Joshua D. Backman

jbackman86@gmail.com

Education:

Candidate, PhD in Human Genetics
University of Maryland, Baltimore
Start Date: 8/27/2012
Defense Date: 8/1/2016
Grade-Point Average: 3.65 (On a 4.0 scale)

BS in General Biology
University of Maryland, College Park
Graduation Date: 5/22/2009
Grade-Point Average: 3.45 (On a 4.0 scale)

Relevant Course Work:

Principles of Epidemiology
Genomics and Bioinformatics
Translational Research
Human Genetics, Biostatistics
Organic Chemistry, General Chemistry
Mechanisms in Biomedical Sciences

Skills and Techniques:

Data Analysis, R, Python, Plink, GCTA, Taqman Genotyping, Next-Generation Sequencing, Aseptic Technique, Pipette, Light microscopy, Cell Culture, Wound Healing Assay, PCR, Nucleotide purification, Protein extraction, DNA quantitation, Western Blot, ELISA Assay

---

Publications:

Backman JD, Lewis JP, Horenstein RB, Spencer SD, Pacanowski MP, Peer CJ, Figg WD, Shuldiner AR, O'Connell JR, Yerges-Armstrong LM. Analysis of Serum Clopidogrel Active Metabolite Concentration Identifies Novel Genetic Variants Associated With Clopidogrel Pharmacokinetics. *Manuscript in preparation.*

Backman, JD, Yerges-Armstrong LM, Horenstein RB, Newcomer S, Shaub S, Morrisey M, Donnelly P, Drolet M, Tanner K, Pavlovich MA, O'Connell JR, Mitchell BD, Lewis JP. Prospective Evaluation of Genetic Variation in PEAR1 Reveals Aspirin-Dependent Effects on Platelet Aggregation Pathways. *Manuscript under review.*

Perry CG., Perry JA, Anforth LE, Pavlovich MA, **Backman JD**, Ryan KA, O'Connell JR, Yerges-Armstrong LM, Shuldiner, AR. From Genotype to Phenotype: Nonsense Variants in *SLC13A1* are Associated with Decreased Serum Sulfate and Increased Serum Aminotransferases. G3. 2016; doi: 10.1534/g3.116.032979.

Fisch AS, Yerges-Armstrong LM, **Backman JD**, Wang H, Donnelly P, Ryan KA, Parihar A, Pavlovich MA, Mitchell BD, O'Connell JR, Herzog W, Harman CR, Wren JD, Lewis JP. Genetic Variation in the Platelet Endothelial Aggregation Receptor 1 Gene Results in Endothelial Dysfunction. PLoS ONE. 2015;10:e0138795.

Nugent KL, Million-Mrkva A, **Backman JD**, Stephens SH, Reed RM, Kochunov P, Pollin TI, Shuldiner AR, Mitchell BD, Hong LE. Familial aggregation of tobacco use behaviors in amish men. Nicotine & Tobacco Research. 2014. doi: 10.1093/ntr/ntu006.

Oral Presentations:

2016 Atherosclerosis, Thrombosis, and Vascular Biology Scientific Sessions, Nashville, TN
2016 Graduate Research Conference, UMSOM, Baltimore, MD

Abstracts:

Backman JD, Lewis JP, Horenstein RB, Spencer SD, Pacanowski MP, Peer CJ, Figg WD, Shuldiner AR, O'Connell JR, Yerges-Armstrong LM. Analysis of Serum Clopidogrel Active Metabolite Concentration Identifies Novel Genetic Variants Associated With Clopidogrel Pharmacokinetics. *American Heart Association Atherosclerosis, Thrombosis, and Vascular Biology Scientific Sessions*, 2016

Backman JD, Lewis JP, Horenstein RB, Spencer SD, Pacanowski MP, Peer CJ, Figg WD, Shuldiner AR, O'Connell JR, Yerges-Armstrong LM. Analysis of Serum Clopidogrel Active Metabolite Concentration Identifies Novel Genetic Variants Associated With Clopidogrel Pharmacokinetics. *University of Maryland Graduate Research Conference*, 2016

Backman JD, Lewis JP, Horenstein RB, Mitchell BD, O'Connell JR, Shuldiner AR, Yerges-Armstrong LM. Genetic Variation in the *SVEP1* Gene Impacts On-Clopidogrel Platelet Aggregation in a Sex-Specific Manner. *American Society of Human Genetics,* 2015

Backman JD, Lewis JP, Horenstein RB, Mitchell BD, O'Connell JR, Shuldiner AR, Yerges-Armstrong LM. Genetic Variation in the SVEP1 Gene Impacts On-Clopidogrel Platelet Aggregation in a Sex-Specific Manner. *Up Close and Personalized, 4th International Congress on Personalized Medicine*, 2015

Backman JD, Shuldiner AR, Lewis JP, Yerges-Armstrong LM. Genome-wide Association Study of Platelet Aggregation Identifies Novel Variant Influencing Clopidogrel Pharmacodynamics. *Pharmacogenomics Research Network.* 2015

Backman JD, Lewis JP, Horenstein RB, Mitchell BD, O'Connell JR, Shuldiner AR, Yerges-Armstrong LM. Genetic Variation in the SVEP1 Gene Impacts On-Clopidogrel Platelet Aggregation in a Sex-Specific Manner. Pharmacogenomics Research Network, 2015

Backman JD, Ning B, Lou W, Perry J, Shi L, Shuldiner AR, Yerges-Armstrong LM. Exome sequencing from extreme responders to aspirin identifies a novel variant associated with platelet aggregation. *University of Maryland Graduate Research Conference*. 2015.

Backman JD, Ning B, Lou W, Perry J, Shi L, Shuldiner AR, Yerges-Armstrong LM. Exome sequencing from extreme responders to aspirin identifies a novel variant associated with platelet aggregation. *American Heart Association EPI/Lifestyle*. 2015.

Backman JD, Million-Mrkva AD, Hong LE, Ehringer MA, Mitchell BD, Shuldiner AR, Ryan KA, Nugent KL, Stephens SH. Characterization and replication of chromosome 15q25 nicotine dependence signals in an old order amish population. *ASHG*. 2013.

Employment Experience:

6/2015-8/2015 PhD Intern
Regeneron Genetics Center, Regeneron, Tarrytown, NY
Conducted analysis of various traits collected from electronic health records of more than 40,000 subjects with exome sequencing. Responsibilities included data munging, computing principal components, and conducting statistical tests. Regularly used R, Python, and Plink. Presented research to Regeneron leadership and CSO George Yancopoulis.

1/2011-7/2012 Associate Scientist
Pharmaceutical Product Development, Inc., Richmond, VA
Conduct an Opsonophagocytic Assay for a multivalent vaccine currently in the clinical testing phase. Use Aseptic technique and follow a strict GLP.

10/2009-8/2012 SAT Prep Instructor
The Princeton Review, Scarsdale, NY

5/2010-9/2010 SAT Prep Instructor
The Princeton Review Korea, Seoul, South Korea
Taught ESL students SAT prep in conjunction with fundamentals of the English language.

12/2009-5/2010 Customer Service Associate
Pitney Bowes Management Services, Tarrytown, NY

9/2008-3/2009 Office Assistant
Pinstripe Property Management, Rockville, MD
5/2007-8/2007 Volunteer
Mt. Sinai Hospital, Manhattan, NY
Shadowed physicians in the Neurology and Rehabilitation Departments. Worked closely with patients and conducted research on their disabilities.

4/2005-8/2008 Truck Driver and Deliveryman
Abbey Ice Company, Spring Valley, NY
Delivered ice and water to various stores and homes. Handled large sums of money and gained valuable experience in communication and customer service. Routinely worked 60 to 70 hour weeks seasonally from May to August.

6/2001-1/2006 Office Assistant
NYDIC Open MRI of America, Montvale, NJ
Scanned and organized documents, data entry, and various clerical duties.

Honors and Activities:

Teaching Assistant, PREV601, Introduction to Biostatistics, Fall 2014
Student Representative, Department of Epidemiology and Human Genetics Curriculum Committee
Student Member, PGRN
Trainee Member, American Heart Association
Trainee Member, American Society of Human Genetics
3 Semesters of Academic Honors at the University of Maryland.
Certified Test Prep Expert for The Princeton Review

***References upon request**

Abstract

Title of Dissertation: Novel Discovery and Functional Characterization of Key Genetic Variants that Influence the Pharmacogenomics of Anti-Platelet Therapy

Joshua David Backman, Doctor of Philosophy, 2016

Dissertation Directed by: Alan R. Shuldiner, Professor, Department of Medicine;
Director, Program in Personalized and Genomic Medicine

Joshua P. Lewis, Assistant Professor, Department of Medicine

Coronary artery disease (CAD) is among the leading causes of death in the United States and worldwide. The most commonly prescribed treatment for the secondary prevention of cardiovascular events in at-risk patients is concurrent administration of clopidogrel and aspirin (DAPT). However, significant inter-individual variability in response has been documented, resulting in sub-optimal therapy in some patients and an increased risk of recurrent events.

While genetic evaluations of *ex vivo* platelet reactivity have identified notable clopidogrel response modifiers such as *CYP2C19**2, heritability estimates suggest most of the genetic determinants of variable DAPT response remain unidentified. We conducted the first genome-wide analysis of clopidogrel active metabolite concentration, a direct measure of clopidogrel pharmacokinetics, and discovered novel loci on chromosomes 3p25 and 17q11that significantly influence clopidogrel metabolism, as well as several other loci with suggestive evidence of association with clopidogrel response. While extension to on-clopidogrel platelet aggregation and ischemic events in an

independent population was inconclusive, this research highlights the utility of clopidogrel active metabolite concentration as a novel endophenotype for clopidogrel efficacy.

In addition to investigating novel antiplatelet response modifiers, we conducted clinical and functional characterization of rs12041331, a well-studied intronic variant in Platelet Endothelial Aggregation Receptor 1 (*PEAR1)* previously correlated with platelet aggregation and cardiovascular event risk. We used a fixed sequence, crossover design to prospectively evaluate platelet reactivity pre- and post- 3 different therapeutic aspirin doses in 67 healthy subjects recruited by rs12041331 genotype. rs12041331 was associated with platelet aggregation before and after aspirin exposure in a pathway- and agonist- specific manner, although higher aspirin dose did not impact platelet reactivity in a genotype-dependent manner. PEAR1 is also suggested to be a modifier of endothelial function. We therefore functionally characterized the impact of *PEAR1* knockdown and rs12041331 on human umbilical vein endothelial cell (HUVEC) behavior *in vitro* and found PEAR1 to have a significant role in endothelial migration, extracellular matrix adhesion, and proliferation.

While further follow-up is necessary, these experiments demonstrate the importance of incorporating multiple measures to evaluate genetic determinants of antiplatelet efficacy, and the results herein have promise to contribute to improved cardiovascular care in the future.

Novel Discovery and Functional Characterization of Key Genetic Variants that Influence the Pharmacogenomics of Anti-Platelet Therapy

by

Joshua D. Backman

Dissertation submitted to the Faculty of the Graduate School of the

University of Maryland, Baltimore in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

2016

For my Dad

Acknowledgements

Special thanks to Drs. Alan Shuldiner, Laura Yerges-Armstrong, and Joshua Lewis. Without the guidance, expertise, inspiration, and words of wisdom from each of you I would not be where I am today. I would also like to extend my gratitude to the support staff of the Shuldiner lab, the University of Maryland Program in Epidemiology and Human Genetics, the Program in Personalized and Genomic Medicine, the Division of Endocrinology, and the Amish Research Clinic. I would also like to thank the Regeneron Genetics Center for an invaluable internship experience, as well as all of the selfless participants of our research studies, without whom none of this work would be possible.

Table of Contents

List of Tables

Chapter 1

Chapter 4

Chapter 5

Chapter 6

List of Figures

Chapter 1

Chapter 4

Chapter 5

Chapter 6

Chapter 1: Background and Significance

Cardiovascular Disease

In 2011, cardiovascular disease was the leading cause of mortality in the United States (U.S.) and worldwide, resulting in more than 785,000 deaths in the U.S alone[15]. In addition, approximately 85 million U.S. citizens are currently living with cardiovascular disease, with direct and indirect costs totaling more than 320 billion[15]. Coronary artery disease (CAD), also known as coronary heart disease and ischemic heart disease, is the most common form of cardiovascular disease, accounting for an estimated 7.4 million deaths globally in 2012[16]. In CAD, an atherosclerotic plaque initially forms, in part, as a result of endothelial dysfunction, and is subsequently followed by cholesterol deposition in the subendothelial lining of the arterial wall. If left untreated, CAD can lead to acute coronary syndromes such as myocardial infarction and angina (Figure 1.1)[10].

Atherosclerosis Progression

Atherosclerosis is a chronic, inflammatory disorder with a complex etiology. In healthy individuals, blood leukocytes typically adhere poorly to the arterial wall. However, a change in endothelial function triggered by elevated

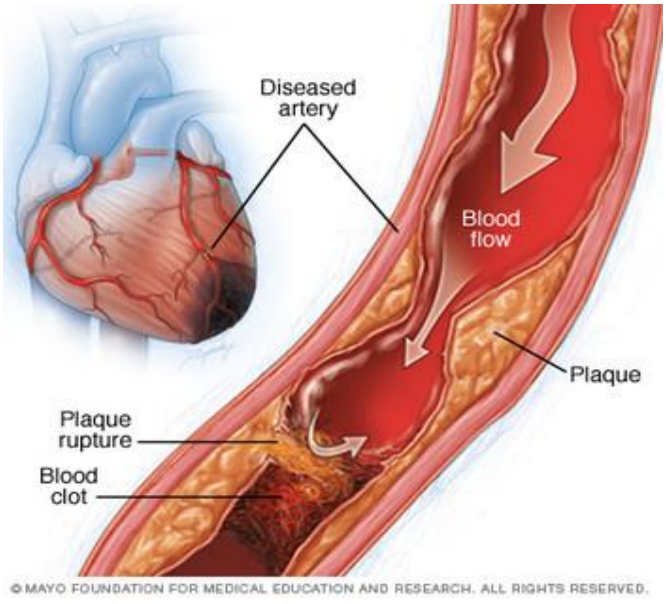


Figure 1.1. Illustration of Coronary Heart Disease. Adapted from Mayo clinic, 2015[10]

LDL levels and other cardiovascular risk factors, at least partially mediated through endothelial nitric oxide synthase (eNOS)[17], leads to abnormal expression of endothelial cell and leukocyte adhesion molecules, which facilitates increased attachment of leukocytes to the endothelium. Concurrent with increased adhesion, changes in endothelial permeability allow LDL particles to traverse the extracellular matrix and begin to accumulate in the intima (Figure 1.2[13]). As LDL deposits in the inner most layer of the artery, chemoattractant mediators are released that direct adherent leukocytes into the intima[13]. Recruited monocytes will differentiate into macrophages and engulf LDL particles to become foam cells (Figure 2[13]). As plaque formation progresses, synthesis of extracellular matrix proteins increases, resulting in smooth muscle cell migration from the media and proliferation in the intima. As smooth muscle cells and foam cells die, lipids, debris, and cholesterol crystals collect in center of the growing plaque, forming a

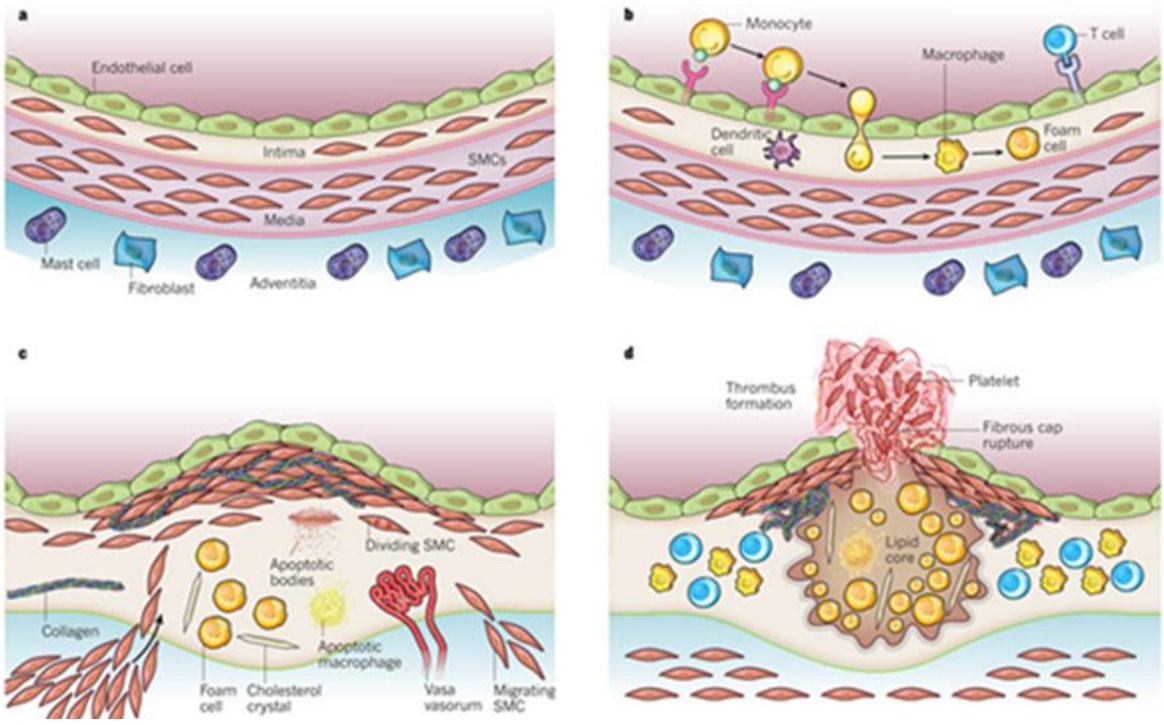


Figure 1.2. Atherosclerotic plaque progression. Adapted from Libby et al, 2011 Nature [13]

necrotic core (Figure 1.2[13]). As this fatty deposit grows and hardens, blood flow to the heart may become partially or fully occluded. In addition, as the plaque grows the fibrous cap becomes thin and prone to rupture, exposing subendothelial proteins that stimulate platelet aggregation, leading to thrombus formation and ultimately an ischemic event[18].

Risk factors such as high lipid and triglyceride levels, smoking, insulin resistance, and hypertension contribute to initial endothelial dysfunction, as well as platelet dysfunction, that predisposes to atherosclerotic plaque development and progression[19]. Though the exact mechanisms are currently unclear and likely complex, emerging evidence over the past decade indicate these risk factors contribute, in part, to alterations in nitric oxide bioavailability, which is recognized as a major precipitant of both endothelial dysfunction and platelet activation [17]. Given this information, further research regarding endothelial and platelet dysfunction is critical to improve our understanding of CAD development, as well as patient response to CAD treatment.

The Endothelium

The endothelium is a semi-permeable monolayer that lines the interior of blood vessels, forming a protective barrier between blood and the surrounding tissue. One major function of the vascular monolayer is maintaining the hemostatic balance between pro- and anti-thrombotic processes[20]. Furthermore, it has a role in other relevant biological processes such as regulating vascular tone, angiogenesis, wound healing, and inflammation. During angiogenesis and wound healing, endothelial cells must be able to migrate from the vasculature, adhere to the extracellular matrix, and proliferate in order to form the new vasculature. If these processes become dysregulated, the health of the endothelium is compromised. In addition, the endothelium is an important regulator of

vasodilation through both nitric oxide-dependent and independent mechanisms. Impaired vasodilation is a prognostic indicator of endothelial dysfunction and coronary artery disease[21]. Indeed, while proper endothelial function is a complex and multifactorial process, reduced nitric oxide synthesis by eNOS is recognized as one of the most important factors in promoting dysfunction of endothelium to date[17]. In addition to promoting reduced vasodilation, nitric oxide also acts to prevent atherosclerosis progression through inhibition of platelet and leukocyte adhesion as well as smooth muscle cell proliferation[22].

Endothelial Dysfunction

There are notable differences between a healthy and unhealthy endothelium. In a healthy state, the endothelium is pro-dilatory, anti-thrombotic, and anti-inflammatory, with high levels of nitric oxide and PGI_2 expression. In addition, a healthier endothelium is indicated by low levels of soluble adhesion factors, oxygen radicals and uric acid (Figure 1.3[23]). Conversely, an unhealthy endothelium has impaired vasodilation, increased oxidative stress, decreased repair capacity, and increased adhesion molecule expression[23]. Given the increased endothelial damage observed in patients with CAD risk[17,21,23-30], the basic functions of endothelial cells for vascular repair are critical in better understanding of CAD pathology. Even in patients that have CAD, therapeutic applications that promote better endothelial function are essential, as variation in endothelial cell migration and adhesion is implicated in stent thrombosis risk after percutaneous coronary intervention (PCI)[31].

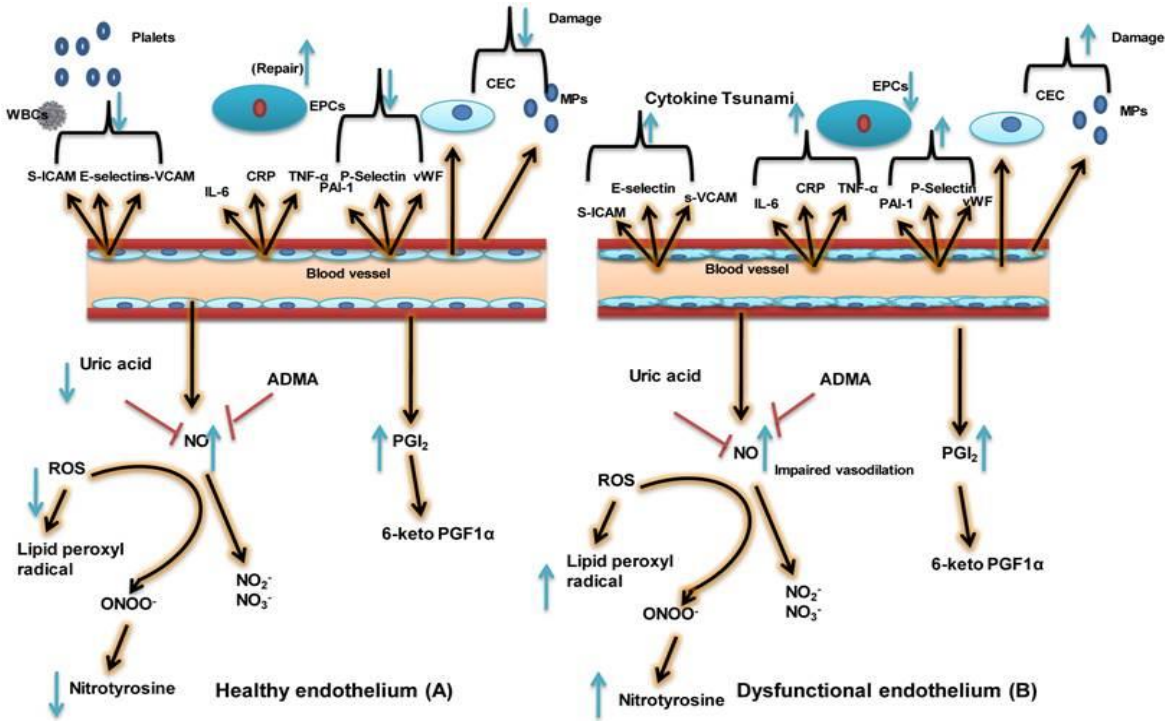


Figure 1.3. Markers of a healthy (a) and unhealthy (b) endothelium. Adapted from Rajendran et al. 2013 International Journal of Biological Science[13]

Endothelial Function Testing

Endothelial dysfunction is an independent prognostic factor for patients with or at risk of developing atherosclerosis, and several methods have been developed to assess endothelial function *in vivo*. The first methods used were invasive, requiring intracoronary or intrabrachial infusion of acetylcholine or other vasodilators, which induce nitric oxide production, the primary driver of endothelium-dependent vasodilation[22]. While these direct measures of endothelial health are still considered the gold standard for detection of endothelial dysfunction, the invasiveness of these procedures makes them unfeasible for routine use. Less invasive techniques, such as flow-mediated dilation (FMD) and peripheral arterial tonometry, while indirectly

assessing endothelial function, have been strongly correlated with the more invasive techniques, and have become standard practice for endothelial health assessment[32,33].

