. 3A). Inhibition of PAR4 blocked the genotype difference at all thrombin concentrations (Fig. 3B). When PAR1 was blocked, there was an apparent genotype effect only at low concentrations (<1.2 nM) and at early time points (~200-400 sec) (Fig. 3C), an effect that is not observed at the fixed 15 min time point used in Fig 2B.

Genotypic Differences in Thrombin-Induced Feedback Mechanisms of Platelet Activation

Activation of both PAR4 and PAR1 induce granule release as a feedback mechanism to enhance and stabilize platelet aggregation, with PAR1 producing reversible aggregation, while PAR4 leads to irreversible aggregation [6, 17, 34]. We therefore considered whether the PAR4 variant might alter granule release. Compared to platelets from rs773902 GG donors stimulated with low concentrations of thrombin, platelets from rs773902 AA donors exhibited a small increase in alpha granule release ($p=0.044$, Fig. 4A) and a more substantial dense granule release ($p=0.02$ at 1 nM thrombin, Fig. 4B).

Platelets with the F2RL3 AA Genotype are Resistant to Desensitization

We considered a number of potential molecular mechanisms that might account for the PAR4 Ala120Thr-dependent difference in thrombin-induced platelet aggregation. First, COS7 cells were transfected with amino-terminally FLAG-tagged PAR4 expression plasmids for each variant, but no significant difference was observed in thrombin cleavage of the two isoforms (Fig. 5A). Second, using HEK293 cells transiently expressing PAR1 and PAR4, we did not observe differences in heterodimerization between the rs773902 AA and rs773902 GG variants as measured by BRET fluorescence (Fig. 5B). Third, because residue 120 of PAR4 is externally facing in the 2nd transmembrane region, and a Thr (polar) substitution for Ala (nonpolar) may be more likely to interact with the lipid bilayer, we considered whether the variant altered localization in lipid microdomains of platelets from different donors. Lipid raft fractions were probed by immunoblots for PAR4, but neither variant was observed to localize in the raft fraction (Fig. 5C). Lastly, PAR4 has been shown to abrogate PAR1 signaling desensitization [35], but the effect of the *F2RL3* rs773902 genotype has not been considered. As shown in Fig. 5D and 5E, rs773902 GG platelets were fully desensitized with 100 μM PAR4-AP, whereas rs773902 AA platelets were minimally affected ($p=0.03$ vs. $p=0.34$, respectively). Compared to rs773902 GG platelets, rs773902 AA platelets required a three-fold higher concentration of PAR4-AP for complete desensitization (Fig. 5F, $p<0.001$). Desensitization of PAR1 or PAR4 had no significant effect on response to the other PAR receptor (Fig. S6). Although there are limitations to the cell line assays (Figs. 5A and 5B), the simplest interpretation of these data is that the rs773902 genotype difference in thrombin sensitivity may be mediated by receptor desensitization and trafficking.

Pharmacogenetic Effect of the Ala120Thr Variant on Antiplatelet Antagonists

Current management of cardiovascular disease is based largely on clinical studies that include primarily white subjects who have a low (~20%) frequency of the rs773902 A allele [9], and we next sought to address whether there is an *ex vivo* pharmacogenetic effect of rs773902 on antiplatelet drugs. Because of inter-individual differences in thrombin EC50s (even within genotype), a thrombin dose-response was performed on each donor's platelets. Platelets from both genotypes were subsequently incubated with varying concentrations of the PAR1 inhibitor vorapaxar, and stimulated with the concentration of thrombin required to cause 80% aggregation (EC80), which averaged 0.59 nM in the 16 donors. There was no significant difference by genotype in the vorapaxar dose-response of aggregation inhibition when stimulated with an EC80 amount of thrombin (Fig. 6A, $p=0.35$). In contrast, when stimulating

platelets with a fixed dose of 1 nM thrombin, the vorapaxar IC50 was 1.8-fold higher for platelets from rs773902 AA donors relative to rs773902 GG donors (Fig. 6B, p<0.0001). These data are consistent with the vorapaxar effect shown in Figs. 2B and 3C.

Since ADP is a critical second messenger released from dense granules, the greater ATP release observed in rs773902 AA platelets (Fig. 4B) raised the possibility that these platelets may be less responsive to $P2Y_{12}$ inhibitors. Platelets from both genotypes were incubated with varying concentrations of the noncompetitive $P2Y_{12}$ inhibitor ticagrelor, and stimulated with either an EC80 amount of thrombin determined for each individual subject, or with a fixed dose of 1 nM thrombin. There was no significant difference in sensitivity to ticagrelor either at an EC80 concentration of thrombin (Fig. 6C), or at 1nM thrombin (Fig. 6D). This indicates that $P2Y_{12}$ function is important for the rs773902 genotype effect at subnanomolar thrombin concentrations. In contrast, the IC50 of the competitive $P2Y_{12}$ antagonist 2-methylthioadenosine 5'-monophosphate (2-MeSAMP) for 1 nM thrombin-induced platelet aggregation was 6-fold higher for platelets from rs773902 AA donors relative to rs773902 GG donors (Fig. S7A). The rs773902 genotype had no effect on the ability of COX inhibition by aspirin to attenuate thrombin-induced platelet aggregation (Fig. S7B).

