

ARTICLE

Prospective Evaluation of Genetic Variation in Platelet Endothelial Aggregation Receptor 1 Reveals Aspirin-Dependent Effects on Platelet Aggregation Pathways

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Genetic variation in the platelet endothelial aggregation receptor 1 (*PEAR1*) gene, most notably rs12041331, is implicated in altered on-aspirin platelet aggregation and increased cardiovascular event risk. We prospectively tested the effects of aspirin administration at commonly prescribed doses (81, 162, and 324 mg/day) on agonist-induced platelet aggregation by rs12041331 genotype in 67 healthy individuals. Prior to aspirin administration, rs12041331 minor allele carriers had significantly reduced adenosine diphosphate (ADP)-induced platelet aggregation compared with noncarriers ($P = 0.03$) but was not associated with other platelet pathways. In contrast, rs12041331 was significantly associated with on-aspirin platelet aggregation when collagen and epinephrine were used to stimulate platelet aggregation ($P < 0.05$ for all associations), but not ADP. The influence of *PEAR1* rs12041331 on platelet aggregation is pathway-specific and is altered by aspirin at therapeutic doses, but not in a dose-dependent manner. Additional studies are needed to determine the impact of *PEAR1* on cardiovascular events in aspirin-treated patients. *Clin Transl Sci* (2017) 10, 102–109; doi:10.1111/cts.12438; published online on 11 January 2017.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✓ *PEAR1* rs12041331 has been previously implicated in altered baseline and on-aspirin platelet aggregation. In addition, the impact of genotype on cardiovascular disease risk seems to be aspirin-dependent.

WHAT QUESTION DID THIS STUDY ADDRESS?

✓ We sought to prospectively evaluate the influence of *PEAR1* rs12041331 on platelet function in an aspirin dose-specific and aggregation pathway-specific manner.

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE?

✓ The platelet aggregation pathways impacted by *PEAR1* rs12041331 are changed by exposure to aspirin, whereby ADP-induced platelet aggregation is influenced prior to drug administration, whereas collagen-induced

and epinephrine-induced platelet aggregation are influenced during aspirin treatment. Furthermore, no genotype-specific differences in platelet function were observed with increasing doses of aspirin.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE

✓ *PEAR1* is a critical signaling component of several pathways that regulate platelet aggregation at baseline and in the context of antiplatelet therapy. Better understanding of the mechanisms by which *PEAR1* influences platelet function may facilitate novel drug-target discovery and influence the way patients with coronary artery disease on aspirin are treated.

Dual antiplatelet therapy with aspirin and clopidogrel is the most commonly used regimen for management of patients with acute coronary syndromes undergoing percutaneous coronary intervention. Although generally effective in most patients, significant interindividual variability in dual antiplatelet therapy response has been well documented, leading to suboptimal therapy in some patients, and

resulting in an increase in cardiovascular event rates.^{1–4} In recent years, substantial effort has been made to identify pharmacogenetic determinants of variable antiplatelet therapy response. Indeed, these investigations have convincingly shown that response to clopidogrel is significantly influenced by genetic variation, most notably *CYP2C19**2, which resulted in an update to the clopidogrel label by the US

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Food and Drug Administration in 2010.³ In contrast, identification of genetic factors that influence aspirin response has remained difficult despite the fact that up to 77% of the variation in aspirin response, as assessed by *ex vivo* platelet aggregometry, is heritable.⁵

One of the few recently identified aspirin response candidate genes is platelet endothelial aggregation receptor 1 (*PEAR1*), which encodes a type 1 membrane protein that has been implicated in megakaryopoiesis, thrombopoiesis, and stabilization of platelet aggregation through enhancement of platelet α IIb β 3 activation.⁶⁻⁸ To date, several retrospective investigations have reported that genetic variation in *PEAR1* significantly influences agonist-induced platelet aggregation at baseline and after aspirin exposure.^{4,9-13} The most consistently and highly associated of these genetic variants is rs12041331, an intronic variant that is believed to modify *PEAR1* expression.^{4,9-11,13} The effects of aspirin on platelet aggregation according to rs12041331 genotype have not been studied prospectively. In this investigation, we prospectively tested the effects of commonly prescribed aspirin doses (81, 162, and 324 mg/day) on agonist-induced platelet aggregation according to rs12041331 genotype in 67 healthy individuals using a fixed-sequence, crossover design. *Ex vivo* platelet reactivity testing was completed using multiple platelet agonists to gain insights regarding the mechanism(s) by which *PEAR1* influences basal and on-treatment platelet reactivity. Urinary thromboxane B₂, a circulating biomarker of platelet function and aspirin responsiveness, was also assessed by *PEAR1* genotype in order to complement our platelet aggregation results.