Specific Measures of Endothelial Function

While FMD of the brachial artery by ultrasound is strongly linked with cardiovascular risk and is the most widely used measure of endothelial function, this phenotype is a composite of multiple endothelial processes, and does not provide insight into specific mechanisms by which an environmental risk factor or genetic polymorphism may impact endothelial function. Given the vast differences between a healthy and unhealthy endothelium, circulating biomarkers may be informative for providing a more specific assessment of endothelial dysfunction. Endothelin-1, for instance, is a circulating protein involved in vasoconstriction and pro-inflammation, and, through these mechanisms, has a major influence on atherosclerosis progression. Elevated endothelin levels have been observed in patients with atherosclerosis and CAD in multiple studies[34,35]. Nitric oxide, as previously described, is the primary promoter of endothelial-dependent vasodilation, and is perhaps the most important molecule for overall endothelial health, with additional cardioprotective benefits through reduced blood pressure, inhibition of platelet aggregation, reduced leukocyte adhesion, and reduced smooth muscle cell proliferation[17,36-38]. Abnormal angiogenesis is another hallmark of endothelial dysfunction, and angiopoietin-2 (Ang-2), a circulating biomarker of angiogenesis, is predictive of CVD outcomes in at-risk patients[24,39]. Endoglin, which acts as a co-receptor for transforming growth factor beta (TGF-β), is another marker of angiogenesis and vascular remodeling, and influences nitric oxide expression through regulation of eNOS. In addition, increased endoglin levels are associated with

inflammation and may contribute to endothelial dysfunction and increased cardiovascular disease risk in the presence of other risk factors [25]. Additionally, elevated serum concentrations of adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) are associated with atherosclerosis progression and cardiovascular disease risk. Although neither measure independently serves as a reliable indicator of future cardiovascular events, VCAM-1 and ICAM-1 are abundantly expressed in areas prone to lesion development, making these proteins among the most commonly measured endothelial biomarkers[21,40].

Modifiers of Endothelial Function

A number of clinical and anthropometric factors influence the endothelium as well as measurement of endothelial function. For instance, gender differences in endothelial dysfunction have long been noted[41-43]. While the prevalence of CAD is higher in men, the incidence of stroke is higher in premenopausal females, and the expression of estrogen and other hormones are likely to contribute to this observation[41,43,44]. In addition, females tend to have higher FMD than men, likely due, at least in part, to smaller vessel diameter[45-47]. Furthermore, racial differences have been noted, with reduced FMD in Africans as compared to Europeans [48,49]. Age is another effecter of endothelial health, with reduced vascular elasticity observed in older adults[47,50]. Smoking and obesity both lead to reduced nitric oxide bioavailability, increased inflammatory signaling, increased oxidative stress, and increased endothelial adhesion molecule expression[26,51]. Statins, on the other hand, though developed and prescribed for lipid lowering, have been noted to improve endothelial health through mechanisms not directly related to a reduction in lipids[52].

In addition to the aforementioned modifiers, genetic variation significantly impacts endothelial function. Previous investigations have shown that heritable variation contributes significantly to inter-individual differences in endothelial function, with around 14% to 16% of total phenotypic variation as measured by FMD due to genetic polymorphisms [47,53]. For example, an analysis of post-occlusive reperfusion of the brachial artery in 122 children identified several polymorphisms in nitric oxide synthase 1 (NOS1) and endothelin-1 (ET-1) that were strongly correlated with endothelial health [54]. In an evaluation of FMD and hyperemic flow velocity in Framingham Heart study participants, a set of variants in endothelial nitric oxide synthase 3 (NOS3) were also found to influence endothelial function[55]. Additional variants in other endothelial regulatory genes such as dimethylarginine dimethylaminohydrolases (DDAH), GTP cyclohydrolase 1 (GCH1), and p22phox (*CYBA*) have also been reported to influence the endothelium[56].

As previously mentioned, CAD has an extremely complex etiology involving the endothelium, platelets, and a number of other tissues and cell types. Further, aspirin, commonly used for CAD treatment, influences both platelets and the endothelium. Response to aspirin is highly variable[57], and could potentially be explained, at least partially, through endothelial mechanisms. Given that thrombus development is closely connected with endothelial function, further investigation regarding the relationship between endothelial function and antiplatelet therapy may unveil novel insights into CAD risk.

Platelet Biology

Platelets, also known as thrombocytes, are discoid shaped, anuclear blood cells approximately 2-3 um in diameter that play a key role in primary hemostasis. They are produced by megakaryocytes, myeloid cells that reside primarily in the bone marrow, in a process called thrombopoiesis. Upon stimulation by thrombopoeitin, megakaryocytes undergo multiple rounds of endomitosis during which the cells becomes polyploid and gain all the necessary organelles, proteins, and molecular components necessary for platelet production[58]. During platelet generation, a mature megakaryocyte will produce platelet precursors called proplatelets from pseudopodia that extend from the cytoplasm in a manner similar to beads on a string [58]. Platelets are released from the ends of the proplatelet shafts leading to cytoplasm-deprived polyploid megakaryocyte degradation. A single megakaryocyte may produce thousands of platelets. Upon generation, platelets will circulate in blood for approximately 10 days[59].

Platelet Activation

Platelets are essential for primary hemostasis, resulting in a hemostatic plug in order to prevent bleeding (Figure 1.4)[2]. Upon vascular injury (e.g. atherosclerotic plaque rupture), platelets will adhere to the damaged endothelium in the first step of hemostatic plug formation. In order to properly adhere, platelets recognize collagen and von Willebrand factor (VWF) exposed by the damaged vasculature. Integrins on the cell surface, specifically the GPIb-V-IX complex, recognize VWF and establish the initial connection to the site of injury[60]. This tethering allows GP IV, GP VI, and the GP Ia/IIa complex on the platelet surface to bind collagen, which promotes platelet activation[60].

Platelets undergo a massive morphological shift upon activation. The membrane of resting platelets contain a number of folds that allows activated platelets to greatly increase their surface area while a concurrent cytoskeletal reorganization gives activated platelets a dendritic appearance (Figure 1.5). Platelets also undergo rapid degranulation of α- and dense granules upon activation, which allows the cell to greatly increase its binding capacity and propagate platelet activation and aggregation in neighboring platelets. α-granules contain a number of adhesive factors, coagulation factors, mitogenic factors, protease inhibitors that are released upon activation. Dense granules contain a number of signaling molecules such as adenosine diphosphate (ADP) and adenosine triphosphate (ATP), which further induce platelet aggregation in neighboring platelets.

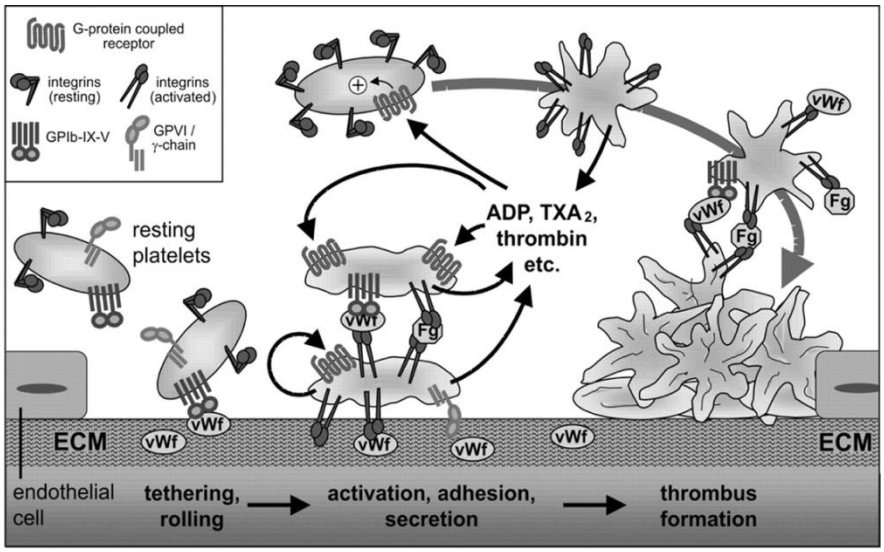


Figure 1.4. Platelet activation pathway. Adapted from Spiel et al, 2008 Circulation[2]

Platelet Aggregation

Platelet aggregation is a complex, biphasic process, which is tightly regulated in order to maintain hemostatic homeostasis. Upon activation, platelets will release a number of pro-thrombotic signaling molecules that propagate the platelet aggregation signal (Figure 1.6). Most commonly, platelets are activated by exposed collagen, a potent

platelet agonist, upon vessel injury. ADP, released from dense granules, stimulates platelet activation and aggregation by binding to the purinergic receptors $P2RY_1$ and $P2RY_{12}$ on the platelet cell membrane. Arachidonic acid (AA), released from platelet membrane phospholipids upon activation of phospholipase A2 (PLA2), is metabolized by cyclooxygenase 1 (COX-1) and thromboxane-A synthase 1 (TBXAS1) into thromboxane A_2 (TXA_2), and activates platelets through TXA_2 binding to the TXA_2 receptor. Platelet activation can also be stimulated by serotonin through the 5HT-2A serotonin receptors, thrombin through protease activated receptor 1 (PAR-1) binding, and epinephrine through alpha-2 adrenergic receptors[61].

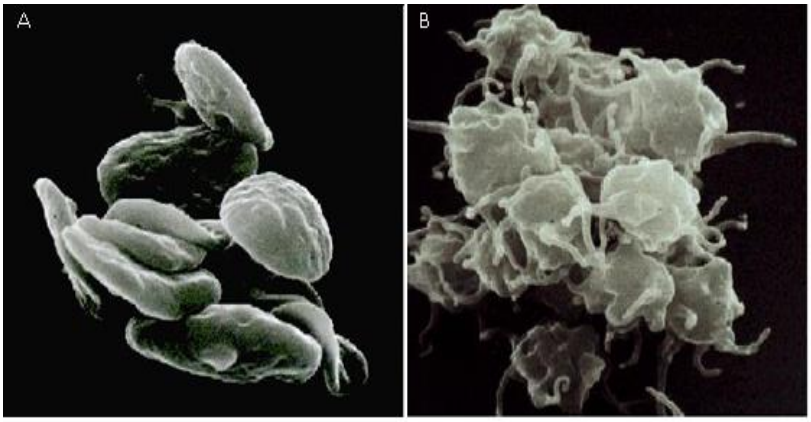


Figure 1.5. Resting (A) vs Activated (B) platelets as seen by electron microscopy. Adapted from Fritsma, Laboratory Evaluation of Hemostasis 2015[9]

In addition to agonist-induced platelet aggregation, platelets can be stimulated directly through contact activation, whether by other platelets, the endothelium, or other blood cell types. Platelet endothelial aggregation receptor 1(PEAR1) for instance, which will be discussed in detail below, is a cell surface protein that induces activation upon contact with neighboring platelets[1]. While each of these agonists, and cell-cell contact, stimulate platelets to varying degrees, the main effect of each is a rapid cytoskeletal reorganization resulting in a shape change, release of α-granules containing coagulation and adhesion factors, release of dense granules containing ADP, and calcium

mobilization, which triggers TXA_2 release, and activation of glycoprotein IIb/IIIa (GPIIb/IIIa) (Figure 1.6[12]). As additional platelets are recruited, GPIIb/IIIa initiates platelet-platelet contact through fibrinogen binding and stabilizes growing aggregates, while coagulation factors reinforce the growing thrombus through fibrin clot formation, trapping the platelets in place, restricting blood flow, and allowing the tissue to heal.

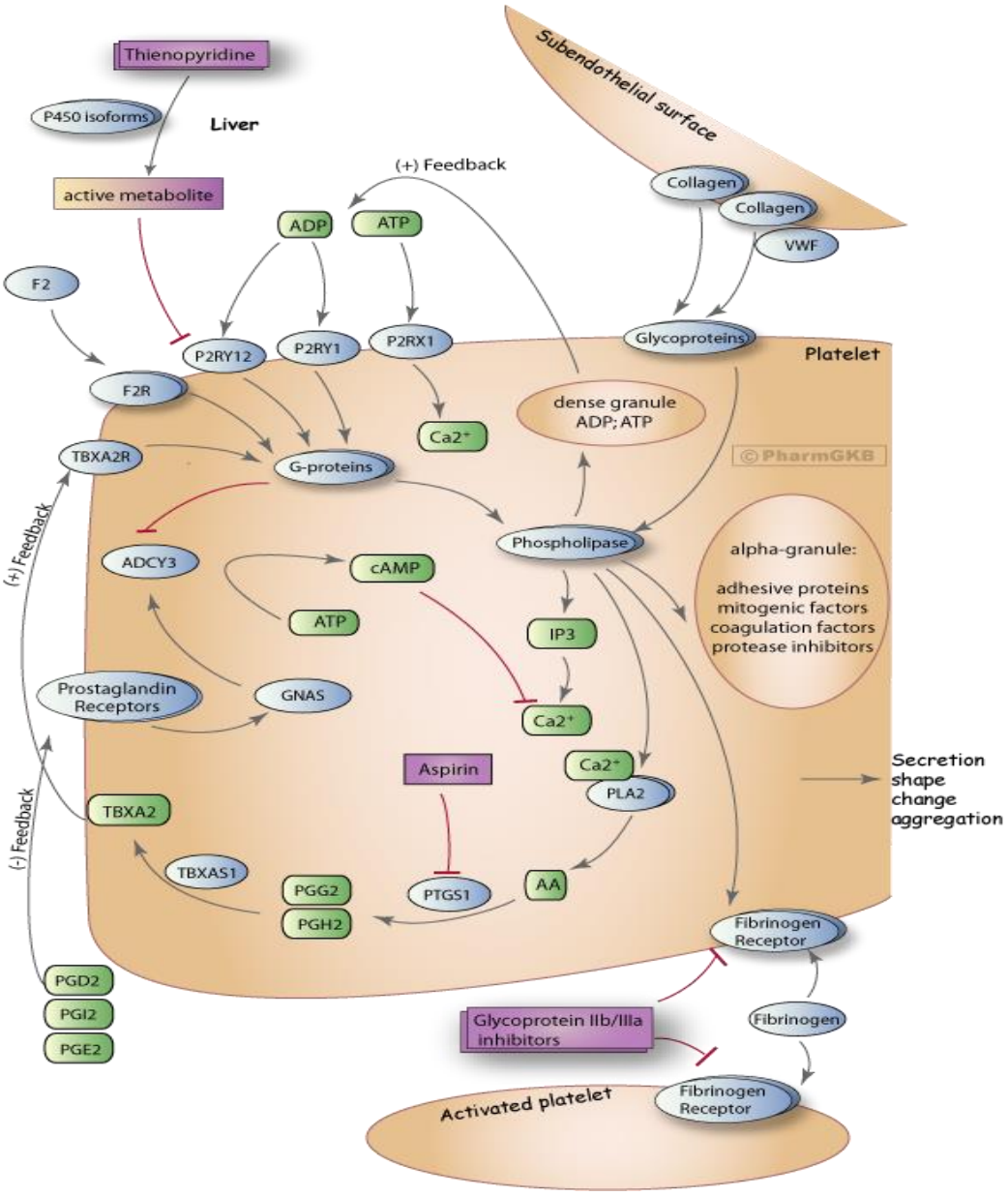


Figure 1.6. Platelet aggregation pathways and antiplatelet therapy targets. Reprinted by permission of the Pharmacogenomics Knowledge Base (PharmGKB) and Stanford University - copyright to PharmGKB (https://www.pharmgkb.org/pathway/PA154444041)[12]

Ex vivo Platelet Function Testing

Reduced platelet count or platelet reactivity may cause a pathologic risk of bleeding. Conversely, elevated platelet count or reactivity may trigger abnormal

thrombus formation. To evaluate platelet reactivity, and further, to evaluate antiplatelet efficacy, a number of different methods, such as flow cytometry and light transmission aggregometry have been utilized. The gold standard for assessment of platelet reactivity is light transmission aggregometry *ex vivo*, which measures platelet aggregation in response to numerous physiological agonists in platelet-rich plasma, as determined by the increase in light transmission through the sample, giving insight into the rate in which agonists form and the maximal percentage of total platelet aggregation. Maximal aggregation as assessed by light transmission aggregometry has been found to be predictive of major adverse cardiovascular events[62]. Additional *ex vivo* tests, such as VerifyNow, VASP, and PFA-100 have also demonstrated utility in predicting cardiovascular outcomes[62], although a high degree of heterogeneity has been observed between different platelet function methods[63]. Despite this correlation, *ex vivo* testing is not routinely used in the clinic for cardiovascular risk prediction or in dosing for antiplatelet therapy[64].

Platelet Biomarkers

In addition to *ex vivo* platelet aggregometry, additional metrics for assessing platelet function are necessary for improving clinical assessment of cardiovascular event risk. Thromboxane A_2 for instance, is rapidly released from activated platelets, and measure of this metabolite or a urinary derivative such at 11-dehyrdo-TXB2 allows the evaluation of platelet activity *in vivo*, as well as the assessment of adherence to antiplatelet therapy[62]. High levels of soluble CD40L (sCD40L) have been correlated with an increased risk of ACS. Following platelet activation, CD40L levels are greatly increased on the platelet surface, followed soon by cleavage that yields high sCD40L in

the blood[65]. Other circulating markers of platelet activation *in vivo* include Matrix metalloproteinases, SCUBE1, GPV, and GPVI[65].

Modifiers of Platelet Function

A number of factors have been found to influence platelet function. For example, platelet hyper-reactivity is greater in Africans than Europeans[66], and higher reactivity has also been observed in females and the elderly[67,68]. In addition, a number of factors including but not limited to lipid levels, hypertension, diabetes, smoking, and endothelial dysfunction all contribute to alterations in native platelet function[69-71]. Furthermore, genetic variation is a major modifier of platelet function, with heritability estimates of 0.44 to 0.62[69]. Through both functional studies and genome-wide approaches, dozens of genes with quite distinct roles have been implicated in altered platelet function, such as those coding for functional receptors (ex. *PEAR1*), regulators of cell morphology (ex. *SVIL*), intracellular signaling proteins (ex. *RAP1B*), and platelet receptor ligands (ex. *GP6*)[72]. Given the importance of atherothrombi formation in cardiovascular disease risk, as well as the role of platelets in the exacerbation of endothelial dysfunction through inflammatory mechanisms[73], a keen understanding of variability in platelet function is critical in CAD prevention and treatment.

CAD treatment

As primary prevention, patients at high risk for atherosclerosis are typically recommended lifestyle modifications such as a change in diet and exercise regimen, and may be prescribed medication to address underlying risk factors, such as hypertension and hyperlipidemia. However, once an atheroma has advanced to the point in which

blood flow is significantly impeded, the patient likely will require a PCI, often with stent placement, or an arterial bypass to re-open or circumvent the obstructed artery. A patient at this stage, whether receiving PCI or after a coronary event, will require antiplatelet therapy to prevent secondary cardiovascular and cerebrovascular events. Atherothrombotic events are the most significant danger for advanced atherosclerosis patients. Given the benefit of antiplatelet therapy in reducing thrombotic event rates in patients with atherosclerosis, a better understanding of the genetic variants that influence on-treatment endothelial function and platelet reactivity will provide novel insights into disease progression and medical management of these patients.

Antiplatelet Therapy

Abnormal thrombus formation is a complex biological process that contributes strongly to the risk of ACS. Reduction in arterial thrombus formation is medically managed through the use of antiplatelet agents such as aspirin and clopidogrel, usually in combination, in order to reduce MI and stroke risk in patients with cardiovascular disease. While anti-coagulants are typically used for venous thrombotic conditions, which are more fibrin-dependent, anti-thrombotic medications are typically used for arterial conditions such as acute coronary syndrome. Depending on clinical indication and stage of disorder, a particular antiplatelet therapy regimen may be advised. For primary prevention, aspirin may be prescribed alone, where it has been demonstrated to reduce myocardial infarction rates by 23 to 32%[74,75]. In patients undergoing PCI or for those following ACS, clopidogrel or another ADP receptor antagonist (i.e. ticagrelor or prasugrel) is typically used in combination with aspirin. Clopidogrel, a second-generation $P2Y_{12}$ inhibitor, is the cheapest ADP receptor antagonist available, and, despite the

advent of potentially more effective therapeutics[76,77], is still widely prescribed[78,79]. In a randomized trial of more than 12,000 ACS patients, it was observed that DAPT with clopidogrel and aspirin reduced the risk of myocardial infarction by 20% as compared to aspirin monotherapy (Figure 1.7)[5]. However, for patients who do not properly metabolize clopidogrel, an alternative and more expensive ADP inhibitor such as ticagrelor or prasugrel would be prescribed instead. Dual antiplatelet therapy with clopidogrel and aspirin has been the most common therapy for secondary prevention in the last decade; however, the risk of pathological bleeding increases when multiple thrombotic pathways are blocked. Other antiplatelet agents include Glycoprotein IIb/IIIa receptor inhibitors, which must be administered intravenously, PAR antagonists, and GPVI antagonists.

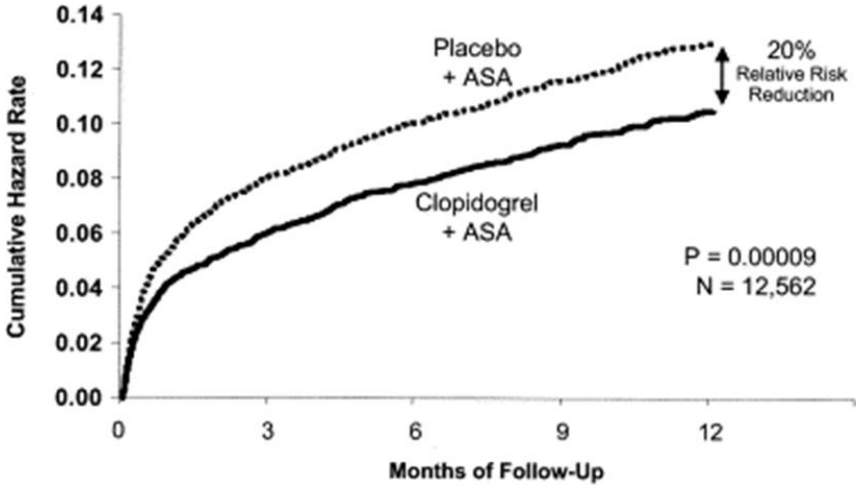


Figure 1.7. Randomized controlled trial of more than 11,000 subjects in the Cure Trial. Adapted from Cure Trial Investigators, et al, 2001 NEJM[5]

Clopidogrel

Clopidogrel is a thienopyridine prodrug that ultimately inhibits platelet aggregation by irreversibly binding to the $P2RY_{12}$ ADP receptor on the platelet surface[80]. Initially, clopidogrel is absorbed, in part, by intestinal P-glycoprotein (encoded by *ABCB1*) before being metabolized in the liver by cytochrome P450 enzymes, most notably CYP2C19, resulting in active metabolite formation and ultimately inhibition of

ADP-stimulated platelet aggregation (Figure 1.8)[11]. Concurrent with activation, clopidogrel is rapidly inactivated, namely by carboxylesterase 1 (CES1), and excreted from the bloodstream. Only about 15% of clopidogrel dose, on average, is available for inhibition of platelet aggregation. Four clopidogrel isomers are found at detectable concentrations in serum, with the H4 isomer being the only active clopidogrel metabolite. However, the extremely short half-life of the clopidogrel active metabolite (30 minutes[81]), in addition to the expertise and equipment required to properly assess metabolite concentrations, has limited the number of studies that have directly analyzed this phenotype. Instead, clopidogrel efficacy is typically assessed indirectly via *ex vivo* platelet aggregation. However, while this is informative, results of *ex vivo* aggregation studies do not correlate perfectly with on-clopidogrel outcomes, and the study of serum clopidogrel active metabolite, as a more proximal measure of clopidogrel pharmacokinetics, may have more utility in clopidogrel efficacy assessment.