F2RL3 Variant Association with Stroke Risk

Public data sets with genome-wide genotype data and atherothrombotic outcomes were examined to determine if individuals with the *F2RL3* rs773902 A allele had an increased risk for platelet-related ischemic clinical outcomes. SiGN was the only large study that included both relevant clinical outcome data and rs773902 genotype data [25]. Table S1 provides the number of patients by genotype and stroke subtype, totaling 6,255. Association analysis demonstrated strong positive associations with stroke risk for the correlates male sex, older age (>=75), smoking, hypertension, and diabetes across the various models, so we analyzed the rs773902 A allele contribution to risk after controlling for these factors as well as an interaction for diabetes status. Table 2 shows that the rs77902 A allele was associated with an increased stroke risk (all TOAST categories) (Odds Ratio increase of 1.166 for each additional copy of A, CI [1.006-1.356], p=0.044); in addition, large artery stroke showed an Odds Ratio increase of 1.232 for each additional copy of A, CI [0.997-1.524], p=0.053. A full listing of the regression models and results are in Table S2, and show the rs773902 A allele demonstrated a modest but consistent pattern of trending for stroke risk across the three dominant, recessive or dose-dependent genetic models.

Discussion

Compared to the platelet PAR1 receptor, the clinical and functional importance of PAR4 has been less well evaluated. In this report we utilize a common and naturally occurring PAR4 functional variant to better define the role of PAR4 in thrombin-induced platelet activation. The major findings in these studies are: (1) The *F2RL3* rs773902 SNP alters *in vitro* platelet reactivity at subnanomolar concentrations of thrombin and at early time points, but genotype differences are overcome at higher thrombin concentrations and longer time points. This indicates both PAR1 and PAR4 contribute to a subnanomolar thrombin response; (2) the enhanced rs773902 AA reactivity is eliminated by $P2Y_{12}$ antagonism with ticagrelor, and at low thrombin concentrations, by vorapaxar; and (3) rs773902 AA platelets are more resistant to receptor desensitization. In addition, we provide evidence that the rs773902 A allele is associated with a risk of stroke in white patients. As with other functional genomic variants [36, 37], rs773902 has the same *in vitro* effect regardless of self-identified race (Fig. 2D-E). Since the frequency of the rs773902 A allele is >3 times higher in blacks of Sub-Saharan East African ancestry than whites, the allele frequency may contribute to racial disparities in the outcomes of cardiovascular disease.

Most studies using heterologous cell systems have shown 1-2 orders of magnitude higher thrombin concentration are required to induce signaling through PAR4 than PAR1 [3, 4]. This, coupled with the lack of a high affinity binding site for α-thrombin [4], have supported the idea that PAR4 cleavage and activation only occur at high thrombin concentrations. Using human platelets, we find that 0.4-0.5 nM thrombin achieves 50% aggregation (EC50) of washed platelets, a finding similar to that reported by Leger et al. [13]. Importantly, the significantly different EC50s between the two PAR4 variants indicates a PAR4 role in platelet aggregation at the steep part of the thrombin dose-response curve. When PAR1 is inhibited by vorapaxar, the thrombin dose-response and time-response data supports persistent genotype effect only at early time points with low concentrations of thrombin stimulation (Fig. 2B, 3C). These thrombin-stimulated platelet aggregation data are consistent with faster G protein binding kinetics demonstrated with purified PAR4 variant proteins pre-incubated with PAR4-AP [33]. Although *in vivo* concentrations of thrombin in the local microenvironment of a forming thrombus are unknown, it is believed that there is a concentration gradient of agonists from the tightly packed core of the thrombus to the outer shell [38-42]. One can imagine that as the thrombin concentrations decrease along this gradient, there is a region where thrombin will activate

rs773902 AA platelets, but not rs773902 GG platelets leading to differences in the size and structure of the thrombus.

The genotype effect on agonist dose response appeared greater for PAR4-AP than thrombin (Figs. 2B and S2). The simplest explanation is that thrombin activation of PAR1 induces left-shift in the curve that overcomes the relative "hypo-reactivity" of rs77902 GG platelets. However, other possible explanations include different PAR4 conformations stabilized by the tethered ligand compared to the activation peptide, which indices coupling to different G proteins [12]. Differential genotype-mediated dimerization with other GPCRs could also signal differently in response to the tethered ligand or activation peptide.

Initial mechanistic studies showed similar surface expression of PAR4, receptor cleavage, dimerization and lipid raft localization between the two *F2RL3* genotypes, and no effect of GPIbα, although deeper investigation into these mechanisms may be warranted. However, significant differences in PAR4 desensitization were observed. It is well-established that stimulation of the PAR receptors in the presence of an inhibitor of aggregation results in "desensitization" of that receptor [43, 44]. Such desensitization has been used as a tool to uncouple PAR4 from PAR1 signaling in response to thrombin, and has been a valuable approach to study post-PAR cleavage signaling events [13, 35, 45, 46]. While the exact mechanism of PAR desensitization is not fully understood, it is thought to involve a combination of post-translational modification and internalization to prevent further downstream signaling. It was recently shown that PAR4Thr120-P2Y$_{12}$ heterodimers mediate PAR4 internalization and enhanced signaling in heterologous cell lines [47, 48]. Because secreted ADP enhances PAR-induced platelet signaling and activation [49], we hypothesized that the Thr120 variant favors PAR4-P2Y$_{12}$ heterodimer formation. Such a mechanism would be consistent with our observed increased dense granule release in Thr120 platelets (Fig. 4B), as well as the inability of the ADP competitive inhibitor 2-MesAMP to overcome the genotype effect of low-dose thrombin activation (Fig. S7). Although co-immunoprecipitation experiments showed increased PAR4-P2Y$_{12}$ heterodimerization in Thr120 expressing HEK293 cells, similar BRET studies did not demonstrate a genotype effect (Fig. S8-S9). Perhaps our experimental conditions did not support a BRET-identified PAR4 variant effect and additional work is needed to address whether Thr120 variant favors PAR4-P2Y$_{12}$ heterodimer formation and to more accurately define a role for ADP and P2Y$_{12}$ in the hyperreactive PAR4 Thr120 phenotype. Nevertheless, our data using a non-competitive P2Y$_{12}$ inhibitor (ticagrelor) demonstrates a *functional* (if not physical) interaction between PAR4 and P2Y$_{12}$ that differs by the PAR4 variant.