MATERIALS AND METHODS

Subjects

We recruited 67 relatively healthy Amish individuals from Lancaster, Pennsylvania, who had previously participated in the Heredity and Phenotype Intervention (HAPI) Heart study,¹⁴ the Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study,³ and targeted family members by known or predicted *PEAR1* rs12041331 genotype between August 2013 and November 2015. Enrolled subjects were rescreened to ensure they met the eligibility criteria, which were identical as those for the original PAPI study.³ The protocol was approved by the University of Maryland, Baltimore Institutional Review Board, and all subjects provided written informed consent.

Study design

The study design used in this investigation is shown in **Figure 1**. Study procedures were performed at the University of Maryland Amish Research Clinic in Lancaster, PA. All participants safely withdrew from medications, vitamins, and supplements at least 1 week prior to, and for the duration of, the study. Any participant who was taking aspirin withdrew from this medication at least 14 days prior to protocol initiation.

During the initial clinic visit, blood samples were obtained from each participant for baseline measures of platelet aggregation and for specimen banking. After samples were obtained, participants received an 81 mg chewable dose of nonenteric-coated aspirin and instructed to take 81 mg/day for the next 6 days. Subjects returned to the clinic on the

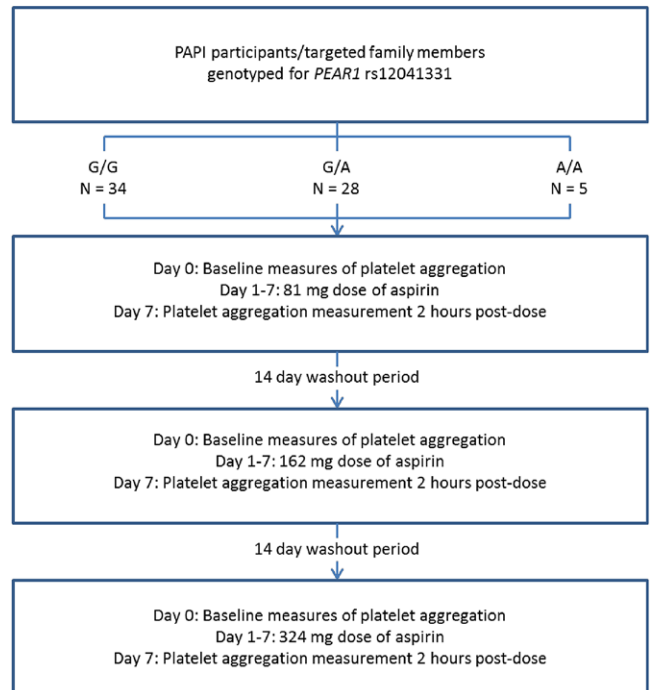


Figure 1 Each study participant underwent 7-day aspirin interventions at a low (81 mg/day), medium (162 mg/day), and high aspirin dose (324 mg/day), with a minimum 2-week washout in between each aspirin regimen. Platelet aggregation was measured at day 0 prior to the first aspirin dose and on day 7 following the final aspirin dose of each treatment period. Blood and urine samples were also obtained at day 0 and day 7 of each treatment period. PAPI, Pharmacogenomics of Anti-Platelet Intervention; *PEAR1*, platelet endothelial aggregation receptor 1.

7th day, on-aspirin measures of platelet aggregation were recorded 2 h after the last 81 mg dose, and samples were banked for future analysis. At this time, participants underwent a washout period of at least 14 days to allow platelet function to return to basal levels, consistent with the average lifespan of platelets (range, 7–10 days).¹⁵ After the washout period, these procedures were repeated for a second time with 162 mg/day of aspirin for 7 days. Again, platelet function was assessed and samples were banked pre-aspirin and post-aspirin administration. Participants then underwent another 14-day washout period. These procedures were repeated for a third time with 324 mg/day of aspirin for 7 days, with platelet function testing and specimen banking pre-aspirin and post-aspirin administration. Medication adherence was confirmed during the study through use of history logbooks and pill counts. Clinic staff was blinded to *PEAR1* genotype throughout the course of the study. Banked samples were stored at -80°C until used.

Genotyping

All subjects were recruited based on known or predicted rs12041331 genotype, which had previously been measured as part of the HAPI Heart or PAPI study for most participants.^{3,14} Genotype of each participant was validated by repeat genotyping of rs12041331 using a TaqMan single nucleotide polymorphism genotyping assay (Applied