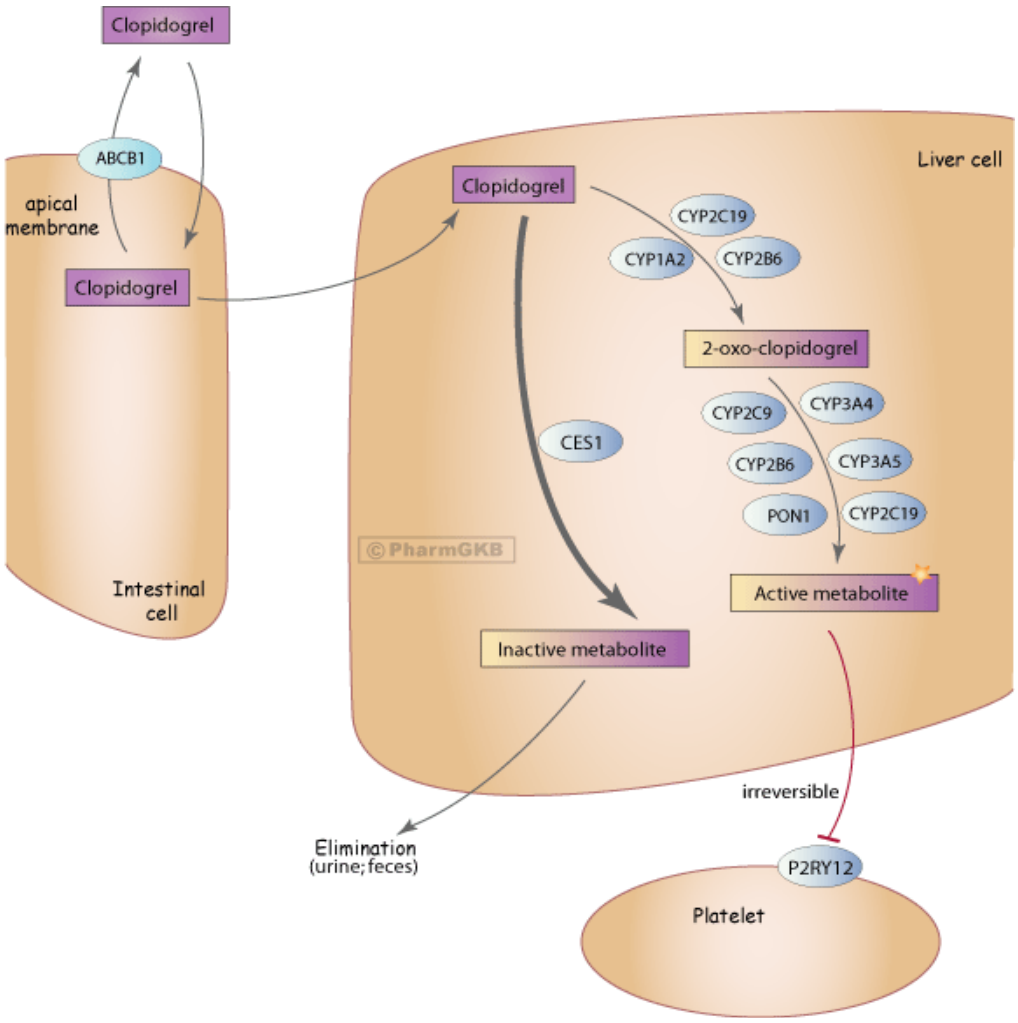


Figure 1.8. Major Proteins Involved in Clopidogrel Transport and Metabolism. Reprinted by permission of the Pharmacogenomics Knowledge Base (PharmGKB) and Stanford University - copyright to PharmGKB (http://www.pharmgkb.org/pathway/PA154424674)[11]

Clopidogrel Effect Modifiers

While clopidogrel is widely prescribed and largely successful at reducing ischemic events, substantial inter-individual variation in responsiveness to clopidogrel has been documented, and an estimated 30% of the patients are considered to be clopidogrel resistant[82](Figure 1.9[7]). In addition to higher reactivity at baseline, as described earlier, women under 50 also have higher on-clopidogrel platelet reactivity than men[68,83]. As previously mentioned, men have higher rates of CAD, while incidence of

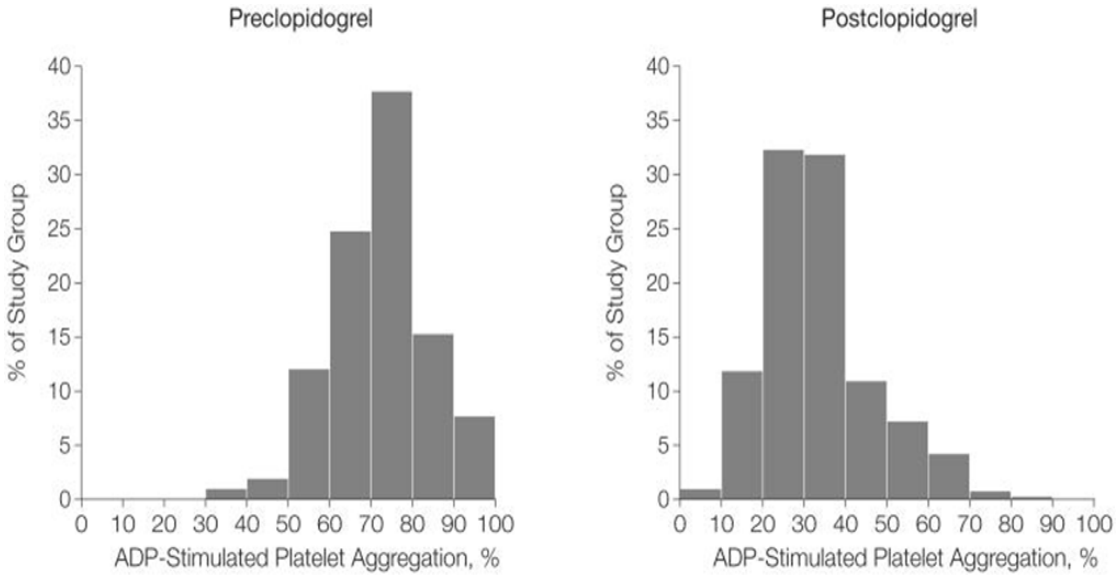


Figure 1.9. Variability in pre and post-clopidogrel ADP-stimulated platelet aggregation in healthy subjects from the PAPI Study. Adapted from Shuldiner et al, 2009 JAMA[7]

stroke is higher in women[44]. Further, while men derive benefit in reduction of stroke, myocardial infarction, and cardiovascular death rates on-clopidogrel, women may only obtain a significant reduction in myocardial infarction risk[84]. However, gender differences in on-clopidogrel clinical outcomes are highly debated[85], and although there is some evidence that estrogen influences on-clopidogrel platelet reactivity[68], further research is warranted to clarify the mechanisms behind the potential antiplatelet response differences noted. Additional comorbidities such as diabetes status, statin use, and patient BMI are also known to significantly impact on-clopidogrel platelet aggregation[86-88]. Furthermore, nicotine users tend to have a modest reduction in platelet reactivity with P2Y12 inhibition as compared to non-users, a phenomenon colloquially referred to as "smoker's paradox"[86,89].

In addition to non-genetic factors, recent studies have indicated that a large percentage of clopidogrel variation, up to 73% based on *ex vivo* platelet reactivity, is

heritable[7]. Therefore, significant effort has been made to identify the genetic factors that influence clopidogrel efficacy (Table 1.1[6]). For example, *ABCB1* C3435T results in reduced clopidogrel active metabolite levels in carriers, and therefore reduced effectiveness of clopidogrel[90]. Carriers of *CES1* G143E, a mutation in the major protein associated with deactivation and clearance of clopidogrel metabolites, have elevated levels of active metabolite, and therefore increased antiplatelet response[91]. The hallmark mutation for clopidogrel efficacy, *CYP2C19**2, a synonymous SNP in the major cytochrome P450 enzyme responsible for clopidogrel bioactivation, has repeatedly been linked to decreased clopidogrel active metabolite formation, increased on-treatment platelet aggregation, and altered clinical events rates in patients with cardiovascular disease, prompting the FDA to update the clopidogrel label to inform clinicians to consider alternative treatment for genetically susceptible patients[7,92-94]. Despite the large effect size for *CYP2C19**2, which accounts for about 12% of the heritable variation in clopidogrel response, as assessed by *ex vivo* platelet aggregation, a majority of the predicted genetic component for clopidogrel response remains unidentified. Furthermore, although additional variants in *CYP2C19* and other candidate genes such as *CYP2C9*, *CYP3A4*, *CES1*, *PON1* and *P2YR12* have been associated with variable on-clopidogrel platelet reactivity, few polymorphisms other than *CYP2C19**2 have been consistently linked with on-treatment reactivity and clinical outcomes[92,95,96].

To date, most studies that examine clopidogrel pharmacogenomics have used pharmacodynamic markers (i.e. platelet aggregation) as an endophenotype. In contrast, few studies have used clopidogrel active metabolite to evaluate pharmacogenomic determinants of clopidogrel efficacy. The evaluation of active metabolite level as a

biomarker is innovative and particularly informative as it enables researchers to focus almost exclusively on clopidogrel pharmacokinetics, with new discoveries leading to a more thorough understanding of the clopidogrel activation pathway. Thus, only by integrating pharmacokinetic parameters such as active metabolite data with pharmacodynamic data will we be better able to understand clopidogrel response variability and identify new genetic factors.

Table 1.1. Candidate SNPs previously associated with clopidogrel response.
Adapted from Gladding et al, 2008 J Am Coll Cardiol Intv[6]

Allele	SNP	dbSNP Reference Sequence No.	Functional Effect	Effect on Clopidogrel Response	Reference #
ABCB1*2	1236C>T	rs1128503	Increased intestinal efflux of drugs	—	—
	3435C>T	rs1045642		Negative	Taubert et al.
	2677G>T/A	rs2032582		—	—
CYP3A4*1B	−392A>G	rs2740574	Reduced enzyme function	None	Angiolillo et al.
CYP3A4*3	23171T>C	rs4986910	Reduced enzyme function	None	Angiolillo et al.
CYP3A4*4	13871A>G	—	Reduced enzyme function	—	—
CYP3A5*1	6986G>A 31611T>C	rs776746	Normal enzyme function	Positive	Suh et al.
CYP3A5*3	6986A>G	—	Nonfunctional enzyme	Negative	Suh et al.
CYP2C19*1	—	—	Normal enzyme function	Normal	Ronkes et al.
					Fontana et al.
					Hulot et al.
CYP2C19*2	19154G>A	rs4244285	Lack of enzyme activity	Negative	Brandt et al.
					Fontana et al.
					Hulot et al.
					Brandt et al.
CYP2C19*4	1A>G	rs28399504	Lack of enzyme activity	—	—
CYP2C19*17	−806C>T	rs12248560	Increased enzyme function	—	—
CYP2C9*1	—	—	Normal enzyme function	Normal	Brandt et al.
CYP2C9*2	3608C>T	rs1799853	Reduced enzyme function	Negative	Brandt et al.
CYP2C9*3	42614A>C	rs1057910	Reduced enzyme function	Negative	Brandt et al.
P2Y12 H2 haplotype	i-T744C	rs2046934	Increased platelet response to ADP	None	Von Beckerath et al.
CES2	g.275A>G	rs1464602	Reduced midazolam clearance	—	—
	Intronic SNP (IVS10-88)	rs3893757	Reduced mRNA gene expression	—	—

Non-platelet effects of clopidogrel

In an advanced atherosclerotic state, endothelial dysfunction promotes a pro-thrombotic environment, and activated platelets release inflammatory proteins that, in turn, promote endothelial dysfunction. Therefore, while clopidogrel is prescribed for its anti-thrombotic properties, reduced platelet activation by clopidogrel may also lead to improved endothelial health. This hypothesis was supported by Heitzer et al., who reported improved endothelial nitric oxide bioavailability in on-clopidogrel CAD patients, which they concluded to be due to reduced levels of oxidative stress and inflammatory biomarkers downstream of platelet $P2Y_{12}$ inhibition[97]. However, it has also been suggested that the positive effects of clopidogrel on adverse cardiovascular outcomes may also include platelet-independent effects. Willoughby et al. recently found microvascular endothelial function, as assessed by peripheral arterial tonometry, was significantly improved after clopidogrel treatment, and no correlation between endothelial function and ADP-induced platelet reactivity at baseline or in response to clopidogrel was observed[98].

While further investigation is warranted into the potential off-platelet effects of clopidogrel, additional metrics to assess clopidogrel efficacy, such as serum clopidogrel active metabolite concentration or endothelial biomarker testing, in conjunction *with ex vivo* platelet reactivity, may facilitate improved prediction of on-treatment outcomes clinically, as well as aid researchers in elucidating novel pharmacogenetics variants for clopidogrel.

Aspirin

Aspirin is indicated for pain relief, fever reduction, reduction of inflammation, and antiplatelet therapy. Cardiovascular disease, as previously noted, involves multiple tissues and cell types, including platelets and endothelium, and aspirin functions to reduce risk through both antiplatelet and anti-inflammatory mechanisms (Figure 1.10[3]). In contrast to clopidogrel, aspirin is ingested in an active form and does not need to be metabolized in order to work. Acetylsalicylic acid, the active ingredient in aspirin, works by inhibiting the cyclooxygenases, and reducing thromboxane and prostaglandin levels. At low doses, aspirin will acetylate COX1, thereby inhibiting arachidonic acid-stimulated platelet aggregation. Arachidonic acid, stored as phospholipids, is released downstream of platelet stimulation by other agonists as a method of positive feedback to induce and reinforce platelet aggregation[99]. As described above, arachidonic acid is metabolized by COX1 and TBXAS1 into thromboxane A_2, ultimately binding to the thromboxane A_2 receptors on platelet cell membranes to stimulate platelet aggregation. In addition, acetylation of COX-2 leads to the release of lipoxins from endothelial cells and the formation of NO-

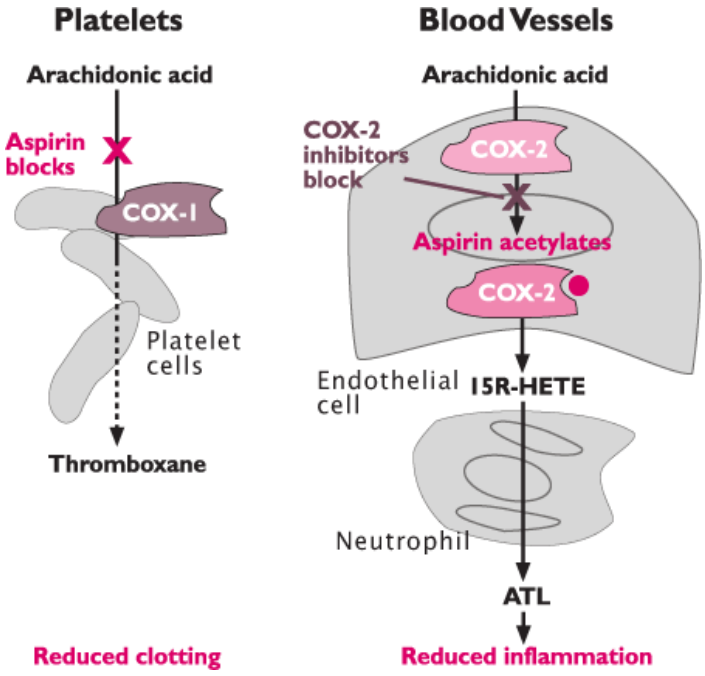


Figure 1.10. Effect of aspirin on platelets and endothelium. Adapted from Chiang et al. PNAS, 2004[3]

radicals, which lead to vasodilation, a reduction in inflammation, and improvement in endothelial health[100].

Pharmacogenomics of aspirin

It is estimated that 10% to 15% of the general population is resistant to aspirin, and similar to clopidogrel, traits such as age, sex, and diabetes status significantly influence aspirin response as assessed by *ex vivo* platelet reactivity assays and cardiovascular event rates[57,82]. Furthermore, the heritability of aspirin responsiveness has been estimated to be up to 77% as measured by thromboglobulin levels and 51% as measured by platelet aggregation[101]. GWAS and candidate gene studies have identified a number of loci associated with post-aspirin platelet response (Table 1.2)[14]. For, example, *GPIIIa*

Table 1.2. Polymorphisms in genes related to aspirin resistance that have had at least two studies published. Adapted from Goodman et al, 2008 British Journal of Clinical Pharmacology[14]

Gene	Polymorphism	Number of studies
COX-1	C22T	6
COX-1	C50T/A842G	8
COX-1	G128A	2
COX-1	C644A	4
COX-1	C714A	4
COX-1	C10427A	2
COX-1	G1446A	2
COX-2	G765C	2
GPIa	C807T	7
GPIbα	C5T	3
GPIIIa	T196C	19
GPVI	T13254C	2
FXIII	G34T	2
P2Y1	C893T	2
P2Y1	A1622G	3
P2Y12	H1/H2	4

P1A1/A2 and *COX1* A842G are two of the more well studied polymorphisms that have been linked to aspirin resistance through analysis of the COX1-dependent platelet aggregation pathway (arachidonic acid mediated)[14,102]. More recently, studies of the non-COX1-dependent platelet aggregation pathways have also yielded new insights into aspirin pharmacogenetics (Table 1.3)[4,103]. However, despite the fact that these genes are in pathways directly targeted by aspirin or are integral in platelet aggregation, results to

Table 1.3. Summary of multipoint linkage signals in African Americans from GeneStar cohort. Adapted from Mathias et al, 2010 BMC Med Genomics[4]

Significance Criteria	Chr	Map position	Marker	Factor
P < 0.00002	5	71.143	D5S628	4B(POST):ADP
	5	80.82	D5S2072	4B(POST):ADP
P < 0.00074	1	160.45	D1S484	2B(PRE):Collagen
	1	216.774	D1S245	4B(POST):ADP
	1	217.215	D1S205	4B(POST):ADP
	2	78.417	D2S2156	5B(POST):ADP PRP
	5	83.18814	D5S1967	4B(POST):ADP
	8	69.341	D8S1737	4B(POST):ADP
	11	154.04129	D11S969	4B(POST):ADP
	17	102.596	D17S940	5B(PP): ADP
	18	21.744	D18S967	7B(POST): ADP/Epinephrine
	19	99.84163	D19S572	4B(PRE):Collagen Lag

POST = Post-ASA Factor, PRE = Pre-ASA Factor, PP = Post-adjusted-for-pre-ASA Factor, Chr = chromosome

Significance criteria as defined by Lander and Kruglyak (1995): suggestive linkage (p-value < 7×10^{-4}) and significant linkage (p-value < 2×10^{-5}).

date have not been consistent, and the impact of these variants on clinical outcomes remains unclear[102,103]

Given the integral role of the endothelium on cardiovascular disease, and the anti-inflammatory effect of aspirin on endothelial health, platelet aggregation studies, while important for investigating the determinants of aspirin's antiplatelet action, are insufficient on their own to assess the genetics of aspirin response variability and the impact on survivability. A more comprehensive approach, including both platelet and endothelial studies, is required. With this in mind, interest is high in one particular candidate gene—*PEAR1*.

PEAR1

Genetic variation in *PEAR1*, a transmembrane protein highly expressed in both endothelial cells and platelets, have repeatedly been associated with altered baseline and on-aspirin platelet aggregation as well as cardiovascular event risk[8,104]. PEAR1 is a platelet contact receptor that, upon ligand binding, becomes phosphorylated, leading to downstream PI3K and AKT signaling and eventually the stabilization of platelet aggregates through activation of glycoprotein aIIbB3 (Figure 1.11[105]). One of the PEAR1 ligands in platelets has recently been identified as FcER1a, the alpha subunit of the IgE receptor[106]. However, the significance of this finding and how it may relate to variable platelet response to aspirin is not yet known. PEAR1 also has a role in megakaryopoeisis and thrombopoesis, with downregulation of PEAR1 expression leading to increased megakaryocyte maturation and platelet production[107].

In a previously published study by our group, the *PEAR1* rs12041331 A-allele was significantly associated with reduced collagen-stimulated platelet aggregation post-

DAPT ($P=7.66x10^{-9}$)[8]. Paradoxically, aspirin-treated rs12041331 A-allele carriers were at increased risk of MI (p=0.02) and cardiovascular outcomes (p=0.05) as compared to GG homozygotes in 1000 stable CAD patients from the INVEST-GENE study. Interestingly, this effect was not observed in untreated patients (Figure 1.12[8]). Given that PEAR1 protein expression is highest in endothelial cells[105], the observed, seemingly contradictory effect could potentially be due to post-aspirin endothelial differences, and not the observed platelet effect.