There are many genomic studies considering associations between vascular ischemic events and platelet gene variants, but few of these were performed with a genotyping platform with good coverage of common variants in PAR4. Our analysis of the SiGN cohort found that individuals bearing the rs773902 A allele show a pattern of greater risk for ischemic stroke. Although our report is the largest study to date of the *in vitro* effects of thrombin on the PAR4 Ala120Thr variant, a larger sample size would enable consideration of other confounding genetic or demographic variables. However, the small but significant association between the rs773902 A allele and ischemic stroke seen in the ~7000 stroke reported in our study (Table 2) and the reduction in bleeding associated with the rs773902 AA genotype seen in ~7000 ischemic coronary patients receiving anti-platelet therapy [14] provide important support for the *in vivo* importance of this genetic variant. Together with our functional data, these clinical association signals warrant further study, including a rs773902-diabetes interaction.

The importance of understanding the physiological effects of the common PAR4-A120T variant is underscored by the critical role of thrombin in platelet thrombus formation and the recent interest in PAR4 inhibition as a therapeutic strategy (e.g., BMS-986141 and BMS-986120) [50-52]. Our results have demonstrated the rs773902 genotype alters *ex vivo* thrombin-induced platelet reactivity, affects receptor desensitization and is associated with a modest stroke risk. These results have potential clinical significance in atherothrombotic disease where the PAR4 variant could modify ischemic or hemorrhagic outcomes despite currently used anti-platelet agents. It will be important for future anti-platelet clinical trials to test for pharmacogenetic interactions with the PAR4 variant and to develop anti-platelet therapies with good efficacy against platelets expressing the rs773902 A allele (predominantly black patients). PAR4 blockade may be beneficial in this regard if the novel compounds are not sensitive to rs773902 genotype effects at low-dose thrombin-induced platelet reactivity. Alternatively, combination anti-platelet therapy that includes a non-competitive, allosteric antagonist of $P2Y_{12}$ may be beneficial.

Addendum

P. F. Bray, L. C. Edelstein and C. A. Shaw designed the overall experimental approach. M. J. Whitley, J. Vesci, M. Stoller, and M. Nieman designed and performed experiments and collected data. D. M. Henke, A. Ghazi and C. A. Shaw performed the statistical genetic analyses. M. J. Whitley, D. M. Henke, A. Ghazi, E. Chen, L. M. Simon, M. Nieman, M. Holinstat, S. E. McKenzie, C. A. Shaw, L. C. Edelstein and P. F. Bray interpreted data. M. J. Whitley, M.

Stoller, C. A. Shaw, L. C. Edelstein, and P. F. Bray drafted the manuscript. M. J. Whitley, M. Nieman, C. A. Shaw, L. C. Edelstein, and P. F. Bray performed critical revision of the manuscript.

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Disclosure of Conflicts of Interest

P. F. Bray, L. C. Edelstein, and M. Holinstat are investors on U.S. Patent 9,789,087, "PAR4 Inhibitor Therapy for Patients with PAR4 Polymorphism." The other authors state that they have no conflict of interest.

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Table 1. Subject Demographics

	AA (n=15)	GG (n=18)	P-value
Age (years, mean ± SD)	41 ± 14	39 ± 13	0.51*
Female (%)	47	83	0.06†
Black race (%)	80	28	0.005†
BMI (kg/m^2, mean ± SD)	30 ± 8	29 ± 6	0.49*
Hb (g/dL, mean ± SD)	11.5 ± 1.6	11.9 ± 1.3	0.49*
MVP (fl, mean ± SD)	10.1 ± 1.8	10.1 ± 1.4	0.98*

WBC (x1000/uL, mean ± SD)	5.5 ± 1.5	5.8 ± 1.6	0.57*
Platelet count (x1000/uL, mean ± SD)	293.5 ± 68	321.7 ± 85	0.31*

Complete blood cell counts were obtained using a Drew Scientific Group Hemovet 950FS (Miami Lakes, FL).

* Students T-test

† Fisher's exact T-test

Table 2. Relationship of rs77902 A to stroke, dosage-dependent model*

Stroke subset	Odds Ratio (95% C.I.)	P value
All stroke	1.166 (1.006 - 1.356)	0.044
Cardioembolic	1.146 (0.954 - 1.379)	0.146
Large Artery Atherosclerosis	1.232 (0.998 - 1.524)	0.053
Small Vessel Occlusion	1.146 (0.946 - 1.390)	0.164
Other	1.105 (0.934 - 1.309)	0.248

* A dose-dependent model is presented here because the *in vitro* platelet function studies showed a dosage effect of the rs77902 A allele.