Table 1 Characteristics of study participants

Trait, units	Total	PEAR1 rs12041331 genotype			P value ^a
		G/G	G/A	A/A	
Number	67	34	28	5	
Age ± SD, year	50.2 ± 1.2	49.7 ± 1.7	51.6 ± 1.9	45.4 ± 4.6	0.86
Sex (n = Female) (%)	28 (42)	15 (44)	10 (36)	3 (60)	0.96
BMI ± SD (kg/m ²)	28.4 ± 0.6	28.0 ± 0.9	29.1 ± 0.9	27.4 ± 2.2	0.41
Hypertension ^b (%)	4 (6)	2 (6)	2 (7)	0 (0)	0.83
Hypercholesterolemia ^c (%)	18 (27)	9 (26)	9 (32)	0 (0)	0.6
Self-reported diabetes (%)	0	0	0	0	N/A
Current smoker (%)	7 (10)	3 (9)	4 (14)	0 (0)	0.96
Hematocrit ± SD (%)	40.3 ± 0.4	40.3 ± 0.5	40.3 ± 0.6	39.9 ± 1.0	0.85
White blood count ± SD (n × 1,000/ μ l)	5.8 ± 0.2	5.5 ± 0.2	6.1 ± 0.3	5.8 ± 0.6	0.12
Platelet count ± SD (n × 100,000/ μ l)	223.1 ± 5.8	206.7 ± 7.3	243.8 ± 8.8	219.2 ± 20.8	0.03

A/A, minor allele homozygotes; BMI, body mass index; G/A, heterozygotes; G/G, major allele homozygotes; N/A, not applicable; *PEAR1*, platelet endothelial aggregation receptor 1.

^aP values represent statistical difference of each trait by *PEAR1* rs12041331 genotype. ^bDefined as systolic blood pressure greater than 140 mm Hg or diastolic blood pressure greater than 90 mm Hg or taking prescription medication for previously diagnosed hypertension. ^cDefined as low-density lipoprotein cholesterol >160 mg/dL or taking prescription medication for previously diagnosed hypercholesterolemia.

Biosystems, Foster City, CA), according to the manufacturer's instructions. The genotype concordance rate of this polymorphism in a subset of duplicate samples was 100% and the overall call rate was 100%.

Platelet aggregation

Maximal platelet aggregation was assessed by optical aggregometry, as previously described.³ Briefly, platelet-rich plasma was isolated from blood samples drawn into 3.2% trisodium citrate anticoagulated tubes (Becton-Dickinson, Franklin Lakes, NJ) and platelet counts were adjusted to 200,000 platelets/ μ l using platelet-poor plasma. Platelet function was assessed using a PAP8E Aggregometer (Bio/Data Corporation, Horsham, PA), according to the manufacturer's instructions after stimulation with collagen (2 μ g/mL), adenosine diphosphate (ADP; 2 μ mol), epinephrine (10 μ M), and arachidonic acid (AA; 1.6 mM), and was expressed as the maximal percentage change in light transmittance using platelet-poor plasma as a referent.

11-dehydrothromboxane B₂ measurement

Measurement of urinary 11-dehydrothromboxane B₂ was performed using a monoclonal 11-dehydrothromboxane B₂ enzyme-linked immunosorbent assay kit (Cayman Chemical, Ann Arbor, MI), according to the manufacturer's instructions. The 11-dehydrothromboxane B₂ concentration was determined photometrically at 405 nm using a PerkinElmer Victor X3 plate reader (PerkinElmer, Shelton, CT) and expressed as pg/mL. All samples were measured in duplicate and expressed as the mean of both measurements.

Statistical analyses

SAS version 9.2 (SAS Institute, Cary, NC) was used to calculate summary statistics, distributions, and frequencies. Association analyses of *PEAR1* rs12041331 were performed using Mixed Model Analysis for Pedigrees (<http://edn.som.umaryland.edu/mmap/>) under an additive genetic model with adjustments for age, sex, and relatedness among study participants. Mixed Model Analysis for

Pedigrees has been extensively used for genomic analyses in the Amish.^{3,4,16–20} Assuming an effect size similar to what was observed in the PAPI study,⁴ we estimate 80% power to detect differences in platelet aggregation of at least 6.75%, as calculated by PS Power.

Urinary concentrations of 11-dehydrothromboxane B₂ were standardized to creatinine and a paired *t*-test was conducted in R to assess the impact of aspirin (324 mg/day) on thromboxane production. Association analyses between *PEAR1* rs12041331 and 11-dehydrothromboxane B₂ levels pre-aspirin and post-aspirin administration (324 mg/day) were performed using Mixed Model Analysis for Pedigrees with age and sex as covariates. Differences between baseline platelet aggregation at study initiation and platelet aggregation after 2-week washout periods were assessed by a repeated-measures analysis of variance in R. The impact of aspirin dose on platelet aggregation as well as aspirin dose-by-genotype interactions were also assessed by repeated-measures analysis of variance in R. All statistical tests were two-sided and *P* < 0.05 was considered statistically significant.