While the role of PEAR1 in platelets has been fairly well characterized, the function of PEAR1 in endothelial cells has only recently begun to be elucidated[108]. As previously mentioned, the health of the endothelium is integral to the progression of cardiovascular disease, especially as it pertains to atherosclerotic plaque formation. Recent evidence by our group suggests that *PEAR1* rs12041331 genotype significantly

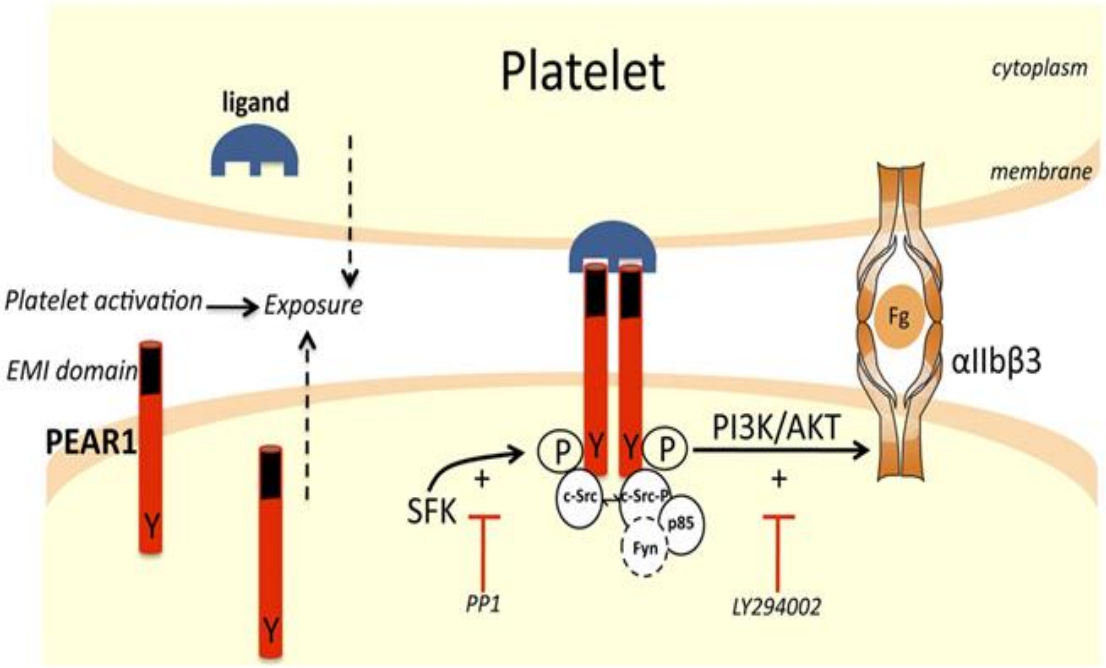


Figure 1.11. PEAR1 activation in Platelets. Adapted from Kauskot et al, 2012 Blood[1]

impacts flow mediated dilation of the brachial artery at baseline[53]. In addition, meta-analysis of publically available microarray data in NCBI GEO suggests PEAR1 significantly influences angiogenesis and wound healing, a finding supported by a PEAR1 knockout study by Vandenbriele et al in 2015[53].

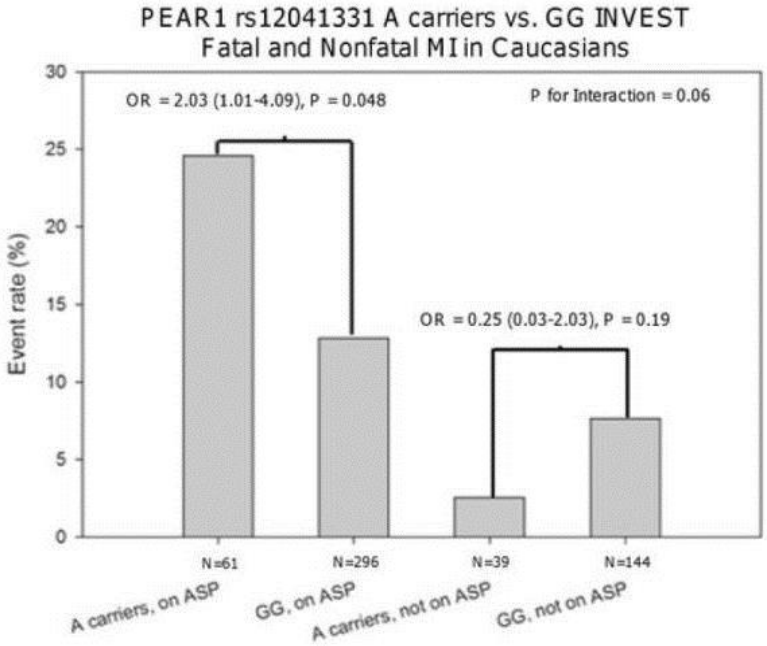


Figure 1.12. Association between *PEAR1* SNP rs12041331 A-allele carrier status and fatal and non-fatal myocardial infarction in aspirin-treated and non-treated European American patients of INVEST-GENES. Adapted from Lewis et al, 2013 Circulation Cardiovascular Genetics[8]

While antiplatelet agents such as aspirin reduce platelet aggregation, they also reduce cardiovascular disease risk through anti-inflammatory effects in other important cell types and tissues such as the vascular endothelium. Thus, given the anti-inflammatory characteristics of aspirin, further study of PEAR1 function in the vascular endothelium, with and without aspirin treatment, will be integral in understanding the role this protein plays in both baseline and on-treatment cardiovascular disease progression.

Summary

The etiology of atherosclerosis and CAD is incredibly complex, and despite the advent of tremendously efficacious antiplatelet therapeutics, cardiovascular disease is still the leading cause of death in the U.S. and worldwide. DAPT with clopidogrel and aspirin is the most widely used antiplatelet regimen, and a high degree of heritable variation in response to both drugs has been noted. To date, a number of clopidogrel response variants have been identified through *ex vivo* platelet aggregation. However, a large proportion of the heritable variation remains unaccounted for and new techniques may be required to elucidate novel pharmacogenomic determinants of clopidogrel efficacy. Further, although aspirin functions through both antiplatelet and anti-inflammatory mechanisms, most studies to date have utilized *ex vivo* platelet aggregation studies to evaluate aspirin response, and a relative dearth of notable pharmacogenetic variants for aspirin have been identified thus far. In this study, we employ novel methodologies to further elucidate the pharmacogenetics of antiplatelet response.

Chapter 2: Specific Aims

Cardiovascular disease is the leading cause of death worldwide with an estimated 17 million deaths annually, a number that is expected to grow to 23 million a year by 2030[15]. While primary prevention of myocardial infarction (MI) and stroke is largely composed of lifestyle modifications, patients with a prior history or current symptoms of cardiovascular disease require prescription treatment to reduce the risk of secondary events. The most commonly used dual antiplatelet therapy (DAPT) for secondary prevention of cardiovascular events in at-risk patients is concurrent administration of clopidogrel and aspirin. Although DAPT is widely prescribed and largely successful in reducing MI and stroke rates, there is significant inter-individual variability in response, resulting in sub-optimal therapy in some patients and an increased risk of recurrent events.

The overall goal of this study was to identify novel genetic determinants of DAPT response. Previous investigations have demonstrated that a number of variants, such as *CYP2C19**2, *ABCB1* C3435T, and *PEAR1* rs12041331, are significantly correlated with altered DAPT response as measured by *ex vivo* platelet aggregation, and significantly influence clinical outcomes[7,8,90] . However, heritability estimates suggest that most of the genetic determinants of variable DAPT response, as assessed by *ex vivo* platelet aggregation, remain unidentified[7,101,104]. Moreover, a majority of the variants that have been associated with altered platelet aggregation following DAPT have not been conclusively linked with clinical phenotypes.

Most large-scale pharmacogenomics studies of DAPT response have focused on platelet aggregation as the primary response phenotype. Though this is a logical sub-

clinical endpoint, additional metrics of DAPT efficacy may have utility in identifying novel variants associated with DAPT response. For example, measurement of clopidogrel active metabolite concentration is may be a more appropriate endophenotype to identify genetic variants associated with pharmacokinetic differences. Moreover, the genetic determinants of on-DAPT endothelial function are not traditionally assessed, but are likely critical in secondary thrombus formation. **I hypothesized that expanding our focus beyond platelet aggregation, by examining measures such as clopidogrel active metabolite concentration and endothelial function, would not only allow us to identify new genetic variants that influence DAPT response but also improve our understanding of currently established variants that significantly impact clinical outcomes.**

In this investigation, I leveraged substantial pre-existing genomic and phenotypic data from the Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study and the International Clopidogrel Pharmacogenomics Consortium (ICPC) in order to identify novel markers of DAPT response with clear clinical implications for cardiovascular outcomes risk. In addition, I aimed to clinically and functionally characterize the relationship between platelet aggregation and endothelial function through evaluation of a newly identified DAPT response effector, *PEAR1* rs12041331.

<u>Specific Aim 1:</u> Identify novel genetic variants that impact clopidogrel response variability.

Aim 1a: Conduct a genome-wide association study of serum clopidogrel active metabolite in 506 subjects from the PAPI Study. Common and rare variation will be

evaluated with Affymetrix genome-wide arrays and the Illumina Exome chip, respectively.

Aim 1b: Analyze candidate SNPs identified in Aim 1a for association with platelet aggregation and clinical endpoints (i.e. major adverse cardiovascular events (MACE), stent thrombosis, and adverse bleeding) in the ICPC, an independent population of more than 4100 DAPT-treated patients.

<u>Specific Aim 2:</u> Clinical outcomes and functional analysis of platelet endothelial aggregation receptor 1 (*PEAR1)* in endothelial cells and platelets.

Aim 2a: Evaluate differences in agonist-induced *ex vivo* platelet aggregation pre- and post-aspirin administration (81, 162, and 324 mg/d for 7d) in 67 healthy subjects stratified by *PEAR1* rs12041331 genotype. The primary clinical phenotype analyzed will be *ex vivo* agonist-induced platelet aggregation pre- and post-aspirin intervention. As secondary outcomes, we will conduct pre- and post-aspirin analysis of additional cardiovascular disease risk biomarkers including Urinary 11-dehydrothromboxane B_2, endothelin-1, endoglin, VCAM-1, and ICAM-1.

Aim 2b: Assess the functional impact of *PEAR1* on endothelial function in human umbilical vein endothelial cells (HUVECs) pre- and post-aspirin exposure. shRNAs will be used to knock down *PEAR1* in order to examine its role *o*n extra-cellular matrix adhesion (CytoSelect™ Cell Adhesion Assay Kit), endothelial migration (Wound Healing Assay), and proliferation (XTT Cell Proliferation Assay Kit).

Chapter 3: Significance and Rationale

The pharmacogenomics of clopidogrel and aspirin have been well studied for more than a decade, yet to date few actionable variants other than *CYP2C19**2 have been definitively linked to high on-treatment platelet reactivity and, in turn, incidence of recurrent cardiovascular events. Furthermore, while *ex* vivo platelet aggregation is the most commonly used method for assessment of antiplatelet efficacy for both research and clinical purposes, no particular methodology is currently established as the clinical standard, and further, there is no defined value for high on-treatment platelet reactivity. In addition, likely due to the complexity of arterial health, ex vivo platelet studies do not correlate perfectly with in vivo function. Inhibition of platelet aggregation is the direct mechanism by which DAPT reduces thrombotic events. However, it is clear platelet-independent effects, particularly for aspirin and potentially for clopidogrel, also influence cardiovascular health.

The work that is presented here is significant in that it accounts for multiple aspects of cardiovascular health including but not limited to *ex vivo* platelet function. The measure of serum clopidogrel active metabolite is a direct measure of clopidogrel pharmacokinetics. This is a more proximal phenotype than platelet aggregation, and may allow the identification of pharmacokinetic variants that do not reach multiple testing correction and would therefore be unidentified in classic platelet aggregation studies. This has implications in better defining the clopidogrel metabolic pathway and improving next-generation drug development for improved patient care in the future.

PEAR1 is one of few aspirin response variants that have been associated with altered cardiovascular outcomes. Given PEAR1 expression is highest in endothelial cells

and platelets, it is expected that PEAR1 may influence pathological disease risk through both tissues. In addition, the increased cardiovascular event risk in *PEAR1* rs12041331A-allele carriers is in the opposite direction than one would expect given the reduced platelet aggregation also observed in A-allele carriers. The analysis of PEAR1 function in the endothelium helps to clarify the mechanisms by which mutations in PEAR1 may impact cardiovascular health. In addition, it was unknown whether the dose of aspirin significantly impacts degree of response by rs12041331 genotype. A dose-response study, as was conducted here, allowed us to assess the feasibility of aspirin dose adjustment based on PEAR1 genotype clinically.

Given the widespread use of these agents, and the high degree of inter-individual variability, the research conducted in this dissertation will contribute to improved, personalized treatment for cardiovascular disease patients at risk of ischemic events in the future.

Chapter 4: Analysis of serum active metabolite identifies novel variants that influence clopidogrel pharmacokinetics

Abstract

Dual antiplatelet therapy (DAPT) with clopidogrel and aspirin is the most commonly used regimen for the secondary prevention of ischemic events in patients with acute coronary syndromes. However, substantial inter-individual variation in response to clopidogrel has been documented, resulting in sub-optimal therapy in some patients and an increased risk of recurrent events. Previous studies indicate that a significant portion of this variation is heritable and a number of genetic variants such as *CYP2C19**2 have been found to influence clopidogrel response. However, a substantial amount of the genetic heritability remains unaccounted for. In this study, we conducted the first genome-wide association study of circulating clopidogrel active metabolite levels in 513 healthy participants of the Pharmacogenomics of Antiplatelet Intervention (PAPI) study in order to more directly measure clopidogrel pharmacokinetics. Consistent with previous investigations, we observed that the *CYP2C19* locus was the strongest genetic determinant of clopidogrel active metabolite formation ($P = 9.5x10^{-15}$). In addition, we identified novel genome-wide significant signals on chromosome 3 (rs187941554, $P = 3.3x10^{-11}$), chromosome 17 (rs80343429, $P = 1.3x10^{-8}$), and chromosome 19 (rs142890248), as well as 6 additional loci that showed suggestive evidence of association ($P \leq 1x10^{-6}$).

Of the 9 novel loci identified, 4 (1p25, 5q35, 7q21, and 17q11) demonstrated nominal associations with on-clopidogrel ADP-mediated platelet aggregation in the

discovery cohort. While none of the identified loci exhibited strong associations with *ex vivo* platelet aggregation in an independent population, variants on chromosomes 1p25, 3p25, and 17q11 showed trends towards variable on-clopidogrel platelet aggregation. Further, the identified variants on chromosome 1p25 also demonstrated trends towards increased on-clopidogrel major adverse cardiovascular event (MACE) risk. While further investigation is warranted to validate the findings in this study, the analysis of non-traditional measures of clopidogrel response, such as clopidogrel active metabolite concentration, holds promise for further elucidating the clopidogrel metabolic pathway, contributing to improved anti-platelet drug development, and ultimately leading to better patient care in the future.

Introduction

Coronary artery disease, the most common form of cardiovascular disease, results in approximately 7.4 million deaths worldwide each year[16]. In order to prevent recurrent cardiovascular events, individuals with advanced cardiovascular disease are frequently prescribed dual anti-platelet therapy, most commonly with clopidogrel and aspirin. Clopidogrel is a thienopyridine prodrug that must be activated *in* vivo in order to exert its anti-platelet function. After absorption in the intestine, the clopidogrel prodrug is transported to the liver where it becomes biologically active through a two-step conversion involving several cytochrome P450 enzymes, most notably CYP2C19. The clopidogrel active metabolite is then transported to the bloodstream, where it reduces platelet reactivity by irreversibly binding the adenosine diphosphate (ADP) receptor, P2YR12, on the surface of circulating platelets[80]. While clopidogrel is largely effective at reducing platelet aggregation and recurrent ischemic events, up to 40% of patients do not receive adequate benefit from traditional clopidogrel doses[109,110]. Furthermore, heritability estimates suggest that up to 73% of the inter-individual variation in clopidogrel response, as measured by *ex vivo* platelet reactivity, is heritable[7]. However, aside from notable variants such as *CYP2C19**2, few polymorphisms have been clearly linked with clopidogrel response, suggesting that most of the genetic variation that leads to inter-individual variability in clopidogrel efficacy remains unidentified[7,91,95,111,112].

Most studies to date have utilized pharmacodynamic measures such as *ex* vivo platelet reactivity or thromboglobulin levels to assess clopidogrel response. However, despite strong scientific interest and multiple published investigations, elucidation of pharmacokinetic factors that influence clopidogrel metabolism remains incomplete. In

this study, we aimed to identify novel genetic variants that impact clopidogrel metabolism by performing the first genome-wide association study of circulating clopidogrel active metabolite concentration. While evaluation of clopidogrel active metabolite, as opposed to *ex vivo* platelet function tests, is a more direct measure of clopidogrel metabolism, it has remained underutilized, likely due to the instability of the clopidogrel active metabolite and intermediate metabolite (2-oxo-clopidogrel), which require rapid derivatization for accurate assessment. Herein, we measured circulating levels of clopidogrel prodrug and active metabolite in 513 healthy Old Order Amish subjects after 8 days of clopidogrel administration (a single 300 mg loading dose followed by 75 mg/d for 7 days). Furthermore, adenosine diphosphate (ADP)-induced platelet aggregation was measured pre-and post-clopidogrel treatment. In order to extend our findings, polymorphisms that significantly influenced clopidogrel active metabolite levels were assessed in 1,400 clopidogrel-treated patients of the International Clopidogrel Pharmacogenomics Consortium (ICPC) in order to evaluate the effect of these variants on ADP-induced platelet aggregation in patients with severe cardiovascular burden as well as to gain insights regarding their impact on clinical endpoints.

Methods

PAPI Study

Participant Characteristics:

As part of the Pharmacogenomics of Antiplatelet Intervention (PAPI) Study, 687 individuals were recruited from the Old Order Amish community of Lancaster, Pennsylvania between August 2006 and January 2012[7,113]. Of these participants, a subset of 513 had clopidogrel active metabolite measured. Participants were over age 20, relatively healthy and agreed to discontinue the use of all prescription medications, supplements, and vitamins at least one week prior to the start of the study. Medical and family history, anthropometry, physical examinations, and blood samples were all taken after an overnight fast (Table 4.1). Exclusion criteria for this study included: blood pressure > 160/95 mmHg, currently pregnant or nursing, platelet count > 500,000/μl or < 75,000/μl, hematocrit < 32%, TSH < 0.05 or > 5.5 mU/L, AST or ALT > 2 times the upper normal limit, creatinine > 2.0 mg/dl, surgery in the past 6 months, history of gastrointestinal bleeding, allergy to clopidogrel or aspirin, inability to safely withdraw from medication use, or any other coexisting malignancy that might interfere with the study[113]. After baseline platelet aggregation measurements were recorded, participants were given a 300-mg oral loading dose of clopidogrel followed by 75 mg per day for 7 days (Figure 4.1). Follow-up platelet aggregation and clopidogrel metabolite measurements were obtained 1 hour following the last dose of clopidogrel (Figure 4.1). Additional information regarding recruitment and design of the PAPI Study has been previously described [7,113]. All study protocols were approved by the institutional review board at the University of Maryland, Baltimore and adhered to the principles of the

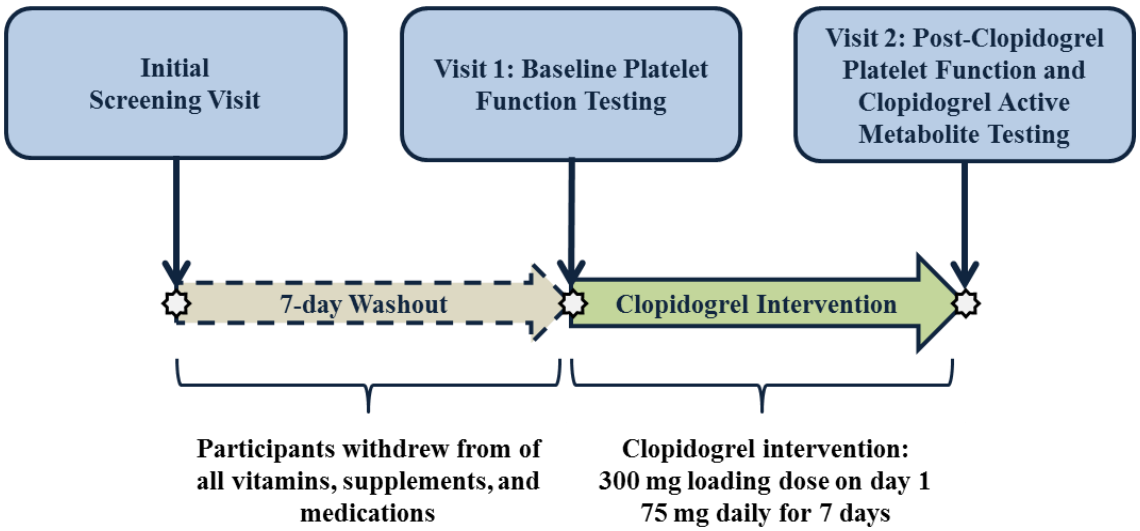


Figure 4.1. Design of the Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study.

Declaration of Helsinki. Written informed consent was obtained from each participant, and subjects were compensated for their participation.

Genotyping:

Genome-wide genotyping was performed with Affymetrix 500k and 6.0 arrays (Affymetrix Inc., Santa Clara, CA, USA), resulting in 410,375 SNPs genotyped in study participants Variants were called using Birdseed V2 and BRLMM. Imputation was performed using the 1000 Genomes Project "Cosmopolitan" reference panel (Phase I version 3, released March 2012) with Impute2[114] and ShapeIt[115] to increase the effective genome coverage to approximately 7.8 million SNPs. Polymorphisms with an imputation quality score greater than or equal to 0.5 and a minor allele frequency of at least 1% were included in this investigation.

Additionally, genotyping was performed in the same subjects using the Illumina Exome Genotyping Chip (Illumina Inc., San Diego, CA, USA), which contains over 240,000 variants that were selected from ~12,000 sequenced genomes and exomes as part

of the NHLBI, GO Exome Sequencing Project's (ESP) SNP selection project[116]. A large proportion of the variants on the exome chip are non-synonymous, splice, and stop variants and were identified in multiple studies to ensure validity. Variants were called using Illumina GenCall Data Analysis software. In total, 44,230 polymorphic variants were identified in the Amish with a total genotyping call rate of 99.83% and a concordance rate of 99.99% in a subset of duplicate samples. Of these, 33,235 polymorphisms were present at a minor allele frequency of at least 1% and were included in this investigation.

We validated our most significant imputation results (rs111985173 and rs80343429) through direct genotyping using a TaqMan® SNP genotyping assay performed on an Applied Biosystems 7900HT Fast Real-Time PCR system according to the manufacturer's protocol (Life Technologies, Foster City, CA, USA). Briefly, a volume of 5 µl consisting of 5 ng of dried genomic DNA, 2.5 µL of TaqMan® Universal PCR Master Mix (2X), 0.06 µL of 40X probe mix (FAM, 3′-TTACAATGTTTCAAATTGTTTTGAA-5′; VIC, 5′-GAAACAGAAGAAGAAGCTTGCAGCC-3′ for rs111985173 and FAM, 3′-GGAATTGAAATATGGCCGGAGCCTG-5′; VIC, 5′-GACATGAAACGGTACAGCTCATTTC-3′ for rs80343429), and 2.4 µL of DNase-free water were mixed in each well of a 384-well genotyping plate (then cycled for qPCR with the following settings: 50^0 for 2 minutes, 95^0 for 10 minutes, 45 cycles of 95^0 for 15 seconds followed by 60^0 for 1 minute. TaqMan® Genotyper Software version 1.3 was used to analyze raw read data using the autocalling method. The overall call rate of

genotyping was greater than 98% for both SNPs. The genotype concordance rate was greater than 100% in a subset of duplicate samples for both SNPs.