Figure Legends

Figure 1. Subject Recruitment and Exclusion Criteria. Healthy subjects were recruited and genotyped for rs773902. Written informed consent was obtained from all participants with the approval of the institutional review board of Thomas Jefferson University in Philadelphia, PA. This flow diagram illustrates who was excluded for recall for the laboratory platelet studies. Failure to aggregate to arachidonic acid was assumed to reflect NSAID use; "healthy" was arbitrarily defined as the use of no more than one prescription medication not known to affect platelet function; 37 were lost to follow-up after genotyping or otherwise not able to be recalled. Of the 33 eligible subjects identified during the 3-year study, at least 20 were available for recall at any given time.

Figure 2. Thrombin dose-response curves for aggregation of rs773902 AA and GG platelets. (**A)** Washed platelets were treated with indicated concentrations of thrombin. Representative aggregation tracings for rs773902 GG homozygotes (left) and rs773902 AA homozygotes (right). **(B-E)** Maximal aggregation (± SEM) assessed at 15 minute fixed time point. **(B)** Combined data for all subjects allowing comparisons among aggregations with no

inhibitor (solid lines), PAR4 inhibition with 400 nM BMS-986120 (dotted line) and PAR1 inhibition with 100 nM vorapaxar (dashed line). Genotype curves were not significantly different after BMS-986120 (P=0.69) or vorapaxar (P=0.53). **(C)** Thrombin dose-response by gender. Effect of race on thrombin dose-response by rs773902 GG **(D)** and AA **(E)** genotypes. Note that the numbers of white rs773902 AA subjects are small because they represent only ~4% of the population, and the numbers of black rs77902 GG subjects are small because they represent only ~6% of the population. Genotype differences between thrombin dose response curves were measured by repeated measure ANOVA.

Figure 3. Kinetics of thrombin-induced aggregation of rs773902 AA and GG platelets. Platelet aggregation shown as mean +/- SEM at different fixed thrombin concentrations but plotted over time. Sampling was performed every 0.5 seconds.

Figure 4. Thrombin-induced granule secretion. Washed platelets were stimulated with the indicated concentrations of thrombin. **(A)** P-selectin was quantified as the percentage of maximal mean fluorescent intensity (MFI) by flow cytometry relative to the MFI of the highest concentration of thrombin. **(B)** ATP release was measured using a Chronolog aggregometer.

Figure 5. Assessment of potential mechanistic effects of rs773902 genotypes. (A) COS7 cells were transfected with FLAG-tagged PAR4 expression vectors for rs773902 A or rs773902 G and incubated with varying concentrations of thrombin. Loss of surface FLAG expression was detected by flow cytometry as a percent of baseline (% max ± SEM). **(B)** HEK293 cells were transfected with HA-PAR1-rLuc (0.5 µg) and PAR4-120T-GFP or PAR4-120A-GFP (0-1 µg), treated with α-thrombin (10 nM) and analyzed for GFP expression, luciferase activity, and bioluminescence resonance energy transfer (BRET). The data are from three independent experiments in which all points were analyzed by global fit to a hyperbolic fit to determine the maximum BRET ($BRET_{max}$: Ala120 0.19 ± 0.05 vs. Thr120 0.22 ± 0.03). The specificity of the interactions determined by BRET were confirmed using rhodopsin (data not shown). (**C)** Platelet lipid raft isolation and immunoblotting for PAR4 and GM1. A representative blot is shown as well as combined data indicating the percent of the total protein per fraction (% total ± SEM). **(D)** Representative tracings for rs773902 GG (left) and AA (right) platelets desensitized with 100 µM PAR4-AP and stimulated with 200 µM PAR4-AP. **(E)** Combined desensitization data for all subjects (Max aggregation % ± SEM). **(F)** rs773902 AA platelets were desensitized

with 100 μM, 200 μM, or 300 μM PAR4-AP and stimulated with 200 μM PAR4-AP (Max aggregation % ± SEM).

Figure 6. Effect of rs773902 genotype and PAR1 and $P2Y_{12}$ inhibition on thrombin-induced platelet aggregation. Washed platelets were incubated with varying concentrations of vorapaxar and stimulated with **(A)** a thrombin EC80 (AA range 0.45 – 0.6 nM; mean: 0.54 nM, GG range 0.45 – 0.55 nM; mean: 0.49 nM), or **(B)** 1 nM thrombin, and percent maximum aggregation ± SEM recorded. A similar experiment was performed with the allosteric $P2Y_{12}$ inhibitor, ticagrelor using **(C)** a thrombin EC80 or **(D)** 1 nM thrombin. IC50s were calculated for ticagrelor and vorapaxar for rs773902 genotypes (mean ± SD). One nM thrombin was chosen since this was the lowest concentration causing the maximal aggregation plateau (Fig. 2B), and would be expected to be sensitive to the effects of ADP. P-values were determined with Students t-test **(B)** and by extra sum-of-squares F-test for the differences in IC50s.