RESULTS

This study used a prospective, genotype-stratified design in which all subjects were studied under three different aspirin doses (81, 162, and 324 mg/day for 7 days) and platelet aggregation at each dose measured in response to four platelet agonists (ADP, collagen, epinephrine, and AA; **Figure 1**). Characteristics of the 67 study participants are shown in **Table 1**. Briefly, these subjects were healthy, middle-aged (mean age = 50.2 years), and had low prevalence of disease (e.g., obesity [mean body mass index = 28.4], hypertension [6.0%], hypercholesterolemia [26.9%], diabetes [0.0%]) and smoking (10.4%). In total, 34 *PEAR1* rs12041331 major allele homozygotes (G/G), 28 heterozygotes (G/A), and 5 minor allele homozygotes (A/A) were recruited. None of the clinical characteristics differed significantly by rs12041331 genotype (**Table 1**) with the exception

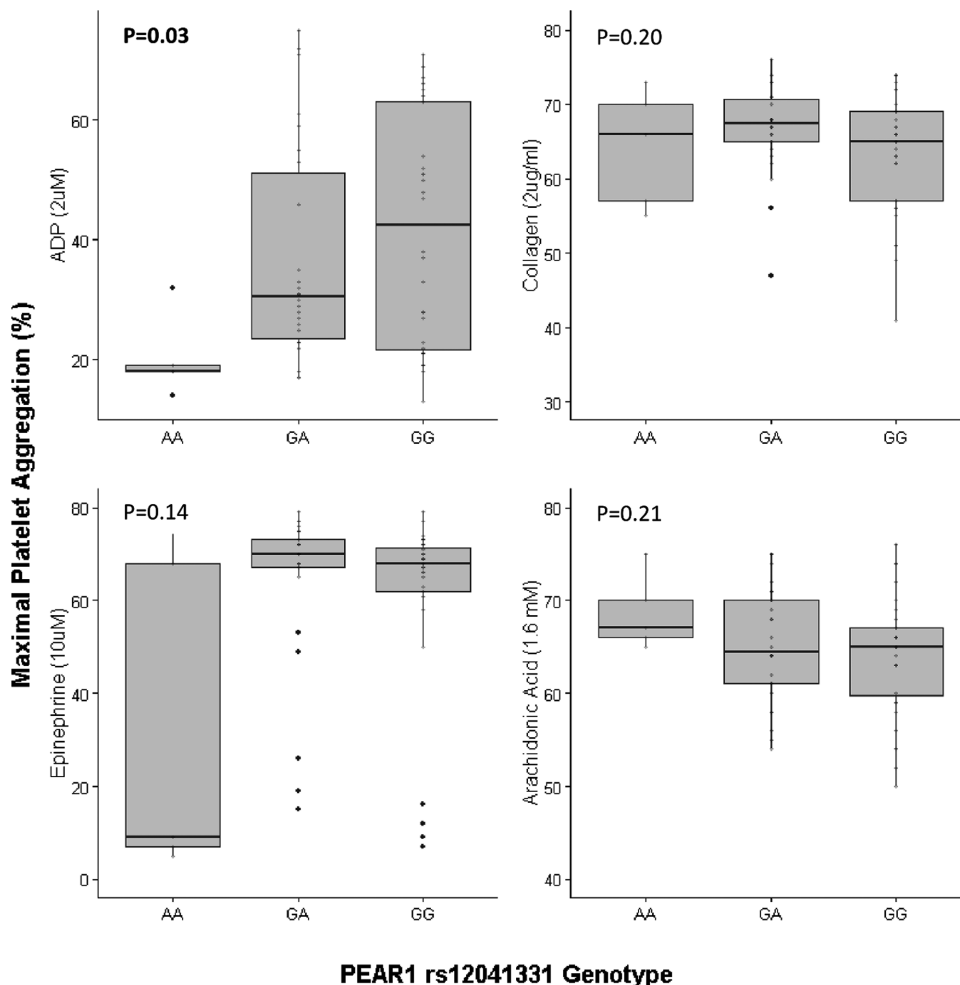


Figure 2 Baseline agonist-induced ex vivo platelet aggregation measurements were obtained in platelet-rich plasma prior to administration of the first aspirin dose. Platelet aggregation is expressed as the percentage of maximal aggregation using platelet-poor plasma as a referent. Adenosine diphosphate (ADP; 2 µM), collagen (2 µg/mL), epinephrine (10 µM), and arachidonic acid (AA; 1.6 mM) were used to stimulate platelet aggregation. The horizontal line within each box indicates the median; the top and bottom borders of each box indicate the interquartile range (IQR). The whiskers extending from each box indicate plus/minus 1.5 IQRs, and the points beyond the whiskers indicate outliers beyond 1.5 IQRs.

of platelet count ($P = 0.03$), which is consistent with the role of *PEAR1* in thrombopoiesis.⁸

To confirm that basal platelet function was restored after each aspirin dose intervention, we compared platelet aggregation measures obtained during the initial visit (prior to any aspirin administration) to measures obtained after the first (~2 weeks after the 81 mg dosing period) and second (~2 weeks after the 162 mg dosing period) washout periods. Indeed, no difference in agonist-induced platelet aggregation was observed between these visits (**Supplementary Table S1**). Similarly, we assessed the impact of aspirin dose on agonist-induced platelet aggregation. As expected, near-complete inhibition of AA-induced platelet aggregation was achieved at 81 mg/day for 7 days with only a modest change at the higher aspirin doses (i.e., at 162 or 324 mg/day) for the same length of time ($P = 0.03$; **Supplementary Table S2**). In addition, the aspirin dose did not significantly influence mean ADP-induced, collagen-induced, and epinephrine-induced

platelet aggregation values ($P = 0.57$, 0.56, and 0.07, respectively).