Clopidogrel Metabolite quantification:

Clopidogrel active metabolite and prodrug levels were assessed in a subset of 513 PAPI Study participants as previously described[91]. Briefly, blood samples from each participant were collected within 1 hour after the last dose of clopidogrel into EDTA tubes containing 2mmol/l (E)-2-bromo-3′-methoxyacetophenone (MPB; Sigma Aldrich, St Louis, Missouri, USA) to derivatize the clopidogrel active metabolite. Plasma levels of clopidogrel MPB-derivatized active metabolite and parent drug were assessed using an ultra-high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) assay with an active metabolite calibration range of 0.1–150 ng/ml and a prodrug range of 0.1-50 ng/ml. Ice-cold acetonitrile (500 µl, Fisher Scientific, Fairlawn, New Jersey, USA) containing 15 ng/ml of the internal standard (IS) ticlopidine (Sigma Aldrich, St. Louis, Missouri, USA) was added to 50 µl of plasma to precipitate plasma proteins. After vortex mixing (30 s) and centrifugation (11,700g for 10 min), 5.0 µl of the resulting supernatant was injected into a Waters Acquity UPLC system (Waters Corporation, Milford, Massachusetts, USA) for chromatographic separation before detection by tandem mass spectrometry (MS/MS) using an AB Sciex Qtrap 5500 (AB Sciex, Foster City, California, USA). Chromatographic separation of the analytes, clopidogrel prodrug, and active metabolite from IS and matrix interferences was carried out using a gradient with 0.1% formic acid (aqueous) and 0.1% formic acid in acetonitrile. Three multiple reaction monitoring transitions were performed simultaneously with dwell times of 200 ms each. Clopidogrel prodrug was identified

selectively by the transition of its prodrug to product ion at m/z 322>212, active metabolite at m/z 504>155, and ticlopidine (IS) at m/z 264>154. Multiple reaction monitoring peak integrations and data analysis were carried out using the MultiQuant algorithm from MultiQuant 4.0 (Analyst; AB Sciex). The total run time was 1.5 min.

Platelet Aggregation:

Platelet-rich plasma (PRP) was isolated from blood samples drawn into tubes containing 3.2% sodium citrate (Becton-Dickinson, Franklin Lakes, NJ, USA) and platelet counts were adjusted to200,000 platelets/μl using platelet-poor plasma (PPP). Platelet function was measured by optical aggregometry with a PAP8E aggregometer (Bio/Data Corporation, Horsham, Pennsylvania) stimulated with ADP (20 μmol/L, Chronolog Corp., Havertown, PA, USA)) and was expressed as the maximal percentage change in light transmittance using PPP as a referent.

GWAS Analysis:

Genome-wide analyses of clopidogrel active metabolite concentration and on-clopidogrel ADP-mediated platelet aggregation were conducted with a variance component method using the analysis program Mixed Model Analysis for Pedigrees (MMAP) (http://edn.som.umaryland.edu/mmap/). MMAP, which has been extensively used for genomic analyses in the Amish [7,8,117,118], accounts for the relatedness of study subjects by conditioning the genotype-phenotype correlations on the phenotypic correlations among relative pairs. The model for the polygenic component was established by constructing a relationship matrix derived from the complete Amish pedigree structure available through published genealogical records maintained by the church [119]. Association analysis for clopidogrel active metabolite was conducted under an

additive model simultaneously adjusted for age, sex, and relatedness among study participants. On-clopidogrel ADP-mediated platelet aggregation was conducted under an additive model and adjusted for age, sex, relatedness, and baseline ADP-mediated platelet aggregation. Variation in clopidogrel prodrug concentration was assessed for each of the top GWAS signals and conditional analyses were conducted with adjustment for the index SNP in each locus. Additionally, a bivariate approach using canonical correlation analysis implemented in plink was used to analyze variants (https://genepi.qimr.edu.au/staff/manuelF/multivariate/main.html).To correct for multiple comparisons a genome-wide significance threshold of $P = 5 \times 10^{-8}$ was used for all tests.

Exome Chip Analysis:

Single variant regression analyses between variants present at a minor allele frequency greater than 0.5% and clopidogrel active metabolite concentrations and ADP-mediated platelet aggregation were conducted using MMAP as described for GWAS analysis. The primary association analysis was under an additive model simultaneously adjusted for age, sex, and relatedness among study participants for active metabolite, and age, sex, relatedness, and baseline aggregation for on-clopidogrel ADP-mediated platelet aggregation. A significance level of $\alpha = 1.5 \times 10^{-6}$ (0.05/33,235 SNPs) was used to correct for multiple comparisons testing. Gene-based burden testing of exonic variants was conducted using sequence kernel association testing ($SKAT^{120}$) and implemented through the MMAP program. In brief, trait residuals are generated by accounting for relatedness and then used to conduct the gene-based burden testing. Genes with at least two missense, nonsense or splice-altering variants were tested. To correct for multiple comparisons, a genome-wide threshold of 6.8×10^{-6} was established (0.05/7,359 genes).

SNP Selection

Correlated SNP sets for loci strongly associated with clopidogrel active metabolite concentration were developed by assessing linkage disequilibrium between the index SNP and all SNPs with a p-value for association less than or equal to 1×10^{-6}. SNPs with D' >= 0.9 with the index SNP in each locus were included in the SNP set. Multiple testing correction for follow-up of top hits in the International Clopidogrel Pharmacogenomics Consortium (ICPC) was determined by the number of correlated SNP sets.

ICPC – extension study

Participant Characteristics and Phenotyping:

The ICPC was developed with the goal of identifying novel genetic determinants of clopidogrel response and clinical outcomes through GWAS and candidate gene approaches. More than 8,800 cardiovascular disease patients on dual-antiplatelet therapy with aspirin and clopidogrel are enrolled in this study through the collaboration of 18 centers from 11 countries. Among these extensively phenotyped patients, 6,280 have provided DNA for genomic analysis. Multiple platelet-related and clinical event phenotypes are available for analysis in ICPC. Platelet aggregation phenotyping was completed at the individual study sites and a standardized phenotype for ADP-mediated platelet aggregation was created and is available for 4,058 subjects with DNA available for genomic analysis. Incidence of major adverse cardiovascular events (MACE), defined as fatal and non-fatal stroke, myocardial infarction, stent thrombosis, and cardiovascular-related death, is available for more than 3,000 ICPC participants. Phenotype and genome-wide genotyping data is curated and available at PharmGKB.

Genotyping:

Genotyping was performed on 2,734 ICPC DNA samples with the Illumina (Illumina, Inc. San Diego, CA USA) Human Omni Express Exome v1.2 Chip, yielding 586,379 SNPs with minor allele frequency greater than 0.05. The total genotyping call rate was 99.96%. Additionally, uncalled genotypes for ICPC were imputed using the 1000 genomes "Cosmopolitan" reference panel (Phase I version 3, released March 2012) with Impute2[114] and ShapeIt[115]. This yielded 9,702,644 SNPs with a minor allele frequency greater than or equal to 1% and an imputation quality score of at least 0.5.

Statistical Analysis:

Analyses of platelet aggregation and clinical endpoints were restricted to European samples of the ICPC. Linear regression analyses were used to determine the relationship between genotype and platelet aggregation. Models are adjusted for age, sex, and first four principal components. ADP-mediated platelet aggregation was measured at each respective study site and then normalized and standardized for analysis with Plink[121]. Principal components for the subjects included in analyses were calculated with GCTA (http://cnsgenomics.com/software/gcta/pca.html)[122]. Logistic regressions to determine the relationship between genotypes and clinical events using age, sex, and the first four principal components as covariates are also completed in Plink.

Results

Participant Characteristics:

In total, 513 subjects (257 males and 256 females) participated in this investigation. All participants were generally healthy, with low incidences of hypertension and diabetes, although BMI and lipid levels were slightly elevated (Table 4.1)[123]. Overall, approximately 10% of participants were active smokers; however, this was driven entirely by men, who smoke at rates comparable with the outbred population[124], while Old Order Amish women, keeping with cultural norms, typically do not smoke[125]. No anthropometric measures were significantly correlated with clopidogrel active metabolite concentration with the exception of BMI and platelet count ($P = 9.6 \times 10^{-3}$ and 3.4×10^{-2}, respectively). On-clopidogrel ADP-mediated platelet aggregation was significantly associated with gender, age, BMI, platelet count, cholesterol levels and triglycerides (6.5×10^{-3}, 1.0×10^{-5}, 3.1×10^{-3}, 1.6×10^{-4}, 1.8×10^{-2}, and 1.5×10^{-2}, respectively).

Table 4.1: PAPI participant characteristics

Characteristics (units)	Total (n=513)	Females (n=256)	Males (n=257)
Age(years)*	44.9 ± 13.2	45.6 ± 13.6	44.2 ± 12.7
BMI (kg/m^2)*†	27.1 ± 4.7	28.2 ± 5.4	26.0 ± 3.6
Platelet Count (thousands)*†	239.7 ± 48.2	245.7 ± 51.3	234.1 ± 44.3
SBP (mmHg)	116.8 ± 12.3	117.0 ± 13.1	116.6 ± 11.4
DBP (mmHg)	70.2 ± 7.1	69.6 ± 7.0	70.8 ± 7.2
Cholesterol (mg/dl)*	208.9 ± 46.8	210.0 ± 49.4	207.6 ± 43.5
LDL (mg/dl)*	135.9 ± 42.7	132.6 ± 44.7	138.6 ± 40.2
HDL (mg/dl)*	58.7 ± 15.2	62.4 ± 15.5	55.6 ± 14.5
Triglycerides (mg/dl)*	71.4 ± 40.2	74.9 ± 42.1	67.3 ± 37.3
Diabetes (%)	0.4%	0.4%	0.4%
Current Smoker (%)	10.3%	0.0%	20.5%

Values are listed as mean ± standard deviation or percent
**Association with on-clopidogrel ADP-stimulated platelet aggregation P < 0.05 in full cohort*
†Association with clopidogrel active metabolite P < 0.05 in full cohort

Heritability and Correlation of clopidogrel-related traits

In concordance with previous estimates, the residual heritability of on-clopidogrel ADP-mediated platelet aggregation was estimated to be 75% after adjustment for age, sex, baseline platelet aggregation, and family relatedness ($P = 2.3 \times 10^{-13}$)[7]. Residual heritability of serum clopidogrel active metabolite concentration after adjustment for age, sex, and relatedness was estimated at 26% ($P = 3.1 \times 10^{-3}$). Clopidogrel prodrug levels were not heritable in this population (0%, P = 1.0).

As expected, clopidogrel active metabolite concentration was significantly correlated with on-clopidogrel ADP-mediated platelet aggregation ($r^2 = 0.12$, $P = 2.2 \times 10^{-16}$). However, clopidogrel prodrug levels were not correlated with on-clopidogrel

ADP-mediated platelet aggregation ($r^2 = 0.001$, P = 0.40) and only modestly correlated with clopidogrel active metabolite concentration ($r^2 = 0.01$, P = 0.01).

GWAS of Serum Clopidogrel Active Metabolite Concentration

Using a genome-wide approach, we tested for association between 7,884,700 SNPs and clopidogrel active metabolite concentration in 513 PAPI Study subjects while simultaneously adjusting for age, sex, and relatedness (Figure 4.2). A locus on chromosome 10 near the *CYP2C9-CYP2C18-CYP2C19* gene cluster was significantly associated with clopidogrel active metabolite concentration (rs137891020, $P = 9.5 \times 10^{-15}$). After adjustment for the known *CYP2C19**2 variant, no variants in this region reached genome-wide significance (Top remaining signal was rs137891020, $P = 9.1 \times 10^{-4}$). In addition to *CYP2C19**2, novel variants significantly impacted clopidogrel active metabolite concentration on chromosome 3p25 (strongest signal was rs187941554, $P = 3.3 \times 10^{-11}$) (Figure 4.3) and chromosome 17q11 (strongest signal was rs80343429, $P = 1.3 \times 10^{-8}$) (Figure 4.4). Another 6 independent loci exhibited suggestive evidence of association ($P \leq 1.0 \times 10^{-6}$) (Table 4.2). Conditional analyses that adjusted for the most significantly associated SNP at each locus fully accounted for the associations observed at all loci (Table 4.3). In addition, all index variants that exceeded genome-wide significance or showed suggestive evidence of association ($P \leq 1.0 \times 10^{-6}$) were tested to see if they impacted on-clopidogrel ADP-mediated platelet aggregation. While no evidence of association was observed between on-clopidogrel platelet aggregation and the chromosome 3p25 locus, rs80343429 on chromosome 17q11 as well as 3 other suggestive loci (rs72392086, rs73407739, and rs6892003 on chromosomes 1q25, 7q21, and 5q32, respectively) reached a nominal level of significance ($P < 0.05$) (Table 4.2).

To verify the significant signals identified through the GWAS of clopidogrel active metabolite, highly associated imputed variants on chromosome 3 (rs111985173, a proxy for rs187941554) and chromosome 17 (rs80434329) were Taqman genotyped in the full cohort of 513. Concordance between imputation and Taqman genotyping was greater than 98% for both SNPs. Accordingly, the association with active metabolite was still quite strong after direct genotyping, with a $P = 5.2 \times 10^{-7}$ for rs111985173 and $P = 1.4 \times 10^{-7}$ for rs80434329.

GWAS of On-Clopidogrel ADP-Mediated Aggregation

A GWAS of *ex* vivo ADP-mediated platelet aggregation in the same 513 PAPI Study subjects again identified *CYP2C19**2 as the strongest determinant of high on-treatment platelet reactivity in the Old Order Amish (chr10:96609093, $P = 1.3 \times 10^{-17}$) (Figure 4.5). After adjustment for *CYP2C19**2, no variants on chromosome 10q24 reached genome-wide significance (Top remaining signal was chr10:96609093, $P = 6.4 \times 10^{-6}$). Furthermore, no other loci were significantly associated with on-clopidogrel ADP-mediated platelet aggregation after correction for multiple testing.

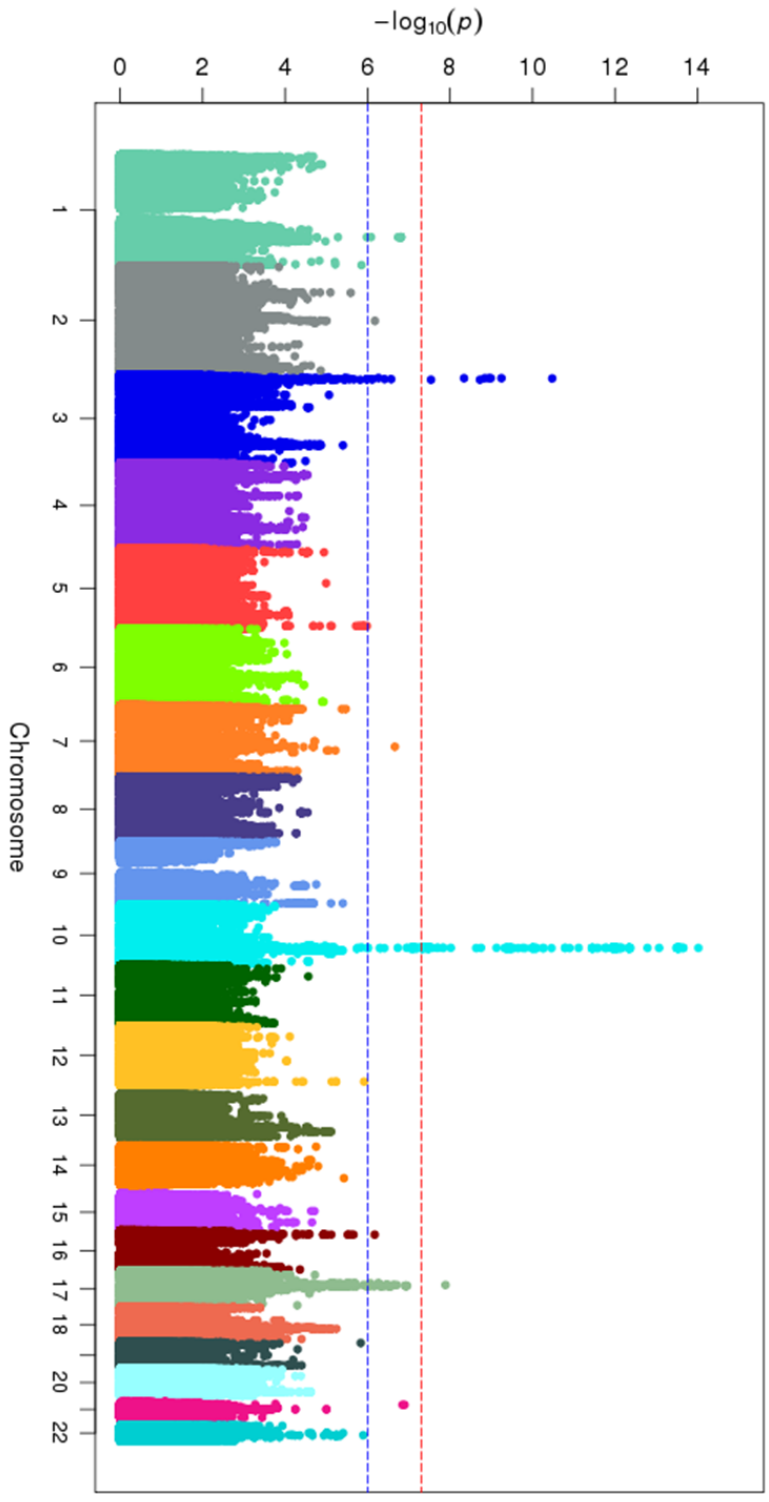


Figure 4.2. Manhattan plot of serum clopidogrel active metabolite concentration as measured by HPLC MS/MS after 7-day 75-mg/day clopidogrel intervention.
N=513. Red line indicates $P < 5 \times 10^{-8}$. Blue line indicates $P < 1 \times 10^{-6}$.

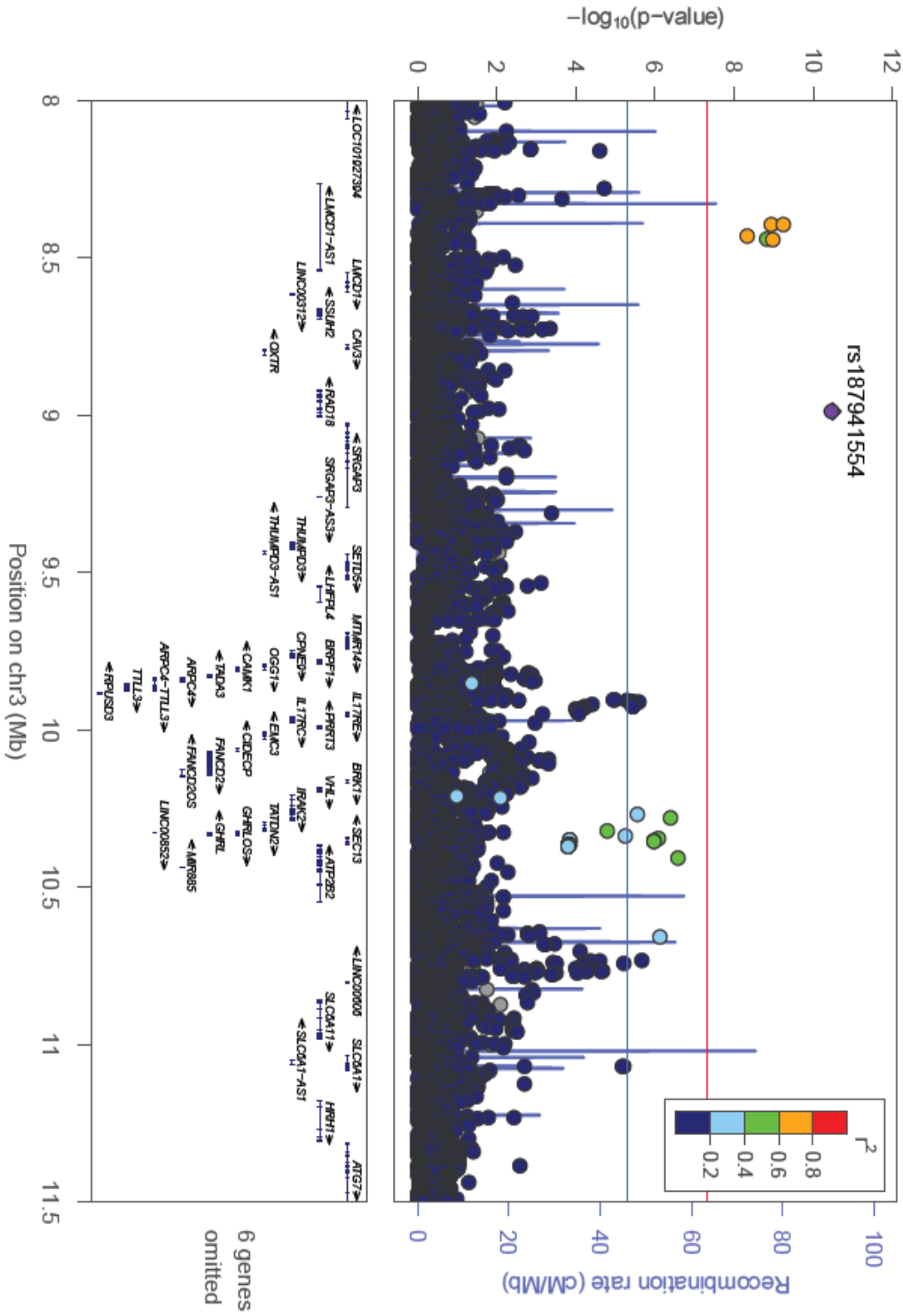


Figure 4.3. LocusZoom plot of the chromosome 3 locus (8MB-11.5MB) identified in the association analysis of clopidogrel active metabolite concentration in the PAPI Study. N=513. Red line indicates $P < 5 \times 10^{-8}$. Blue line indicates $P < 1 \times 10^{-6}$.

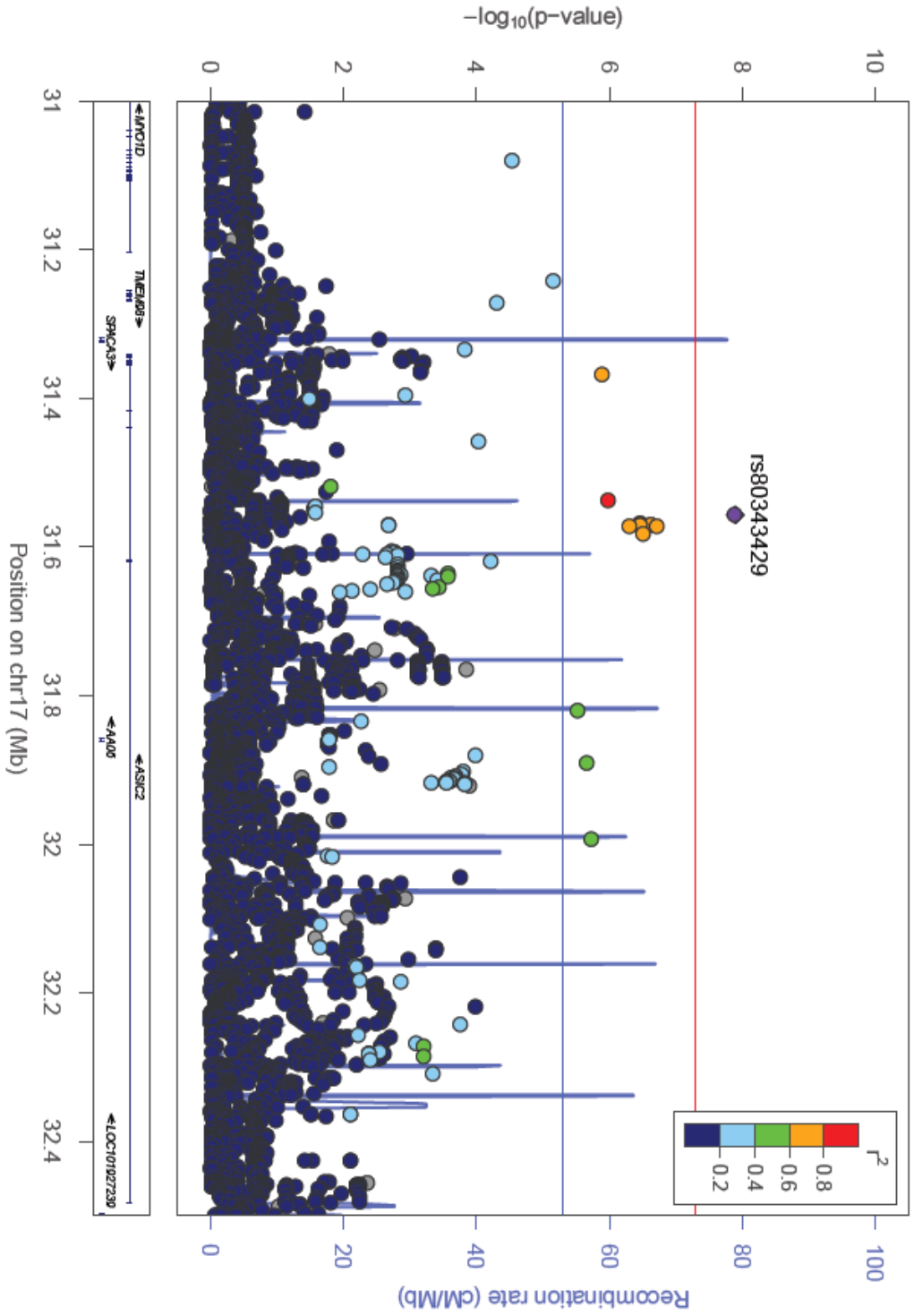


Figure 4.4. LocusZoom plot of the chromosome 17 locus (31MB-32.5MB) identified in the association analysis of clopidogrel active metabolite concentration in the PAPI Study. N=513. Red line indicates $P < 5 \times 10^{-8}$. Blue line indicates $P < 1 \times 10^{-6}$.