Fig. 1

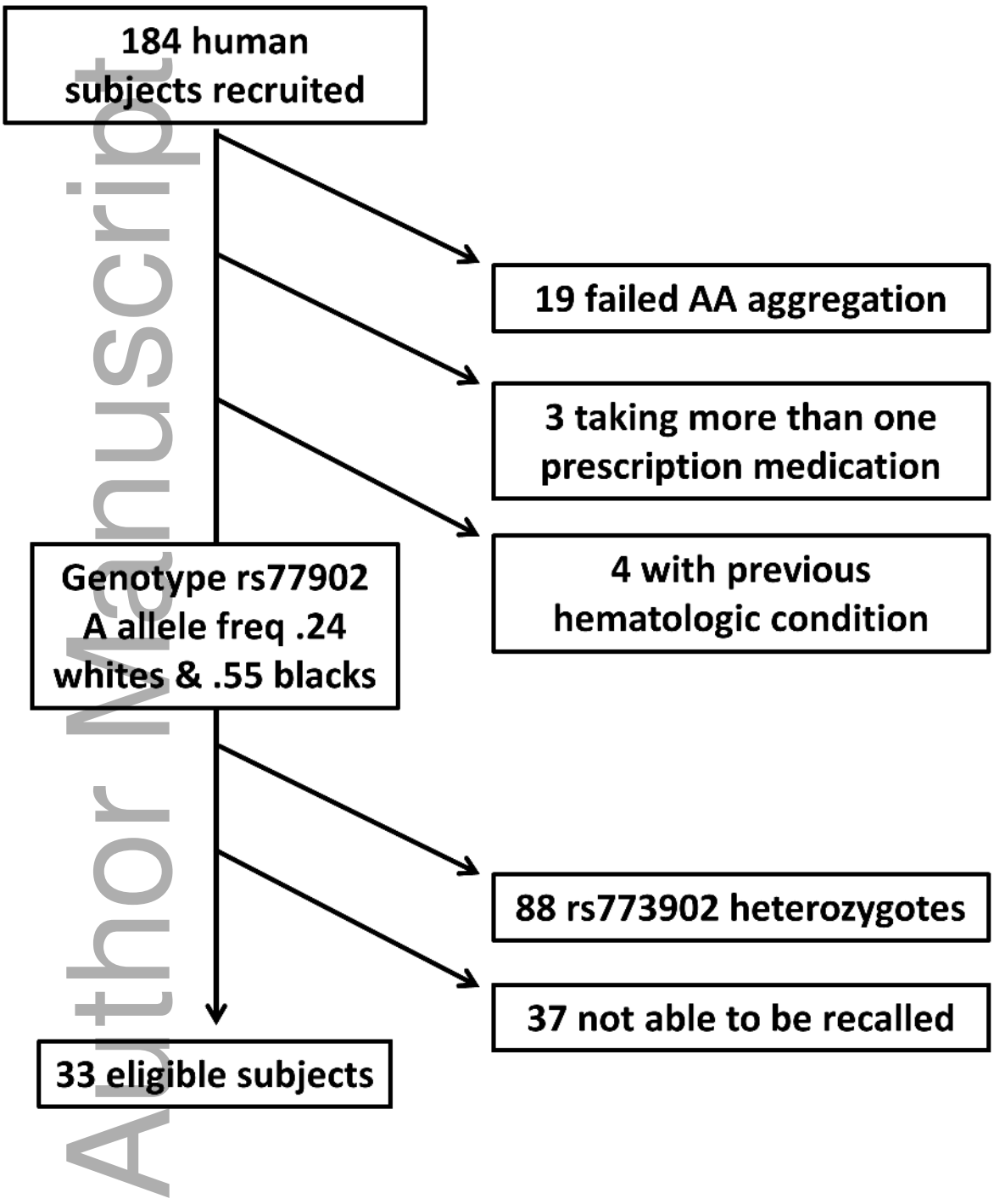


Fig. 2

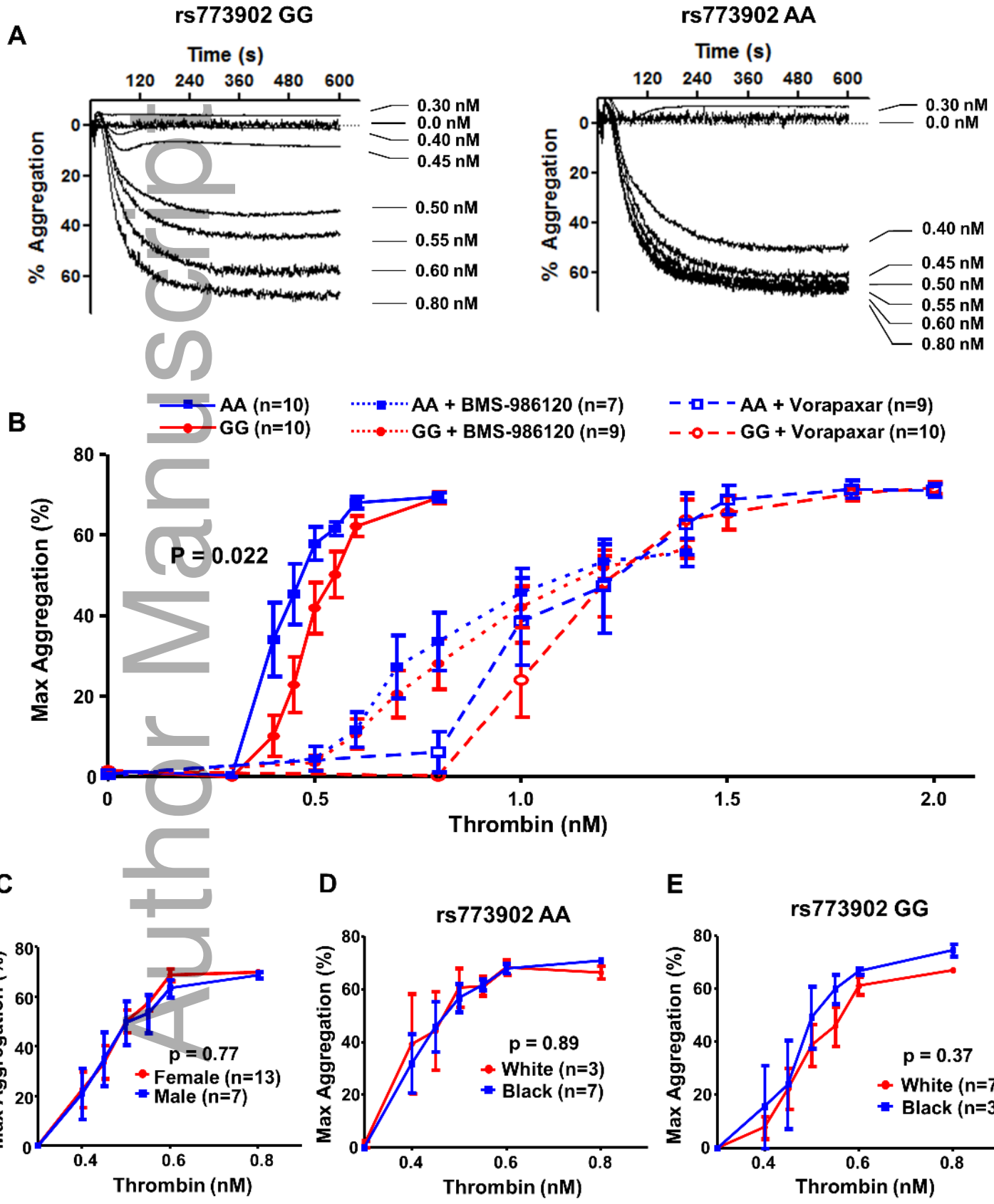


Fig. 3

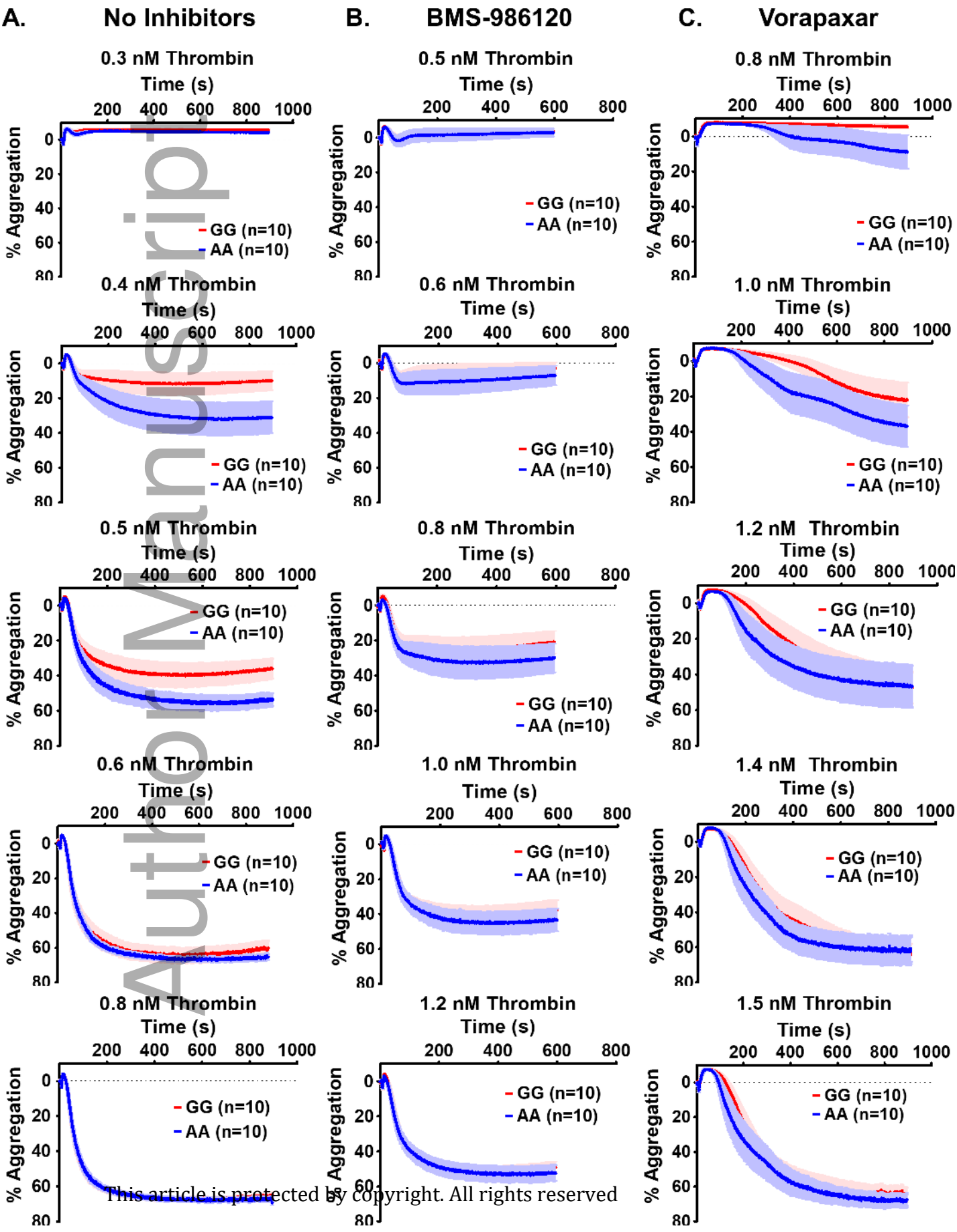