Prior to aspirin administration, we tested whether *PEAR1* rs12041331 was associated with maximal percentage of platelet aggregation after stimulation with ADP, collagen, epinephrine, and AA. Given that urinary levels of 11-dehydrothromboxane B₂ were also measured before and after administration of 324 mg of aspirin (see below), baseline measures of platelet aggregation recorded prior to administration of 324 mg of aspirin are reported in **Figure 2** and genotype-stratified analysis of all baseline data are shown in **Supplementary Figure S1**. We observed that subjects who carried the minor allele of rs12041331 had significantly reduced ADP-stimulated platelet aggregation compared with noncarriers (mean maximal platelet aggregation = 20.2, 37.0, and 41.8 for rs12041331 genotypes A/A, G/A, and G/G, respectively; $P = 0.03$). In contrast, we observed no evidence of association between rs12041331 and collagen, as

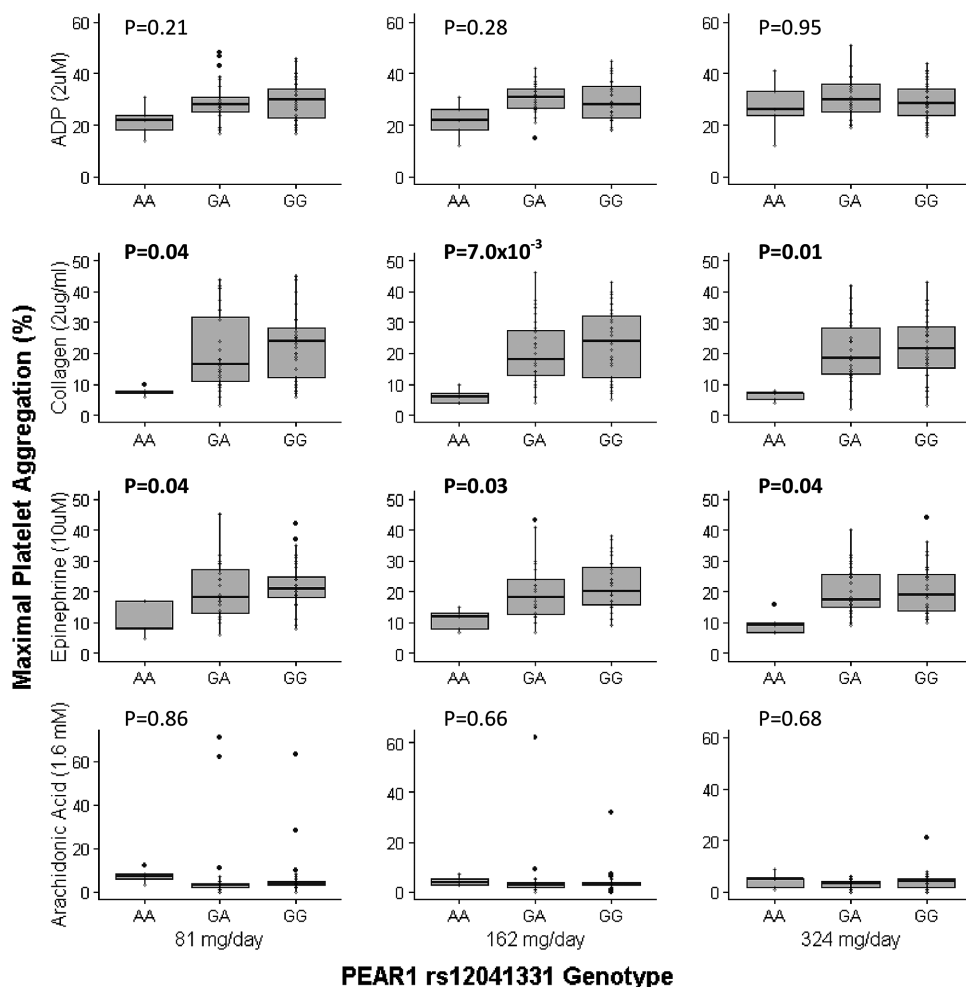


Figure 3 On-aspirin agonist-induced *ex vivo* platelet aggregation measurements were obtained in platelet-rich plasma on day 7 of each treatment period 2 h following administration of the final aspirin dose. Aspirin doses of 81 mg, 162 mg, and 324 mg were administered for 7 days. Platelet aggregation is expressed as the percentage of maximal aggregation using platelet-poor plasma as a referent. Adenosine diphosphate (ADP; 2 μ M), collagen (2 μ g/mL), epinephrine (10 μ M), and arachidonic acid (AA; 1.6 mM) were used to stimulate platelet aggregation. The horizontal line within each box indicates the median; the top and bottom borders of each box indicate the interquartile range (IQR). The whiskers extending from each box indicate plus/minus 1.5 IQRs and the points beyond the whiskers indicate outliers beyond 1.5 IQRs.

well as AA-induced platelet aggregation ($P = 0.20$ and 0.21 , respectively). Although a reduction in epinephrine-induced platelet aggregation was observed in participants who were homozygous for the rs12041331 minor allele (maximal platelet aggregation = 33.6, 64.0, and 60.8 for *PEAR1* rs12041331 genotypes A/A, G/A, and G/G, respectively), this comparison did not reach statistical significance ($P = 0.14$).