Table 4.2. Regions in genome with suggestive associations ($P \leq 1.0 \times 10^{-6}$) with clopidogrel active metabolite concentration. Abbreviations: MAF, minor allele frequency; Chr:Pos, chromosome and position; P, p-value.

SNP	Chr:Pos	MAF	Gene	Beta—Active Metabolite	P—Active Metabolite	Beta—On-Clopidogrel Platelet Aggregation	P—On-clopidogrel Platelet Aggregation
rs111985173	3:8441942	0.01	LMCD1-AS1	16.8±2.7	1.00×10^{-9}	-0.9 ± 3.4	8.00×10^{-1}
rs80343429	17:31556681	0.05	ASIC2	8.1±1.4	1.30×10^{-8}	-3.5 ± 1.7	4.30×10^{-2}
rs79172967	21:18571112	0.02		15.5±2.9	1.30×10^{-7}	-2.2 ± 3.7	5.50×10^{-1}
rs72392086	1:185033816	0.04	RNF2	7.6±1.4	1.60×10^{-7}	-5.0 ± 1.8	5.32×10^{-3}
rs73407739	7:92491283	0.01	LOC101927497	16.8±3.2	2.20×10^{-7}	-11.9 ± 3.9	2.23×10^{-3}
rs181524103	2:123209400	0.03		12.0±2.4	6.60×10^{-7}	-3.1 ± 3.0	3.10×10^{-1}
rs138852022	16:9155981	0.01		17.0±3.4	6.70×10^{-7}	-6.9 ± 4.1	9.30×10^{-2}
rs6892003	5:174191465	0.29		-3.3±0.7	1.00×10^{-6}	2.2 ± 0.9	9.67×10^{-3}

Table 4.3. Top remaining SNP in each locus associated with clopidogrel active metabolite in the PAPI Study after conditioning on the original index SNP.
Abbreviations: MAF, minor allele frequency; Chr:Pos, chromosome and position; P, p-

SNP	Chr:Pos	MAF	Gene	Beta—Active Metabolite	P—Active Metabolite
rs6766130	3:11625202	0.08	VGLL4	-5.31±1.2	8.96×10^{-6}
rs116990978	17:32869119	0.03		5.58±1.8	2.17×10^{-3}
rs2823968	21:18050085	0.39		-2.23±0.7	1.62×10^{-3}
rs145982435	1:185352761	0.02		6.38±2.5	9.89×10^{-3}
rs73219929	7:92990291	0.01	CCDC132	14.6±4.3	6.44×10^{-4}
rs296072	2:123650738	0.4		1.85±0.6	3.94×10^{-3}
rs35614103	16:9155981	0.06		5.3±1.7	1.83×10^{-3}
rs1422909	5:174679840	0.04		-5.91±2.3	1.04×10^{-2}

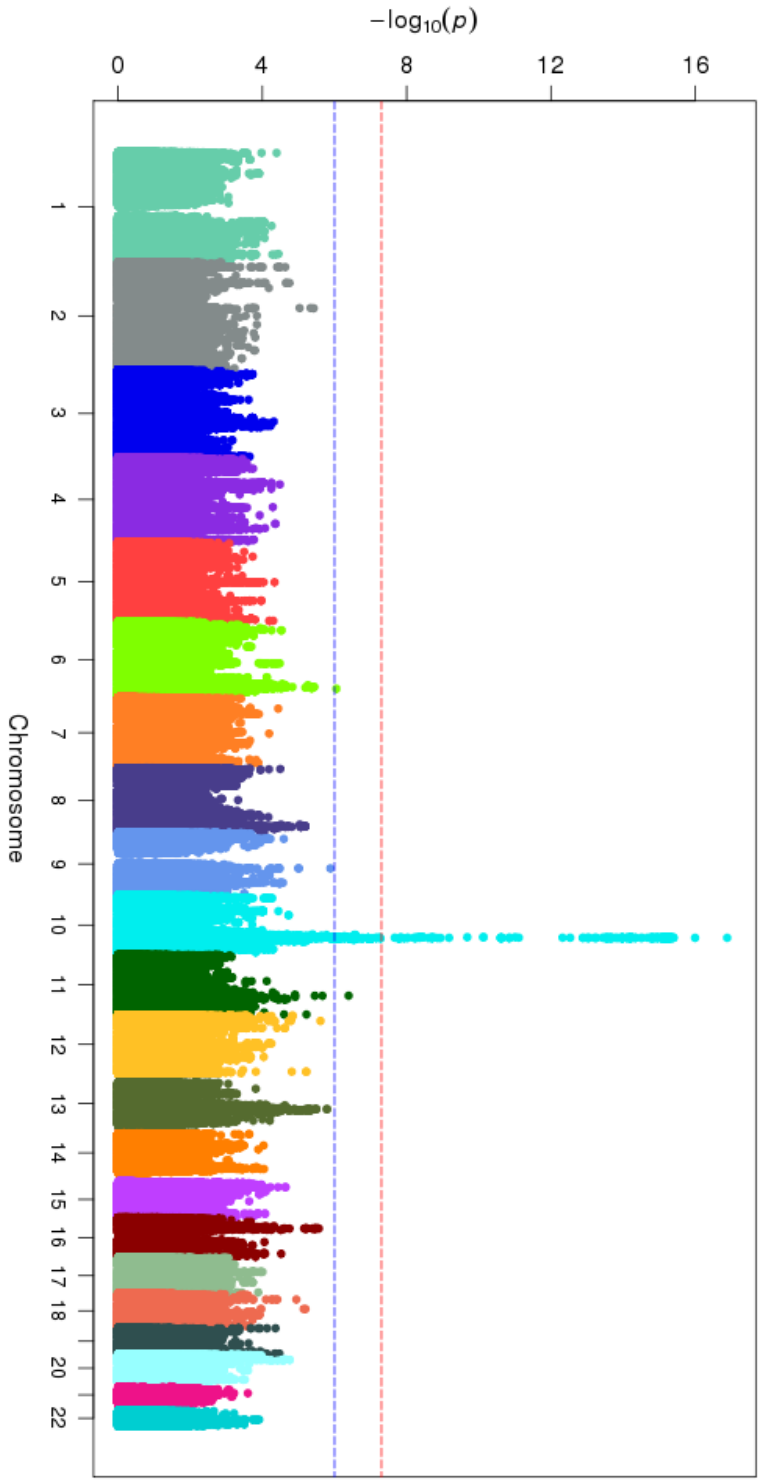


Figure 4.5. Manhattan plot of on-clopidogrel ADP-mediated platelet aggregation as measured by PAP8E light aggregometry after 7-day 75-mg/day clopidogrel intervention. N=513. Red line indicates $P < 5 \times 10^{-8}$. Blue line indicates $P < 1 \times 10^{-6}$.

ExWAS Results

In addition to the GWAS, we also performed an exome-wide association study (exWAS) of clopidogrel active metabolite levels. In agreement with the GWAS results, exWAS analysis revealed that the *CYP2C19* locus was the strongest determinant of clopidogrel active metabolite concentration (rs12772169, $P = 2.59 \times 10^{-13}$; Figure 4.6). After adjustment for *CYP2C19**2, no significant association was observed at this locus (Top remaining signal was rs35637370, $P = 1.0 \times 10^{-5}$). In addition, a genome-wide significant signal was observed on chromosome 19p13 (rs142890248, $P = 7.05 \times 10^{-7}$) and a near-significant association signal was observed on chromosome 17q12 (rs192868542, $P = 2.0 \times 10^{-6}$). rs142890248, a rare missense variant (MAF = 0.006, Arg556His) located within *MISP*, exhibited no association with on-clopidogrel ADP-mediated platelet aggregation (P = 0.91). rs192868542, which is in moderately high LD with the chromosome 17q11 index SNP identified in the GWAS of clopidogrel active metabolite (rs80343429, $r^2 = 0.52$, D' = 0.83), was also not associated with on-clopidogrel ADP-mediated platelet aggregation (P = 0.19).

The exWAS of *ex vivo* on-clopidogrel ADP-mediated platelet aggregation identified the *CYP2C19* locus as the only genome-wide significant signal (Figure 4.7, rs1126545, $P = 1.62 \times 10^{-14}$), with adjustment for *CYP2C19**2 fully accounting for this association (Top remaining signal was rs1126545, $P = 2.0 \times 10^{-4}$). No other locus revealed association with on-clopidogrel ADP-mediated platelet aggregation after accounting for multiple testing.

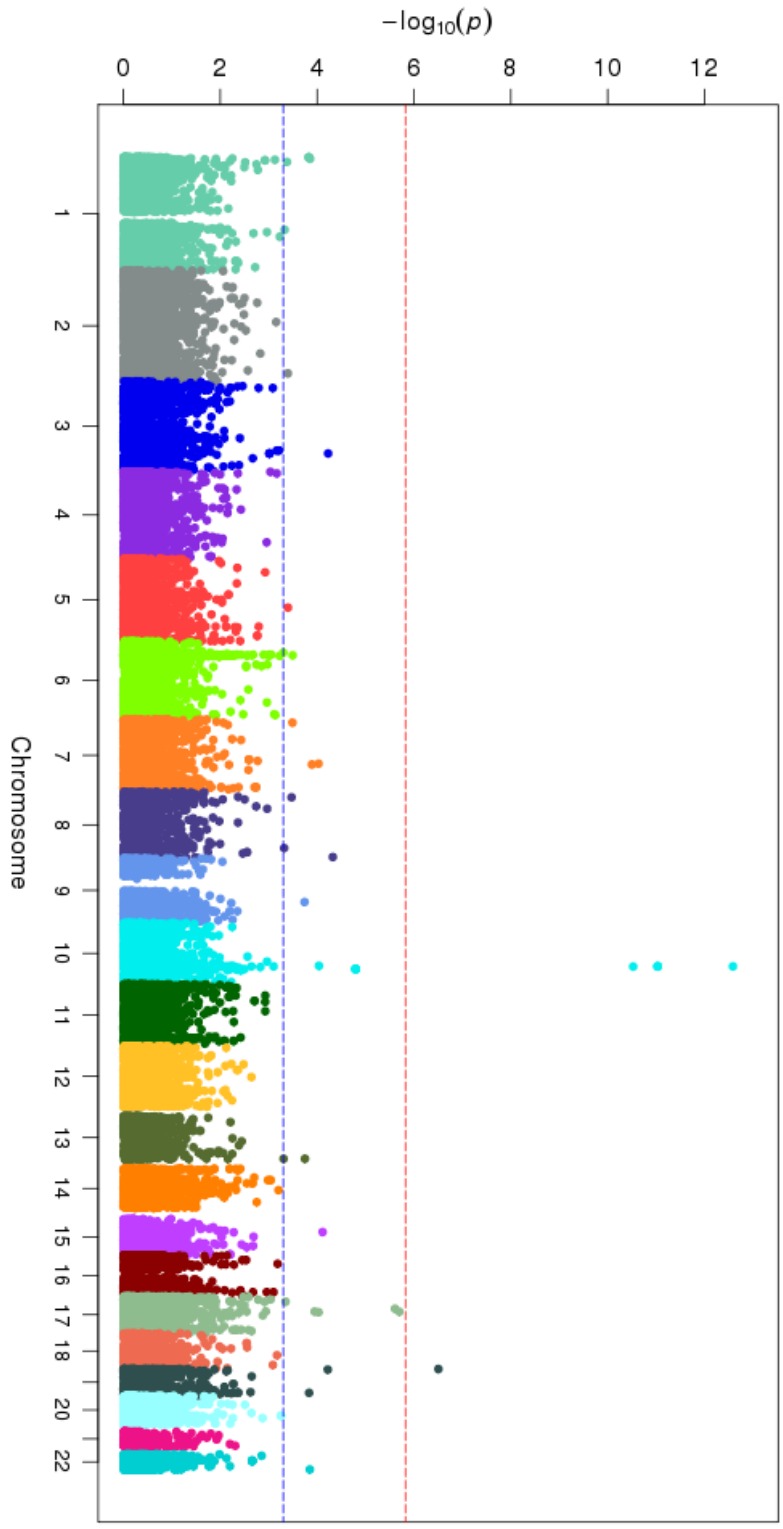


Figure 4.6. ExWAS for clopidogrel active metabolite. N=513. Covariates include age, sex, baseline ADP-mediated platelet aggregation, and relatedness. Red line indicates $P < 5 \times 10^{-8}$. Blue line indicates $P < 1 \times 10^{-6}$.

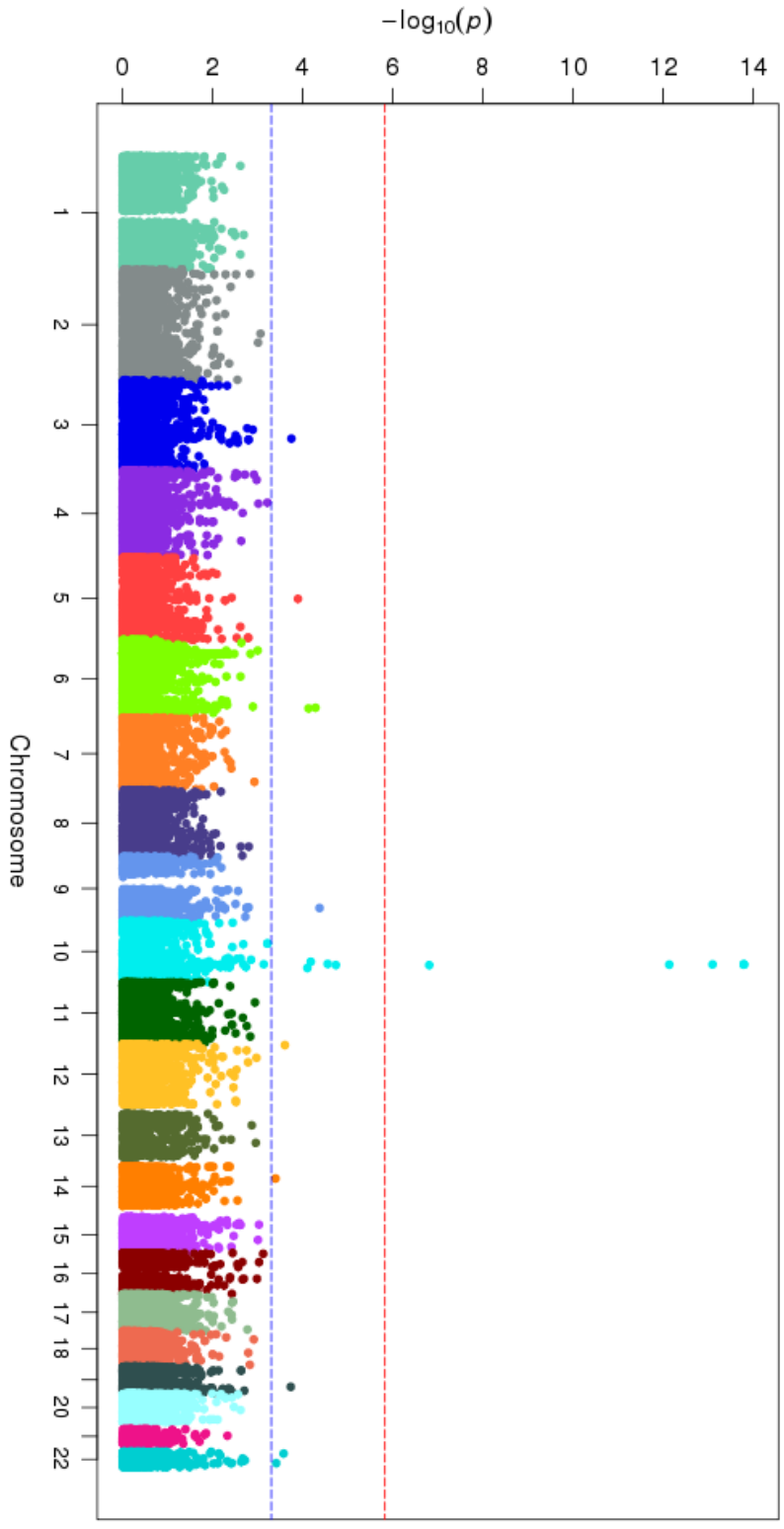


Figure 4.7. ExWAS for on-clopidogrel ADP-mediated Platelet Aggregation. N=513. Covariates include age, sex, baseline ADP-mediated platelet aggregation, and relatedness. Red line indicates $P < 5 \times 10^{-8}$. Blue line indicates $P < 1 \times 10^{-6}$.

Gene-Based Burden Test

SKAT was used to evaluate the association between sets of exome chip variants located within 7,359 RefSeq genes with at least 2 polymorphic variants and clopidogrel response. Surprisingly, no gene near the *CYP2C19* locus was among the top association signals for clopidogrel active metabolite with SKAT. However, this is likely explained by the observation that the most strongly associated *CYP2C19**2 proxy alleles from the exWAS analysis of clopidogrel active metabolite did not map to defined gene regions and were thus excluded from SKAT. The strongest SKAT association signal for clopidogrel active metabolite was *CRYBA1*, a gene on chromosome 17q11, which reached genome-wide significance ($P = 4.4 \times 10^{-6}$, Table 4.4). Specifically, 2 variants in *CRYBA1* were tested using SKAT, rs142631461 and rs117757092. Interestingly, one of these two SNPs (rs142631461) was strongly associated with clopidogrel active metabolite levels in our single variant exWAS analysis ($P = 2.7 \times 10^{-6}$). Moreover, rs142631461 was in partial LD with rs192868542, the chromosome 17q12 index SNP from the clopidogrel active metabolite exWAS ($r^2 = 0.28$, D' = 0.64) as well as rs80343429, the chromosome 17q11 index SNP from the clopidogrel active metabolite GWAS ($r^2 = 0.30$, D' = 0.79). As expected, including rs192868542 as a covariate in the association model abrogated the CRYBA1 SKAT signal ($P = 1.6 \times 10^{-4}$). While no genes reached genome-wide significance for association with on-clopidogrel ADP-mediated platelet aggregation, the top signal was *ENTPD1-AS1*, a gene on chromosome 10q24 near the *CYP2C9-CYP2C18-CYP2C19* cluster ($P = 2.3 \times 10^{-5}$) (Table 4.5). Adjusting for *CYP2C19**2 greatly diminishes this association ($P = 8.2 \times 10^{-3}$).

Table 4.4. SKAT with exome data for clopidogrel active metabolite. Abbreviations: cMAF, cumulative minor allele frequency; N SNPs, Total number of polymorphic variants in gene.

Gene	Chr.	Position	cMAF	N SNPs	P-value
CRYBA1	17	27573875-27581502	0.029	2	4.39E-06
MLF1	3	158288953-158324249	0.865	2	7.74E-05
ZNF528	19	52901121-52921657	0.079	2	8.37E-05
OTOL1	3	161214596-161221730	0.389	2	1.22E-04
LONRF1	8	12579406-12612992	0.276	2	1.31E-04
SLFN11	17	33677329-33700720	0.606	3	1.48E-04
TNFRSF14	1	2487805-2495188	0.405	3	2.37E-04
IFT46	11	118415243-118436791	0.130	2	2.50E-04
DKK3	11	11984543-12030629	0.345	2	3.22E-04
ST3GAL5	2	86066271-86116157	0.207	2	3.22E-04

Table 4.5. SKAT with exome data for on-clopidogrel ADP-mediated Platelet Aggregation. Abbreviations: cMAF, cumulative minor allele frequency; N SNPs, Total number of polymorphic variants in gene.

Gene	Chr.	Position	cMAF	N SNPs	P-value
ENTPD1-AS1	10	97678695-97849992	0.230	3	2.32E-05
KIAA1257	3	128689782-128712986	0.031	2	7.86E-05
ZNF518A	10	97889472-97923517	0.137	4	9.57E-05
KIAA1161	9	34368907-34376894	0.333	3	1.03E-04
C10orf71	10	50507187-50535537	1.589	11	2.45E-04
FOXM1	12	2966847-2986321	0.238	5	3.01E-04
PVR	19	45147098-45169428	0.128	3	3.02E-04
LOC100507424	12	2945982-2968961	0.235	3	3.02E-04
LINC00871	14	46533362-46971104	0.453	2	4.65E-04
ACSM5	16	20420856-20452281	0.144	5	1.24E-03

Bivariate analysis

A bivariate analysis for on-clopidogrel ADP-mediated platelet aggregation and clopidogrel active metabolite utilizing canonical correlation association generated nearly identical results to the clopidogrel active metabolite GWAS. Again, the strongest determinant of clopidogrel active metabolite level was *CYP2C19**2 ($P = 3.4 \times 10^{-23}$). In addition, strong evidence of association was observed on chromosomes 3p25 and 17q11 corresponding to the top association signals identified by the clopidogrel active metabolite GWAS ($P = 2.2 \times 10^{-9}$ and 3.1×10^{-8}, respectively) (Figure 4.8).