Fig. 4

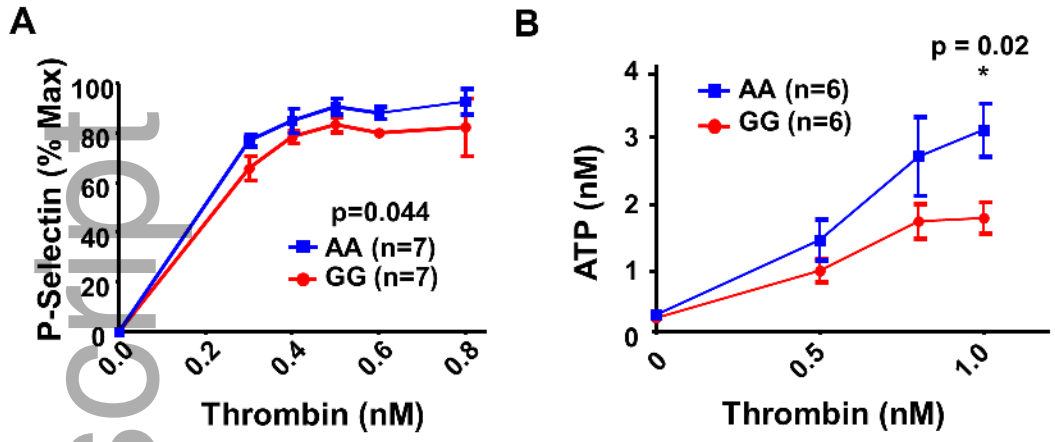


Fig. 5

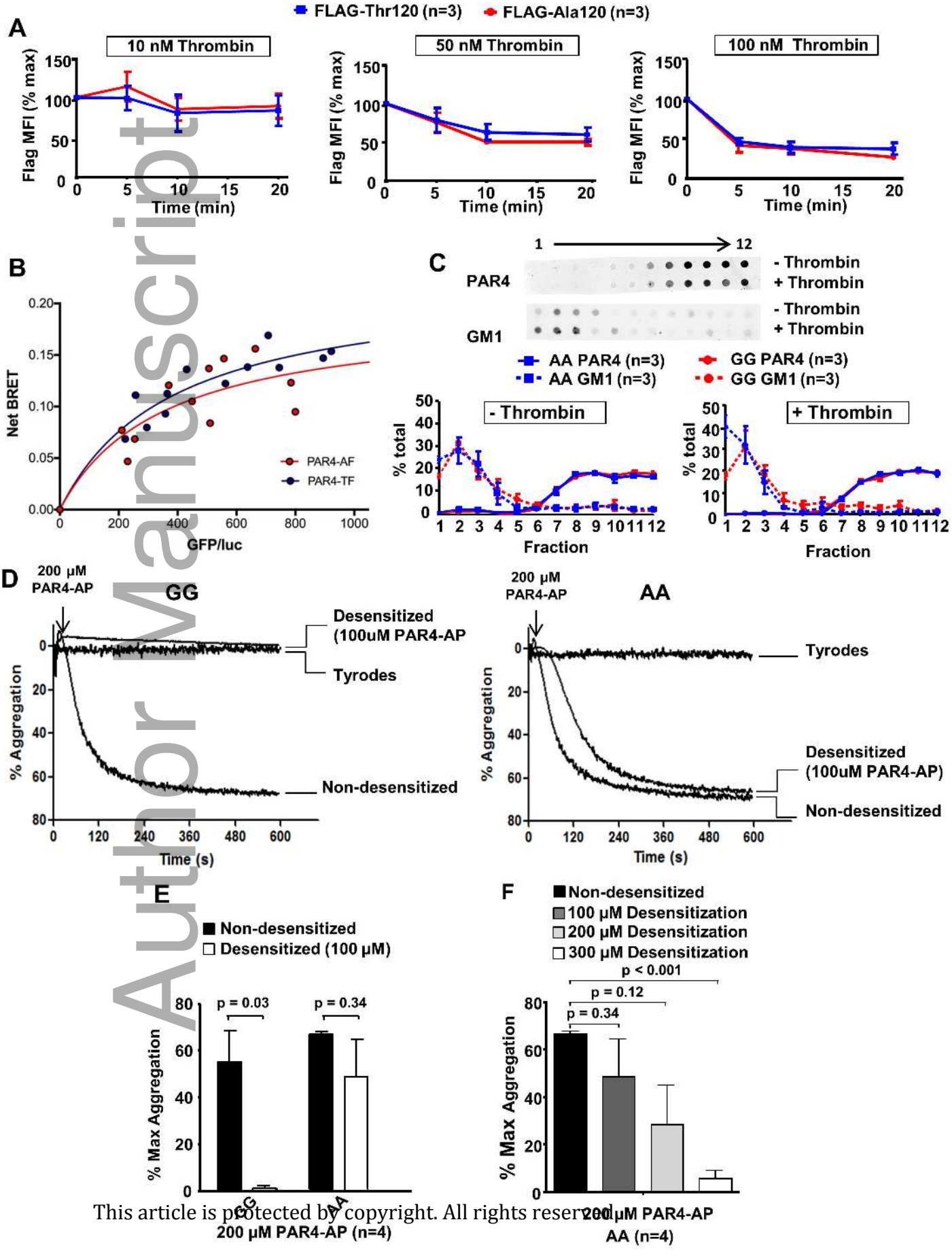
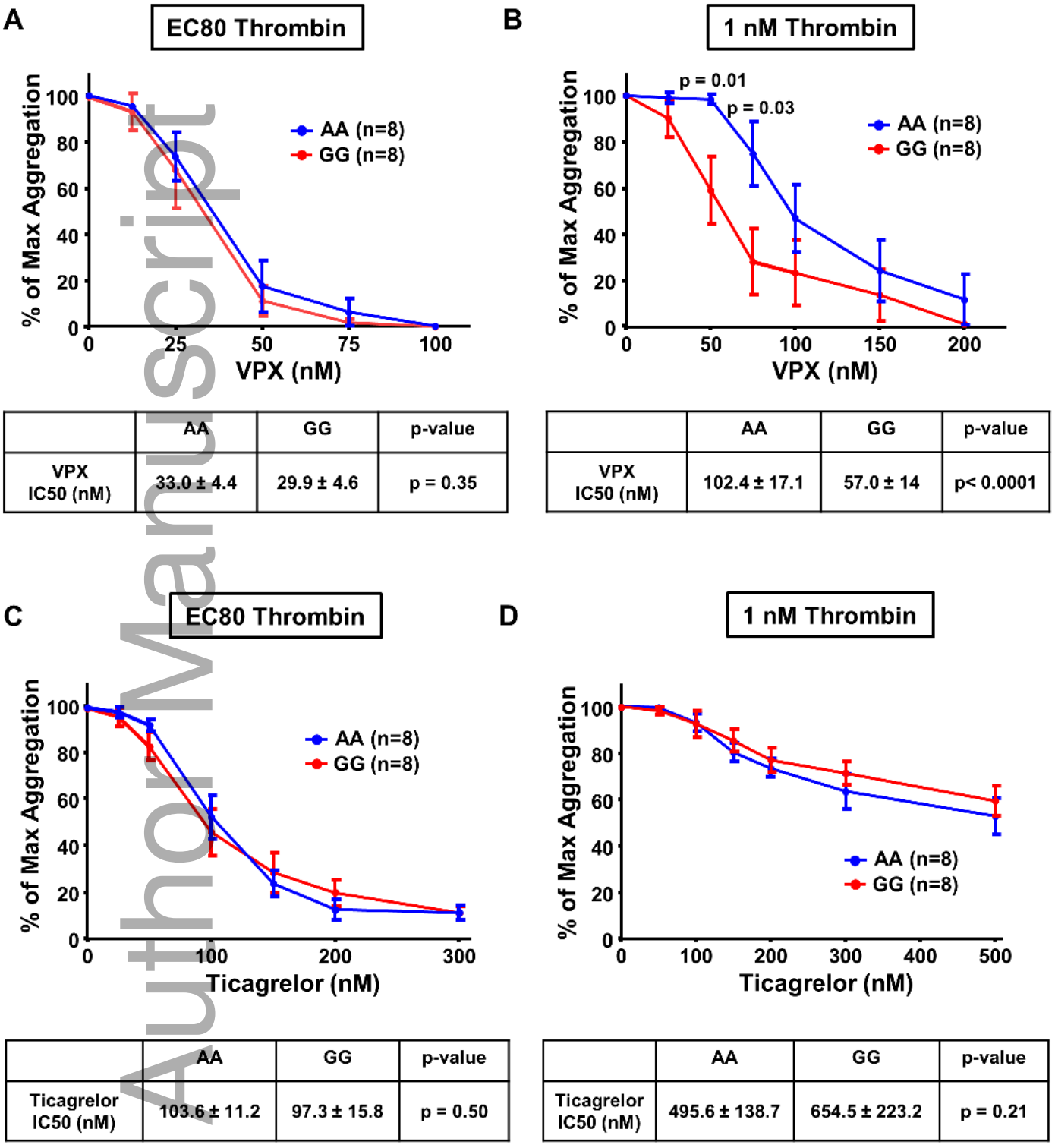


Fig. 6



	AA	GG	p-value
VPX IC50 (nM)	33.0 ± 4.4	29.9 ± 4.6	p = 0.35

	AA	GG	p-value
VPX IC50 (nM)	102.4 ± 17.1	57.0 ± 14	p< 0.0001

	AA	GG	p-value
Ticagrelor IC50 (nM)	103.6 ± 11.2	97.3 ± 15.8	p = 0.50

	AA	GG	p-value
Ticagrelor IC50 (nM)	495.6 ± 138.7	654.5 ± 223.2	p = 0.21