Interestingly, we observed different patterns of association between rs12041331 and agonist-induced platelet aggregation after aspirin treatment (**Figure 3**). Unlike pre-aspirin measurements, we observed no evidence of association between rs12041331 and ADP-stimulated platelet aggregation after administration of 81, 162, and 324 mg of aspirin for 7 days ($P = 0.21$, 0.28 , and 0.95 , respectively). In contrast, we observed strong evidence of association between this polymorphism and collagen-stimulated platelet aggregation regardless of aspirin dose ($P = 0.03$, 7.0×10^{-3} , and 0.01 for

aspirin doses of 81, 162, and 324 mg, respectively). Furthermore, subjects who carried the rs12041331 minor allele had significantly reduced on-aspirin epinephrine-induced platelet aggregation compared with noncarriers ($P = 0.04$, 0.03 , and 0.04 for aspirin doses of 81, 162, and 324 mg, respectively). Similar to pre-aspirin measurements, AA-induced platelet aggregation was not influenced by rs12041331 regardless of aspirin dose ($P = 0.86$, 0.66 , and 0.68 for aspirin doses of 81, 162, and 324 mg, respectively).

We also performed interaction analyses to evaluate the influence of increasing doses of aspirin on agonist-induced platelet aggregation in the context of *PEAR1* rs12041331 genotype. Regardless of the agonist used, we observed no interaction between aspirin dose and rs12041331 genotype ($P = 0.89$, 0.15 , 0.77 , and 0.55 when ADP, collagen, epinephrine, and AA were used as an agonist, respectively), indicating an aspirin dose above 81 mg had no major impact on degree of reduction in agonist-induced platelet

Table 2 Urinary 11-dehydrothromboxane B₂ levels by *PEAR1* rs12041331 genotype

	G/G homozygotes	G/A heterozygotes	A/A homozygotes	P value
Pre-aspirin administration	2463 ± 261	2154 ± 217	1748 ± 432	0.16
Post-aspirin administration ^a	474 ± 59	361 ± 33	365 ± 92	0.09

PEAR1, platelet endothelial aggregation receptor 1.

Urinary 11-dehydrothromboxane B₂ levels ± SDs are expressed as pg/mg creatinine.

^aMeasurements were recorded before and after administration of 324 mg/day aspirin for 7 days.

aggregation by *PEAR1* rs12041331 genotype (**Supplementary Table S3**).

Finally, we assessed urinary 11-dehydrothromboxane B₂ levels pre-aspirin and post-aspirin administration in the total population as well as by rs12041331 genotype (**Table 2**). As expected, levels of 11-dehydrothromboxane B₂ were significantly reduced after aspirin exposure ($P = 2.2 \times 10^{-16}$), with near-complete inhibition of 11-dehydrothromboxane B₂ achieved after aspirin exposure for 7 days. The 11-dehydrothromboxane B₂ levels were not significantly different by rs12041331 genotype before ($P = 0.09$) or after aspirin exposure ($P = 0.16$).

DISCUSSION

Although aspirin is the most frequently used antiplatelet medication globally, there is a high degree of interindividual variation in patient response to this agent when considering cyclooxygenase (COX)-independent pathways of platelet function and occurrence of major adverse cardiovascular events. This is reflected in the lack of consensus regarding the appropriate dose of aspirin to be administered clinically, despite updated guidelines and numerous studies that indicate high-dose aspirin has no additional benefit but a reduced safety profile as compared with low-dose aspirin.^{5,21–25} In past years, substantial effort has been made to identify the genetic and nongenetic determinants of aspirin response, and although these investigations have increased our understanding, identification of *bona fide* aspirin response candidate polymorphisms have remained elusive. Multiple factors contribute to lack of genetic replication, including study design, participant population, clinical indication, and several other confounding variables. Furthermore, the ways in which aspirin response is defined (e.g., inhibition of platelet aggregation vs. rates of cardiovascular outcomes) as well as the use of multiple platelet function tests has made it difficult to compare results across investigations. Given that aspirin irreversibly acetylates COX resulting in the inhibition of thromboxane A₂ formation from AA, traditional tests of on-aspirin platelet function include stimulation of platelets with AA as well as assessment of urinary 11-dehydrothromboxane B₂, a stable metabolite of thromboxane A₂. However, an increasing body of evidence suggests that evaluation of non-COX-dependent platelet pathways complement traditional aspirin response approaches and can aid in the identification of factors that lead to variation in response to this drug.^{4,5,21,26}