Extension in the ICPC

In order to evaluate the impact of the newly identified variants on clopidogrel efficacy in an independent population consisting of cardiovascular disease patients, a credible set of SNPs for each genome-wide and exome-wide significant locus was developed based on linkage disequilibrium in participants of the PAPI Study. Initially, all SNPs with a $D' \geq 0.9$ with the index SNP on chromosomes 3p25, 17q11, and 19p13 (rs187941554, rs80343429, and rs142890248, respectively) were included in this analysis. 12 SNPs near chromosome 3p25 between 7.7 MB and 11.8 MB, 8 SNPs near chromosome 17q11 between 31.5 MB and 31.6 MB, and 1 SNP on chromosome 19p13 were followed up. On chromosome 3p25, 1 SNP (rs147119990) was nominally associated with on-treatment platelet aggregation ($P = 0.04$, Table 4.6). In addition, the eight SNPs in the 17q11 correlated set, all in high LD with each other in the ICPC ($D' \geq 0.99$, $r^2 \geq 0.69$), showed nominal evidence of association with platelet aggregation in the ICPC ($P \leq 0.05$, Table 4.6). No association was observed between rs142890248 (chromosome 19p13) and platelet aggregation in the ICPC. Of note, the direction of

effect for the chromosomes 17q11 and 3p25 variants associated in the ICPC cohort were in the opposite direction from what was observed in the PAPI Study. Furthermore, we extended follow-up analysis in the ICPC cohort to the other 6 loci that showed suggestive evidence of association ($P \leq 1.0 \times 10^{-6}$) for clopidogrel active metabolite concentration in the PAPI Study. While no variants surpass multiple testing correction ($P \leq 0.05/9 = 0.0055$), several SNPs in the chromosome 1q25 set displayed trending associations ($P <= 0.07$), with the same direction of effect as the discovery cohort results (Table 4.6).

Association with Clinical Endpoints

In addition to assessing *ex vivo* platelet aggregation in patients of the ICPC, we also examined the incidence of major adverse cardiovascular events. No SNPs in the credible set on chromosome 3p25, chromosome 17q11, or chromosome 19p13 had a nominal association with MACE (Table 4.6). Interestingly, the chromosome 1q25 credible set that trended towards increased platelet aggregation also showed a trend with MACE (Table 4.6).

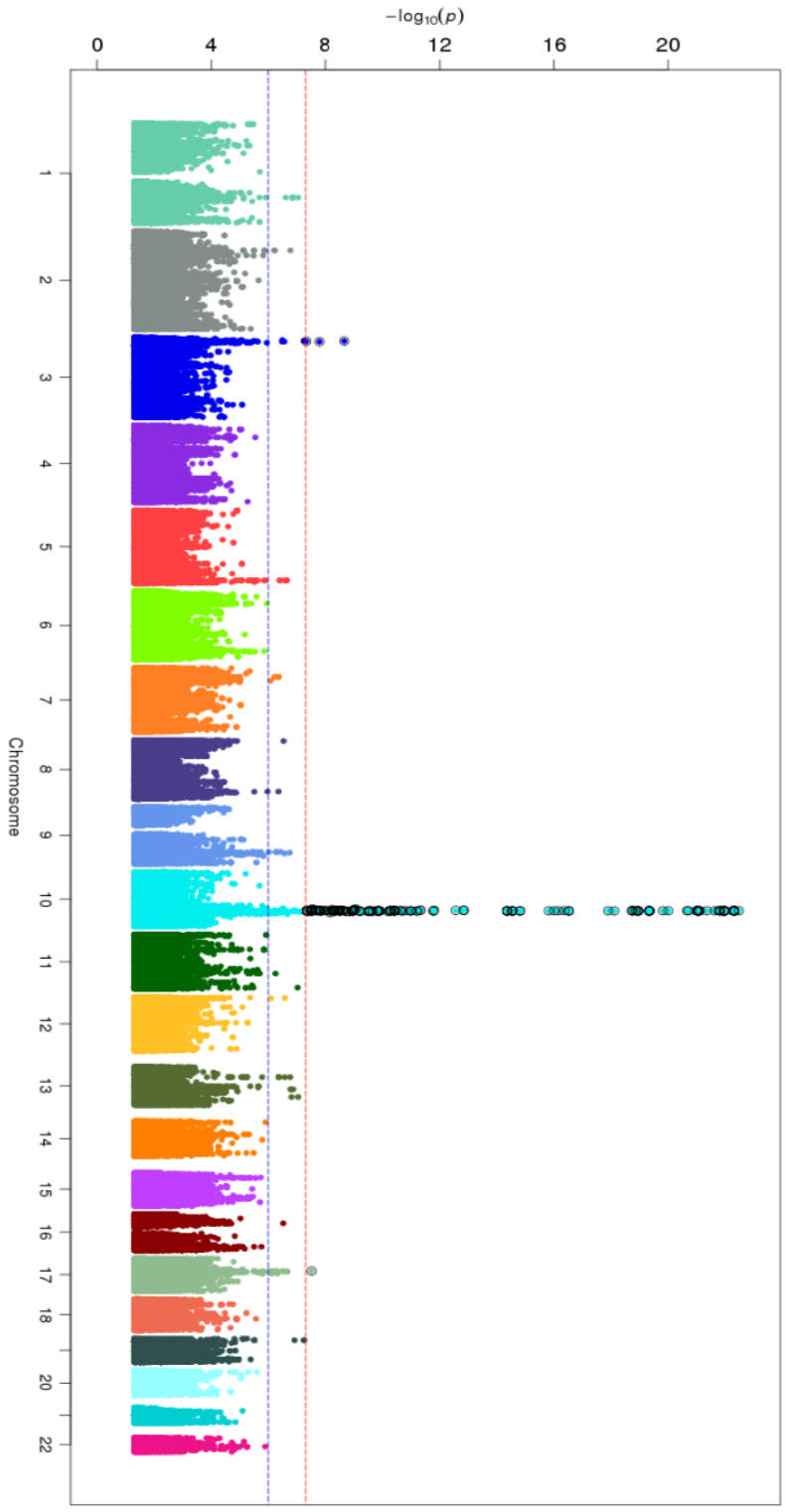


Figure 4.8. GWAS for bivariate analysis with clopidogrel active metabolite and on-clopidogrel ADP-stimulated Platelet Aggregation. N=513. No adjustment for relatedness, age, or sex was included in analysis. Red line indicates $P < 5 \times 10^{-8}$. Blue line indicates $P < 1 \times 10^{-6}$.

Table 4.6. Credible SNP set associations in ICPC. Abbreviations: MACE, major adverse cardiovascular events; BP, chromosomal position; D', coefficient of linkage disequilibrium; MAF, minor allele frequency; N, sample size; P, p-value; OR, odds ratio.

SNP	Chr.	BP	Minor Allele	D'	MAF	Platelet Aggregation			MACE		
						N	BETA	P	N	OR	P
rs148515972	3	7744038	A	1	0.0148	2247	-0.12	0.31	1160	1.65	0.44
rs140967106	3	8392736	G	1	0.0057	2193	0.35	0.09	1129	1.71	0.62
rs149772317	3	8392814	A	1	0.0034	2212	0.21	0.46	1144	1.15×10^{-6}	0.99
rs77545247	3	8428699	C	1	0.0275	2098	0.11	0.27	1091	0.68	0.60
rs184395014	3	8437893	T	1	0.0148	2159	0.22	0.09	1121	0.65	0.68
rs111985173	3	8441942	G	-	0.0057	2194	0.30	0.15	1128	1.50	0.71
rs187941554	3	8987233	A	1	0.0002	2238	-0.95	0.35	1155	1.69×10^{-6}	0.99
rs115267786	3	10278444	G	1	0.0029	2211	0.35	0.25	1140	1.56×10^{-6}	0.99
rs11923545	3	10405061	T	1	0.0021	2188	0.45	0.18	1126	1.33×10^{-6}	0.99
rs114622784	3	10657758	G	1	0.0035	2160	-0.18	0.49	1109	3.35×10^{-6}	0.98
rs147119990	3	11791617	A	1	0.0049	2216	0.46	0.04	1141	5.73×10^{-6}	0.98
rs73813043	3	11797104	T	1	0.0059	2206	0.07	0.74	1134	2.14	0.51

SNP	Chr.	BP	Minor Allele	D'	MAF	Platelet Aggregation			MACE		
						N	BETA	P	N	OR	P
rs319761	17	31567750	G	1	0.12	2245	0.09	0.03	1159	1.21	0.48
rs412242	17	31569646	T	1	0.13	2221	0.10	0.02	1146	1.20	0.49
rs319759	17	31569677	T	1	0.13	2221	0.10	0.02	1146	1.20	0.49
rs319757	17	31569872	T	1	0.13	2221	0.10	0.02	1146	1.20	0.49
rs319755	17	31569899	G	1	0.13	2219	0.11	0.02	1145	1.19	0.51
rs319754	17	31570172	C	1	0.13	2208	0.10	0.02	1144	1.21	0.47
rs319750	17	31571877	T	1	0.13	2247	0.10	0.03	1161	1.20	0.50
rs1497360	17	31581412	A	1	0.09	2230	0.10	0.05	1153	1.61	0.09

Table 4.6 continued

SNP	Chr.	BP	Minor Allele	D'	MAF	Platelet Aggregation			MACE		
						N	BETA	P	N	OR	P
rs75339668	1	184936189	A	0.99	0.03	2147	-0.15	0.07	1106	1.21	0.70
rs72739693	1	185030234	C	1	0.10	2240	-0.09	0.07	1158	1.62	0.06
rs12030761	1	185030385	A	1	0.04	2059	-0.05	0.54	1063	1.58	0.23
rs10911673	1	185034502	C	1	0.10	2243	-0.09	0.06	1159	1.62	0.06
rs2378958	1	185042400	G	1	0.10	2249	-0.10	0.06	1161	1.65	0.05
rs72739698	1	185042711	T	1	0.10	2233	-0.09	0.07	1154	1.59	0.07
rs72739699	1	185042955	G	1	0.10	2248	-0.10	0.05	1160	1.65	0.05

SNP	Chr.	BP	Minor Allele	D'	MAF	Platelet Aggregation			MACE		
						N	BETA	P	N	OR	P
rs181524103	2	123209400	G	-	0	2249	-	-	1161	-	-

SNP	Chr.	BP	Minor Allele	D'	MAF	Platelet Aggregation			MACE		
						N	BETA	P	N	OR	P
rs1848326	5	174190130	T	1	0.21	2145	-0.02	0.59	1111	0.95	0.82
rs4867777	5	174190638	A	1	0.21	2206	-0.03	0.42	1143	0.99	0.98
rs6873128	5	174191419	T	1	0.21	2197	-0.03	0.43	1139	0.98	0.95
rs6892003	5	174191465	A	-	0.23	2189	-0.04	0.29	1134	1.08	0.74
rs34799282	5	174194805	G	1	0.20	2173	-0.03	0.47	1128	1.02	0.93
rs13182985	5	174194968	T	1	0.21	2188	-0.03	0.37	1135	1.08	0.75
rs6870098	5	174195202	C	1	0.21	2192	-0.03	0.38	1138	1.09	0.72
rs13172820	5	174196542	C	1	0.21	2191	-0.04	0.33	1139	1.09	0.71

SNP	Chr.	BP	Minor Allele	D'	MAF	Platelet Aggregation			MACE		
						N	BETA	P	N	OR	P
rs73407739	7	92491283	T	-	0.03	2010	-0.02	0.81	1030	1.54	0.39

Table 4.6 continued

SNP	Chr.	BP	Minor Allele	D'	MAF	Platelet Aggregation			MACE		
						N	BETA	P	N	OR	P
rs138852022	16	9155981	G	-	0.03	2136	-0.12	0.17	1107	1.31	0.59

SNP	Chr.	BP	Minor Allele	D'	MAF	Platelet Aggregation			MACE		
						N	BETA	P	N	OR	P
rs142890248	19	758613	A	-	0.005	2116	0.11	0.57	1103	1.2 x 10-6	0.98

SNP	Chr.	BP	Minor Allele	D'	MAF	Platelet Aggregation			MACE		
						N	BETA	P	N	OR	P
rs79172967	21	18571112	T	1	0.01	2054	-0.18	0.27	1068	2.3 x 10-6	0.98
rs75958703	21	18587290	G	-	0.01	2088	-0.25	0.17	1083	1.8 x 10-6	0.98

Discussion

In this study we conducted the first genome-wide analysis of circulating clopidogrel active metabolite concentration, a direct measure of clopidogrel pharmacokinetics, to identify novel genetic variants that influence clopidogrel metabolism. Clopidogrel, a prodrug that must be metabolized *in vivo* in order to be biologically active, is among the most widely distributed and efficacious anti-platelet agents available, and the contribution of genetic variation to patient response, estimated at 73% by *ex vivo* platelet aggregation [7], has been highly studied for more than a decade[126,127]. To date, the majority of studies have utilized platelet function tests that are based on *ex vivo* ADP-mediated platelet aggregation, a pharmacodynamic measure, in order to examine clopidogrel response. Interestingly however, the majority of variants that have been identified through platelet function testing influence clopidogrel pharmacokinetics. For instance, *ABCB1* C3435T, a missense mutation in a gene integral in the intestinal absorption of the clopidogrel prodrug, results in reduced clopidogrel active metabolite levels in carriers, and therefore reduced effectiveness of clopidogrel treatment [111,112]. Carriers of *CES1* G143E, a mutation in the major protein responsible for the deactivation and clearance of clopidogrel and its metabolites, have elevated levels of the active metabolite, and therefore increased anti-platelet response [91]. The hallmark mutation for clopidogrel efficacy, *CYP2C19**2, a nonsynonymous SNP in the major cytochrome P450 enzyme responsible for clopidogrel bioactivation, has repeatedly been linked to decreased clopidogrel active metabolite formation, increased on-treatment platelet aggregation, and altered clinical events rates in patients with cardiovascular disease, prompting the FDA to update the clopidogrel label to inform clinicians to

consider alternative treatment for genetically susceptible patients[7,92,93,95,128]. However, apart from these polymorphisms, few variants have been identified that are consistently linked to clopidogrel efficacy. Therefore, based on current heritability estimates, a large proportion of the genetic variants that impact clopidogrel response remain unknown. Given this information, we hypothesized that the genome-wide analysis of clopidogrel active metabolite concentration would facilitate the identification of novel variants that affect clopidogrel metabolism that may be missed in genome-wide studies focusing solely on pharmacodynamic measures such as platelet function testing.

The active metabolite of clopidogrel is highly unstable, with a half-life of approximately 30 minutes[129], and must be derivatized in order to be accurately measured [130], which has likely limited the widespread use of this phenotype in large studies. Through combining genome-wide genotyping and assessment of clopidogrel active metabolite in a relatively large sample of subjects, we successfully identified novel loci on chromosomes 3p25 and 17q11 that are significantly correlated with clopidogrel metabolism in the Old Order Amish, as well as several other loci that may warrant further investigation. Consistent with our hypothesis, 5 of the 9 loci strongly associated with clopidogrel active metabolite exhibit, at minimum, a trending association with on-clopidogrel platelet aggregation. However, none of these variants were associated with ADP-mediated platelet aggregation at a genome-wide level and would not have been identified in traditional investigations that solely utilize platelet function testing. Furthermore, none of the pharmacokinetic variants identified in this study were correlated with differing starting concentrations of the clopidogrel parent drug. Gene-level testing using SKAT again identified the chromosome 17q11 locus as a modifier of clopidogrel

active metabolite levels. Additionally, we utilized a bivariate approach with canonical correlation analysis, which has been demonstrated to increase analytical power in the genetic analysis of pleiotropic or correlated traits[131]. The results of this analysis lended further support for the chromosomes 3p25 and 17q11 loci identified by clopidogrel active metabolite GWAS.

This investigation was conducted in the Old Order Amish, a closed, founder population with a high degree of consanguinity that, in many ways, is ideal for genetic investigations of complex traits. However, due to the unique characteristics of this population, genetic drift has resulted in reduced haplotype diversity that can inhibit causal variant identification, particularly in gene dense regions. Indeed, we observed that multiple SNPs are in high linkage disequilibrium with the index SNP of each significantly associated locus. Further, the majority of the most significantly associated variants are located in intergenic or intronic regions, and *in silico* functional predictors offer little assistance in variant prioritization. Therefore, in an attempt to validate newly identified loci, we assessed the relationship between a correlated set of variants for each of the top associated loci and on-clopidogrel platelet aggregation as well as MACE in the ICPC, the largest cohort of on-clopidogrel cardiovascular disease patients assembled to date. While nominal associations were observed with on-clopidogrel platelet aggregation in the ICPC at chromosomes 3p25 and 17q11, the effects were in the opposite direction from what would have been expected based on the PAPI Study active metabolite associations. While this is a surprising finding, it is not impossible given potential differences in haplotype structure, and further follow-up is warranted. Potentially more

interesting, a cluster of SNPs in the chromosome 1p25 suggestive loci had trending associations with both platelet aggregation and MACE in the ICPC.

While they may not be causal, the most highly associated polymorphisms were identified in, or near, genes with potential roles in clopidogrel response. For instance, in the PAPI Study analysis of clopidogrel active metabolite, a cluster of highly associated SNPs in the chromosome 3p25 locus were within the gene regions of *RAD18, LMCD1-AS1*, and *ATP2B2*. *RAD18,* which encodes for an ubiquitin ligase involved in post-replication DNA repair[132], and *LMCD1-AS1,* an antisense RNA gene[133], do not have clear roles in drug metabolism *in vivo*. However, *ATP2B2,* which codes for a plasma membrane calcium transporter[134], is an interesting candidate that has previously been implicated in clopidogrel response[135] and is also expressed in the liver[136]. In work presented at the American College of Cardiology Scientific Sessions in 2012, Price et al. identified a mutation in *ATP2B2* that influenced clopidogrel efficacy, hypothesizing that differential calcium flux in platelets leads to altered clopidogrel pharmacodynamics[135,137]. It remains to be seen whether these are in fact the same signals.

In this investigation, we also observed that genetic variants on chromosome 17q11 significantly impacted levels of clopidogrel active metabolite in the PAPI Study and nominally associated with on-clopidogrel ADP-mediated platelet aggregation in the ICPC. All 8 of the associated SNPs are clustered in *ASIC2*, a gene that codes for a member of the degenerin/epithelial sodium channel superfamily, which has been previously implicated in acid-sensing in the gastrointestinal tract[138]. To date, no role for ASIC2 in clopidogrel absorption has been established. However, one plausible

mechanism is altered clopidogrel uptake, as has been seen with multiple drugs in patients with gastrointestinal disorders[139].

While, only exhibiting a suggestive significance level in the PAPI Study analysis of clopidogrel active metabolite ($P = 1.6 \times 10^{-7}$), the chromosome 1p25 locus is perhaps the most interesting association observed. In addition to exhibiting nominal evidence of association with on-clopidogrel platelet aggregation in the PAPI Study ($P = 4.6 \times 10^{-3}$), a cluster of SNPs in the chromosome 1p25 correlated set were the only variants to demonstrate trending associations for both platelet aggregation and MACE in the ICPC. In both the PAPI Study and the ICPC, the most strongly associated variant is located within Ring Finger Nuclease 2 (*RNF2*), which encodes a polycomb group protein important for transcriptional regulation of other genes[140]. Interestingly, RNF2 interacts with P-glycoprotein, a key enzyme for the intestinal absorption of clopidogrel[141]. Previous investigations have shown that RNF2 protein levels are negatively correlated with P-glycoprotein expression in both insect and human models[141]. Further research is warranted to determine the causality of the *RNF2* variants in our study.

This study provides the first analysis of genome-wide genetic variation that influences clopidogrel active metabolite concentration, enabling us to identify novel clopidogrel variants that otherwise would not be found in a study exclusively examining on-clopidogrel platelet aggregation. Rather surprisingly, correlation between clopidogrel active metabolite and on-clopidogrel ADP-mediated platelet aggregation was not as high as expected, with an r^2=0.12. Further, only 4 of the 9 index SNPs identified from the active metabolite GWAS and exWAS exhibited nominal associations with platelet aggregation, although 1 other locus did trend in that direction. One partial explanation is

that active metabolite is a direct measure of clopidogrel pharmacokinetics, whereas, *ex vivo* platelet aggregation is a composite phenotype with significantly more heritable variation due to both pharmacokinetic and pharmacodynamic effects. Indeed, the heritability of clopidogrel active metabolite was estimated at 26%, while the heritability of on-clopidogrel ADP-mediated platelet aggregation was estimated at 75% in this study. Another plausible contributor to the relatively low concordance between these traits is the differential impact of environment and anthropometric values on the pharmacodynamic and pharmacokinetic measures. Whereas age, BMI, platelet count, total cholesterol, LDL, HDL, and triglycerides were all associated with on-clopidogrel ADP-mediated platelet aggregation, only BMI and platelet count were associated with clopidogrel active metabolite in our study. While a number of studies have examined the influence of comorbidities and patient characteristics on clopidogrel response through *ex vivo* platelet aggregation, few, if any, reports to date have directly examined the influence of these environmental factors on clopidogrel pharmacokinetics. Further, the reduced concordance could potentially be due to the methods used to measure each phenotype. The active metabolite was measured at a single time-point 1 hour after the final clopidogrel dose (C_{max}), which given the short half-life of clopidogrel only reflects response to a single clopidogrel dose. On the other hand, the active metabolite irreversibly binds the platelet ADP-receptor, which means that measurement of platelet aggregation reflects the cumulative impact of a 7-day clopidogrel intervention.

There are some limitations of this study that should be considered. Imputation, while useful for fine mapping, can lead to false positive results, particularly with rare variants. In order to address this potential limitation, we verified the accuracy of our

imputed genotype data through Taqman genotyping of our two strongest signals and showed high genotype concordance (>98%) for both variants. However, direct genotyping of the other 6 suggestive loci in the PAPI study was not completed. While in this study we conducted the first analysis of clopidogrel active metabolite in a large scale population, our ability to replicate our findings is limited due to the unavailability of a true replication cohort. Alternatively, we utilized the ICPC, the largest cohort of on-clopidogrel cardiovascular disease patients with complete genotype and phenotype information available to evaluate the impact of the variants identified herein on platelet aggregation and clinical endpoints. This study is also potentially limited by the methodology used to isolate and quantify the clopidogrel active metabolite. The derivatization process with MPB does not differentiate between the racemic H4 (active) and H3 (inactive) stereoisomer of clopidogrel metabolites, potentially leading to some measurement error. While, high concordance between H3 and H4 has previously been noted[142], recent evidence indicates that the metabolism of H3 and H4 are, to an extent, distinct[143]. While it is unlikely, it is therefore possible that mutations that have been identified in this study to influence active metabolite but not platelet aggregation might differentially impact the production of H3, not but H4. Further research examining the production of the H4 metabolite versus the H3 metabolite would be informative. Finally, the unique genetic substructure of the Amish population could result in a lack of translatability of findings to non-Amish populations. However, this has not been the case in the past[7,8,117,118],

Inter-individual variation in response to clopidogrel has been noted since the drug was first approved by the FDA in 1997, with non-modifiable factors such as race, sex,

and age, as well as modifiable exposures such as obesity, smoking, and interactions with other drugs such as statins significantly influencing patient response[83,84,86,87,109]. In addition, pharmacogenetic variants significantly influence clopidogrel efficacy. However, while the identification of polymorphisms such as *CYP2C19**2 has informed clinicians to alter treatment plans and improved outcomes in a percentage of cardiovascular disease patients, there is still large 'missing' heritability that undoubtedly leaves a number of patients at unnecessarily high risk of recurrent ischemic events. In this study, we aimed to identify novel genetic variants that significantly influenced formation of the clopidogrel active metabolite. Indeed, a number of novel variants with potential effects on clopidogrel efficacy were identified. While these results still need further verification in an independent population, this study demonstrates the utility of directly assessing clopidogrel metabolites, and has implications for improved next-generation drug design and improved anti-platelet treatment for at risk patients in the future.