PEAR1 was identified in 2005 as a type 1 transmembrane receptor that is highly expressed in platelets and endothelial cells, among other cell types.⁷ In platelets, *PEAR1* acts as a contact receptor that, upon ligand binding, becomes phosphorylated in an Src family kinase-dependent manner, leading to downstream PI3K and AKT signaling, ultimately resulting in the stabilization of platelet aggregates through secondary activation of glycoprotein α IIb β ₃.^{6,7} Continual refinement of the biological processes that are influenced by *PEAR1* in platelets and other cell types are consistent with the results of genetic investigations of *PEAR1* (see below) and allow for better understanding of *PEAR1*-dependent changes in platelet aggregation before as well as after antiplatelet treatment.

To date, multiple investigations have convincingly shown that genetic variation in *PEAR1* significantly influences agonist-induced platelet aggregation at baseline^{9–13,27} as well as after treatment with aspirin,^{9,28} prasugrel,²⁹ and dual antiplatelet therapy with aspirin and clopidogrel.^{4,30} Although several polymorphisms that influence platelet aggregation have been identified in this gene, rs12041331 seems to be the most highly and consistently associated of these variants.^{4,9–11,13} The rs12041331 polymorphism results in G to A substitution in intron 1 and has been previously implicated in reducing *PEAR1* expression.⁹ In a genome-wide meta-analysis of basal platelet function in over 4,500 European and African American participants, Johnson et al.¹³ showed that *PEAR1* rs12041331 was among the strongest determinants of basal ADP-induced and epinephrine-induced platelet aggregation ($P = 3.8 \times 10^{-16}$ and 4.9×10^{-19} , respectively) but did not report associations with collagen or collagen lag time. Similarly, evaluation of Caucasian Americans of the GeneSTAR cohort revealed that *PEAR1* rs12041331 was among the strongest determinants of platelet aggregation pre-aspirin administration, accounted for ~15% of the total phenotypic variation in platelet function, and sequencing of the full *PEAR1* gene in 104 individuals confirmed that rs12041331 accounted most strongly for the relation between *PEAR1* and platelet aggregation.¹¹ Surprisingly, however, a different pattern of association emerged post-aspirin administration (81 mg/day for 14 days), in which significant associations were observed with collagen-induced and epinephrine-induced platelet aggregation but not when ADP was used as a platelet agonist.⁹ To complicate matters further, in African Americans of the GeneSTAR cohort, in which the minor allele frequency of this variant is substantially higher compared with Caucasian participants (0.37 vs. 0.09, respectively), rs12041331 was significantly associated with ADP-induced, collagen-induced, and epinephrine-induced platelet aggregation pre-aspirin and post-aspirin exposure. In our own genome-wide association studies of dual antiplatelet therapy response (aspirin and clopidogrel) in 565 healthy Amish participants of the PAPI study, *PEAR1* rs12041331 was the strongest modifier of collagen-stimulated platelet aggregation ($P = 7.7 \times 10^{-9}$).⁴ We also observed that African American and Caucasian patients with non-emergent percutaneous coronary intervention who carried the minor allele of rs12041331 had reduced 1-year survival (hazard ratio = 3.97; $P = 0.04$ and hazard ratio = 2.62; $P = 0.06$, respectively).

and that Caucasian patients with stable coronary artery disease on aspirin experienced higher rates of myocardial infarction compared with noncarriers (odds ratio = 2.03; $P = 0.05$). Interestingly, rs12041331 was not associated with myocardial infarction in patients with stable coronary artery disease who were not treated with aspirin.⁴

Although several retrospective studies have independently shown that *PEAR1* rs12041331 influences platelet aggregation and cardiovascular outcomes, others have not.³¹ In addition, differences in participant populations (e.g., healthy patients vs. patients with cardiovascular disease), study design (e.g., presence vs. absence of antiplatelet agents), as well as utilized platelet function tests (i.e., the agonist used to stimulate platelet aggregation) have made it challenging to definitively determine the platelet activation pathways influenced by *PEAR1*. To better define the influence of *PEAR1* rs12041331 on platelet aggregation pathways, we, for the first time, prospectively evaluated this polymorphism before and after aspirin administration (81, 162, and 324 mg/day) using multiple platelet agonists (i.e., ADP, collagen, epinephrine, and AA). Prior to aspirin administration, we showed a strong relationship between *PEAR1* genotype and ADP-stimulated platelet aggregation but not with collagen-induced platelet aggregation, consistent with the results observed in nontreated patients in both the Framingham Heart Study and GeneSTAR cohorts.^{9,13} Interestingly, after aspirin treatment, *PEAR1* rs12041331 substantially influenced collagen-induced platelet aggregation but not ADP-induced platelet aggregation, suggesting a potential shift in the platelet pathways impacted by *PEAR1* in the presence of aspirin. Although we found that on-aspirin epinephrine-induced platelet aggregation was altered by *PEAR1* genotype, consistent with prior reports, we failed to observe such a relationship prior to aspirin administration. This latter observation is in contrast to previous investigations; however, it should be noted that we did observe a non-significant reduction in platelet aggregation in rs12041331 minor allele homozygotes after stimulation with epinephrine prior to aspirin treatment (maximal platelet aggregation = 33.6, 64.0, and 60.8 for *PEAR1* rs12041331 genotypes A/A, G/A, and G/G, respectively). Importantly, we did not observe evidence of association between AA-induced platelet aggregation and urinary 11-dehydrothromboxane B₂ levels with rs12041331 pre-aspirin or post-aspirin administration. In other words, assessment of non-COX-dependent biological pathways, in addition to canonical pathways, provided useful insights regarding the determinants of variable aspirin response, consistent with previous investigations.^{4,5,9,21,26,28,30}

Given the prior observation that aspirin-treated patients with coronary artery disease who carried the minor allele of *PEAR1* rs12041331 experienced higher rates of myocardial infarction compared with nontreated carriers,⁴ we, for the first time, prospectively investigated the potential interaction between therapeutically used aspirin doses and *PEAR1* genotype on agonist-induced platelet aggregation. However, we did not observe any such interaction, nor did we observe any impact of aspirin dose on degree of inhibition of platelet aggregation in the entire cohort overall, consistent with recent US guidelines that favor low-dose aspirin antiplatelet therapy.²⁵

Although it is increasingly clear that genetic variation in the *PEAR1* gene substantially impacts agonist-induced platelet aggregation, it is still unclear by what mechanism they may influence cardiovascular event rates. For example, the minor allele of rs12041331 leads to reduced on-treatment platelet aggregation yet higher rates of thrombotic outcomes.⁴ It has been speculated that the additive effect of aspirin therapy in addition to reduced platelet aggregation in carriers of the rs12041331 minor allele may lead to unstable thrombi and subsequent plaque rupture; however, no evidence exists to support this hypothesis. Alternatively, it is possible that reduced platelet aggregation in minor allele carriers is unrelated to the increased cardiovascular event risk. *PEAR1* expression in the endothelium is sixfold higher than expression in platelets.⁷ Vandenbrielle et al.³² recently showed that *PEAR1* is a negative regulator of neoangiogenesis and wound healing.³² Furthermore, Fisch et al.¹⁶ showed through meta-analysis of publically available microarray data that *PEAR1* expression is highly correlated with several genes and phenotypes that are integral to endothelial function and that *PEAR1* rs12041331 is significantly associated with flow-mediated dilation of the brachial artery, a widely used noninvasive measure of endothelial function. Additional mechanistic studies both in the endothelium and in platelets will be required to clarify the role of *PEAR1* in cardiovascular disease risk in both the presence and absence of aspirin.

We would like to acknowledge some limitations of our investigation. Given the moderate sample size of our cohort, it is possible that we may have been statistically underpowered for some of our association analyses. However, the effect sizes of *PEAR1* rs12041331 on platelet aggregation in this study are similar to the effect sizes observed in previous investigations.^{4,9} In addition, our study design evaluated *PEAR1* genotype in the context of acute aspirin administration. Given the methods used in this investigation (i.e., *ex vivo* platelet aggregation) and average lifespan of platelets (7–10 days), we do not believe that platelet aggregation results would differ considerably between short-term and long-term aspirin exposure. However, if the relationship between *PEAR1* genotype and platelet aggregation is significantly impacted by *PEAR1* activity in the endothelium, where it is highly expressed, it is possible that chronic aspirin exposure might influence our findings. Finally, we conducted our investigation in healthy Amish participants. Although our results are consistent with previously conducted retrospective analyses in white populations, these results may not be generalizable to participants of different race/ethnicities or to patients with cardiovascular disease. Additional evaluation of the influence of *PEAR1* rs12041331 genotype in these groups will be needed to confirm our findings.

In summary, we prospectively investigated the impact of a recently identified aspirin response polymorphism pre-aspirin and post-aspirin administration using several important platelet agonists. Although no difference in platelet function was observed using traditional tests of aspirin response (i.e., AA-induced platelet aggregation and circulating levels of urinary thromboxane B₂), *PEAR1* genotype significantly impacted non-COX-dependent platelet aggregation pathways. Furthermore, exposure to aspirin led to a shift in the platelet aggregation pathways influenced by

rs12041331, whereby ADP-stimulated platelet aggregation was affected prior to aspirin administration yet collagen-induced and epinephrine-induced platelet aggregation were impacted post-aspirin exposure. Continual investigation will be necessary to better understand the dynamics of *PEAR1*-mediated platelet aggregation *in vivo* as well as the implications of these findings on adverse event rates in aspirin-treated patients with cardiovascular disease.

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