Chapter 5: Prospective Evaluation of Genetic Variation in PEAR1 Reveals Aspirin-Dependent Effects on Platelet Aggregation Pathways

Abstract

Background: Genetic variation in the Platelet Endothelial Aggregation Receptor 1 (*PEAR1*) gene is implicated in altered on-aspirin platelet aggregation and increased cardiovascular event risk. rs12041331 has been the most highly and consistently associated SNP with these phenotypes.

Methods: We tested the effects of aspirin administration at three commonly prescribed aspirin doses (81, 162, and 324 mg/d) on agonist-induced platelet aggregation according to rs12041331 genotype in 67 healthy individuals using a fixed-sequence, crossover design. We further examined the impact of rs12041331 on circulating levels of 11-dehydrothromboxane B_2 and different platelet aggregation pathways pre- and post-aspirin administration.

Results: Prior to aspirin administration, rs12041331 minor allele carriers had significantly reduced ADP-induced platelet aggregation compared to non-carriers (P=0.03) although genotype was not associated with collagen- or epinephrine –induced platelet aggregation (P=0.20 and 0.21, respectively). In contrast, rs12041331 genotype 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. Neither arachidonic acid-induced platelet aggregation nor urinary 11-dehydrothromboxane B_2 levels were associated with *PEAR1* genotype pre- or post-aspirin administration. Increasing the dose of aspirin did not influence platelet aggregation in a genotype-dependent manner.

Conclusions: The influence of *PEAR1* rs12041331 on platelet aggregation is pathway-specific and is altered by aspirin at therapeutic doses. Additional studies are needed to determine the impact of *PEAR1* on cardiovascular events in aspirin-treated patients.

Introduction

Dual antiplatelet therapy (DAPT) with aspirin and clopidogrel is the most commonly used regimen for medical management of patients with acute coronary syndromes undergoing percutaneous coronary intervention. While generally effective in most patients, significant inter-individual variability in DAPT response has been well-documented, leading to sub-optimal therapy in some patients and resulting in an increase in cardiovascular event rates[7,8,90,144]. In recent years, substantial effort has been made to identify the 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 U.S. Food and Drug Administration in 2010[7]. In contrast, identification of genetic factors that influence response to aspirin has remained difficult despite the fact that up to 77% of the total variation in aspirin response, as assessed by *ex vivo* platelet aggregometry, is heritable[101].

One of the few recently identified and promising 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 αIIbβ3 activation[1,105,107]. To date, several retrospective investigations have independently reported that genetic variation in *PEAR1* significantly influences agonist-induced platelet aggregation at baseline as well as after aspirin exposure[8,61,104,145-147]. The most consistently and highly associated of these genetic variants is rs12041331, an intronic variant that is believed to modify PEAR1 expression[8,61,104,145,147]. 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 aspirin administration at three commonly prescribed aspirin doses (81, 162, and 324 mg/d) on agonist-induced platelet aggregation according to rs12041331 genotype in 67 healthy individuals using a fixed-sequence, crossover design. In addition, *ex vivo* platelet reactivity testing was completed using multiple platelet agonists to examine the impact of *PEAR1* rs12041331on different platelet aggregation pathways pre- and post-aspirin administration to gain insights regarding the mechanism(s) by which PEAR1 influences basal and on-treatment platelet reactivity. Urinary thromboxane B_2, a circulating biomarker of platelet function and aspirin responsiveness, was also assessed by *PEAR1* genotype in order to complement our platelet aggregation results.

Methods

Subjects

We recruited 67 relatively healthy Old Order Amish individuals from Lancaster, Pennsylvania who had previously participated in the Heredity and Phenotype Intervention Heart (HAPI) Study (between 2003 and 2006) [148], the Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study (between 2006 and 2012) [7], and targeted family members. The 67 subjects recruited for this substudy included 34 *PEAR1* rs12041331 major allele homozygotes (GG), 28 heterozygotes (GA), and 5 minor allele homozygotes (AA), all enrolled 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. Subjects were generally healthy Amish men and women and between 20 and 70 years of age. Exclusion criteria included: platelet count $< 100{,}000$ mm^3 or $> 500{,}000$ mm^3, hematocrit < 25 or > 50, blood pressure > 140/90, TSH < 0.40 or > 5.50, creatinine > 2.0, AST or ALT > 2.0x normal, allergy to aspirin, planned warfarin or dabigatran therapy during the study period, pregnant or breast feeding, history of myocardial infarction, coronary artery bypass surgery, unstable angina, or angioplasty, history of gastrointestinal bleeding, a life-threatening bleeding event, bleeding diathesis, or coagulopathy, type 2 diabetes, surgery in the past 3 months or planned surgery in the next 12 months, any other co-existing malignancy or other condition that would place participants at unacceptable risk. Medical and family history, anthropometric measures, physical examinations, and blood and urine samples were taken after an overnight fast. Complete blood count with platelet numbers and levels of serum lipids were assayed by Quest Diagnostics (Horsham,

Pennsylvania, USA) and low density lipoprotein cholesterol levels were calculated using the Friedewald equation.

The protocol was approved by the University of Maryland, Baltimore Institutional Review Board and adhered to the principles of the Declaration of Helsinki. All subjects provided written informed consent. Participants were compensated for their participation.

Study Design

This study employed a prospective, genotype-stratified design in which all subjects were studied under three different aspirin doses (7 days of aspirin followed by 14-day washout period) and platelet aggregation at each dose measured in response to four different agonists. Figure 5.1 provides an overview of the study design. 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 subjects who were taking aspirin discontinued this medication at least 2 weeks prior to the study.

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 non-enteric-coated aspirin under direct supervision and were instructed to take 81 mg/d for the following 6 days. Subjects returned to the clinic on the 7^{th} day and on-aspirin measures of platelet aggregation were recorded 2 hours 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 (7-10 days) [149]. After the washout period, subjects presented back to the clinic

and blood samples were drawn for repeat measures of baseline platelet function and sample banking. Participants then received a 162 mg dose of aspirin under direct supervision and were instructed to take this dose for the following 6 days, consistent with what was done for the 81 mg dosing period. On day 7, subjects returned to the clinic and received the final 162 mg aspirin dose. Two hours after the last aspirin dose, blood samples were obtained for platelet function testing and banking. Participants then underwent another 14 day washout period. These procedures were repeated for a 3rd time with 324 mg/d of aspirin for 7 days. Again, platelet function was assessed and samples were banked pre- and post-aspirin administration. Medication adherence was confirmed during the study through usage 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 *PEAR1* rs12041331 genotype, which had previously been measured as part of the HAPI or PAPI Study for most participants [8,148]. Genotype of each recruited participant was validated by repeat genotyping of *PEAR1* rs12041331 using a TaqMan SNP genotyping assay (Applied Biosystems, Foster City, California) 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 (%MPA) was assessed by optical aggregometry as previously described [7]. Briefly, platelet-rich plasma (PRP) was isolated from blood

samples drawn into 3.2% trisodium citrate anticoagulated tubes (Becton-Dickinson, Franklin Lakes, NJ) after discarding the initial 4 ml of blood and platelet counts were adjusted to 200,000 platelets/μl using platelet-poor plasma (PPP). Platelet function was assessed using a PAP8E Aggregometer (Bio/Data Corporation, Horsham, Pennsylvania) according to the manufacturer's instructions after stimulation with collagen (2 μg/ml), adenosine diphosphate (ADP; 2 μmol), epinephrine (10 μM), and arachidonic acid (1.6 mM) and was expressed as the maximal percentage change in light transmittance using platelet-poor plasma as a referent.

Biomarker Assessment

Thromboxane A2, which induces positive feedback during platelet aggregation downstream of the COX1-dependent platelet aggregation pathway, is metabolized to 11-dehydrothromboxane B2, which can be screened in urine to assess COX1-dependent platelet aggregation as well as participant adherence to the aspirin protocol[65]. Measurement of urinary 11-dehydrothromboxane B_2 was performed using a monoclonal 11-dehydrothromboxane B_2 enzyme-linked immunosorbent assay kit (Cayman Chemical, Michigan, USA) according to the manufacturer's instructions. Briefly, 25 μl of urine was incubated with 25 μl assay buffer, 25 μl 11-dehydrothromboxane B_2-acetylcholinesterase conjugate as a competitive inhibitor, and 25 μl mouse monoclonal antibody. 11-dehydrothromboxane B_2 concentration was determined photometrically at 405 nm using a PerkinElmer Victor X3 plate reader (PerkinElmer Inc., 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 Inc., Cary, North Carolina) was used to calculate summary statistics, distributions, and frequencies. Association analyses of *PEAR1* rs12041331 were performed using Mixed Model Analysis for Pedigrees (MMAP; http://edn.som.umaryland.edu/mmap/) under an additive genetic model with adjustments for age, sex, and relatedness amongst study participants. Under the additive model, the variant was coded as 0, 1, and 2 for G/G, G/A, and A/A, respectively. MMAP, which has been extensively used for genomic analyses in the Amish [7,8,53,91,117,118,128], accounts for the relatedness of study subjects by conditioning the genotype-phenotype correlations on the phenotypic correlations among relative pairs. The model for the polygenic component was established by constructing a relationship matrix derived from the complete Amish pedigree structure available through published genealogical records maintained by the church [119].

Urinary concentrations of 11-dehydrothromboxane B_2 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 B_2 levels pre- and post-aspirin administration (324 mg/day) were performed using MMAP as described above with age and sex as covariates. Differences between baseline platelet aggregation at study initiation and platelet aggregation following two-week washout periods were assessed by a repeated-measures ANOVA in R. The impact of aspirin dose on platelet aggregation as well as aspirin dose-by-genotype interactions were also assessed by a repeated-measures ANOVA in R. All statistical tests were 2-sided and $P < 0.05$ was considered statistically significant.

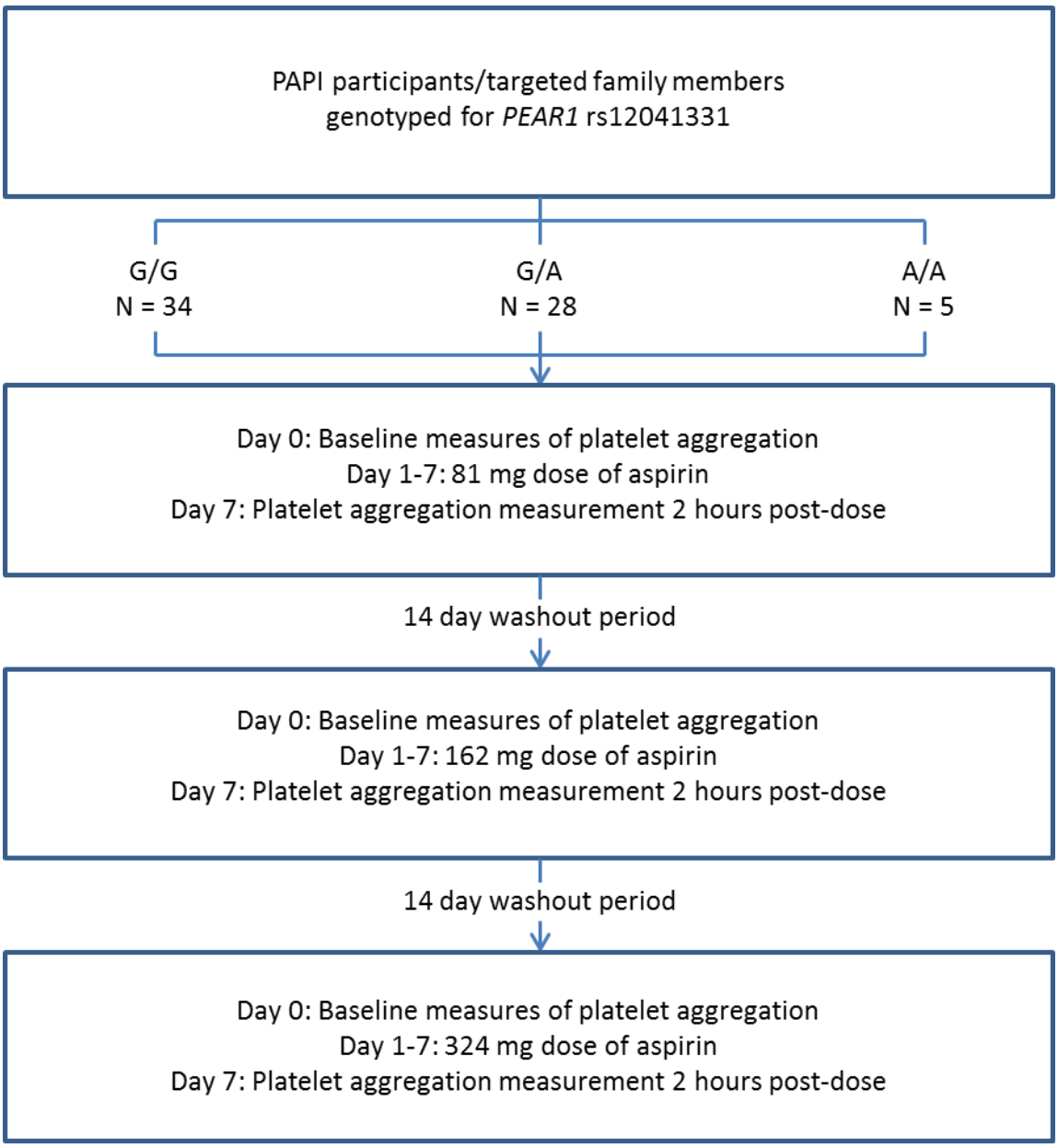


Figure 5.1. PEAR1 Study design. Each study participant underwent 7-day aspirin interventions at a low (81mg/day), medium (162mg/day), and high aspirin dose (324mg/day), with a minimum 2-week washout in between each aspirin regimen. Platelet aggregation was measured at Day 0 prior to the first aspirin 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. Abbreviations: Pharmacogenomics of Anti-Platelet Intervention, PAPI; platelet endothelial aggregation receptor 1, PEAR1.

Results

Characteristics of the 67 study participants are shown in Table 5.1. Briefly, these subjects were healthy, middle-aged (mean age = 50.2 years), and had low prevalence of disease (e.g. obesity [mean BMI = 28.4], hypertension [6.0%], hypercholesterolemia [26.9%], diabetes [0.0%],) and smoking (10.4%). In total, we recruited 34 *PEAR1* rs12041331 major allele homozygotes (G/G), 28 heterozygotes (G/A), and 5 minor allele homozygotes (A/A). None of the clinical characteristics differed significantly by rs12041331 genotype (Table 5.1), with the exception of platelet count (P = 0.03), which is consistent with previous investigations [107].

To confirm that basal platelet function was restored after each aspirin dose intervention, we compared agonist-induced platelet aggregation measures obtained during the initial clinic 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 clinic visits (Table 5.2). Similarly, we assessed the impact of aspirin dose (81, 162, and 325 mg/day for 7 days) on agonist-induced platelet aggregation. As expected, near-complete inhibition of arachidonic acid-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/d) for the same length of time (P=0.03, Table 5.3). In addition, aspirin dose did not significantly influence mean ADP-, collagen-, 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 arachidonic acid. Given that urinary levels of 11-dehydrothromboxane B_2 were also measured before and after administration of 325 mg of aspirin for 7 days (see below), baseline measures of platelet aggregation recorded prior to administration of 325 mg of aspirin are reported in Figure 5.2 and genotype-stratified analysis of all baseline data are shown in Figure 5.3. We observed that subjects who carried the minor allele of this variant had significantly reduced ADP-stimulated platelet aggregation compared to non-carriers (mean maximal platelet aggregation [MPA] = 20.2, 37.0, and 41.8 for *PEAR1* 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 well as arachidonic acid-induced platelet aggregation (P = 0.20 and 0.21, respectively). While a reduction in epinephrine-induced platelet aggregation was observed in participants who were homozygous for the rs12041331 minor allele (MPA = 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 *PEAR1* rs12041331 and agonist-induced platelet aggregation after aspirin treatment (Figure 5.4). Unlike pre-aspirin measurements, we observed no evidence of association between *PEAR1* 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 minor allele of rs12041331 had significantly reduced on-aspirin epinephrine-induced platelet aggregation compared to non-carriers (P = 0.04, 0.03, and 0.04 for aspirin doses of 81, 162, and 324 mg, respectively). Similar to pre-aspirin measurements, arachidonic acid-induced platelet aggregation was not influenced by *PEAR1* 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 in order 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 to stimulate platelet aggregation, we observed no interaction between aspirin dose and rs12041331 genotype (P = 0.89, 0.14, 0.76, and 0.54 when ADP, collagen, epinephrine, and arachidonic acid was used as an agonist, respectively), indicating an aspirin dose above 81 mg had no major impact on degree of reduction in agonist-induced platelet aggregation by *PEAR1* rs12041331 genotype (Table 5.4).

Finally, we assessed urinary 11-dehydrothromboxane B_2 levels pre- and post-aspirin administration in the total population as well as by *PEAR1* rs12041331 genotype (Table 5.5). As expected, levels of 11-dehydrothromboxane B_2 were significantly reduced after aspirin exposure ($P = 2.2x10^{-16}$), with near-complete inhibition of 11-dehydrothromboxane B_2 achieved after aspirin exposure for 7 days. 11-dehydrothromboxane B_2 levels were not significantly different by *PEAR1* rs12041331 genotype before (P = 0.09) or after aspirin exposure (P = 0.16).

Table 5.1. Characteristics of Study Participants

Trait (units)	Total	*PEAR1* rs12041331 Genotype			P-Value[‡]
		G/G	G/A	A/A	
Number (n)	67	34	28	5	-
Age ± SD (yr)	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[*] (%)	4 (6)	2 (6)	2 (7)	0 (0)	0.83
Hypercholesterolemia[†] (%)	18 (27)	9 (26)	9 (32)	0 (0)	0.60
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 x 1000/μl)	5.8 ± 0.2	5.5 ± 0.2	6.1 ± 0.3	5.8 ± 0.6	0.12
Platelet count ± SD (n x 100,000/μl)	223.1 ± 5.8	206.7 ± 7.3	243.8 ± 8.8	219.2 ± 20.8	**0.03**

Abbreviations: BMI, body mass index; N/A, not applicable; PEAR1, platelet endothelial aggregation receptor 1; SD, standard deviation

** Defined 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*

[†]Defined as LDL-cholesterol greater than 160 mg/dl or taking prescription medication for previously diagnosed hypercholesterolemia

[‡]P-values represent statistical difference of each trait by PEAR1 rs12041331 genotype

Table 5.2. Mean maximal platelet aggregation (%) during washout periods

Agonist	Baseline visit	Washout visit 1	Washout visit 2	P-value
Arachidonic acid	65.3	64.4	64.9	0.79
ADP	40.6	40.2	38.1	0.08
Collagen	65.9	65.3	64.8	0.39
Epinephrine	63.4	62.5	60.0	0.15

Table 5.3. Mean maximal platelet aggregation (%) by aspirin dose

Agonist	81 mg /day	162 mg /day	324 mg/day	P-value
Arachidonic acid	7.9	4.6	3.8	**0.03**
ADP	28.7	29.2	29.7	0.57
Collagen	20.7	20.8	20.2	0.56
Epinephrine	21.0	20.1	19.8	0.07

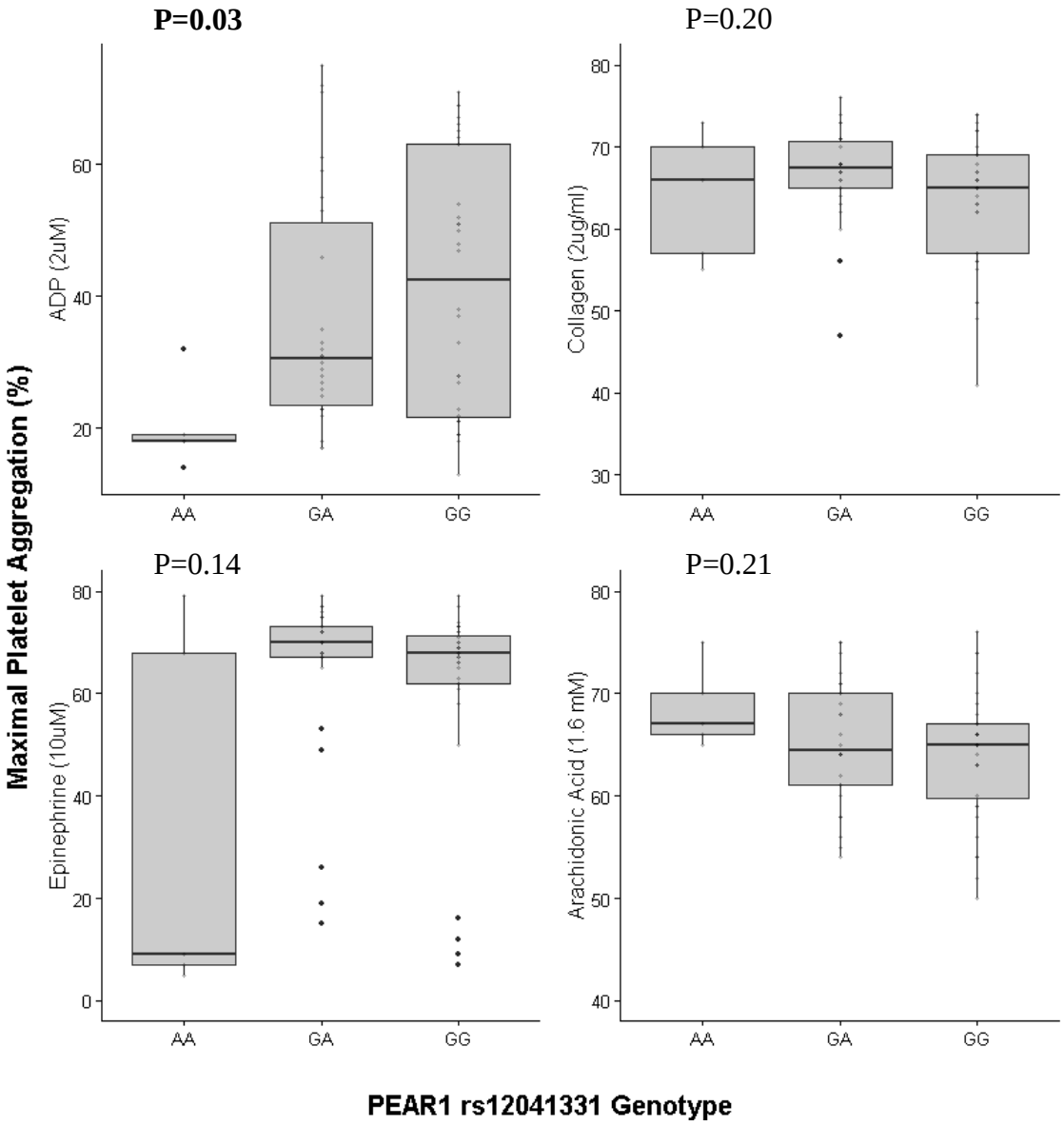


Figure 5.2. Baseline agonist-induced *ex vivo* platelet aggregation measurements were obtained in platelet-rich plasma prior to administration of the first aspirin dose. Platelet Aggregation is expressed as the percentage of maximal aggregation using platelet-poor plasma as a referent. Adenosine diphosphate (2 µM), collagen (2 µg/ml), epinephrine (10 µM), and arachidonic acid (1.6 mM) were used to stimulate platelet aggregation.

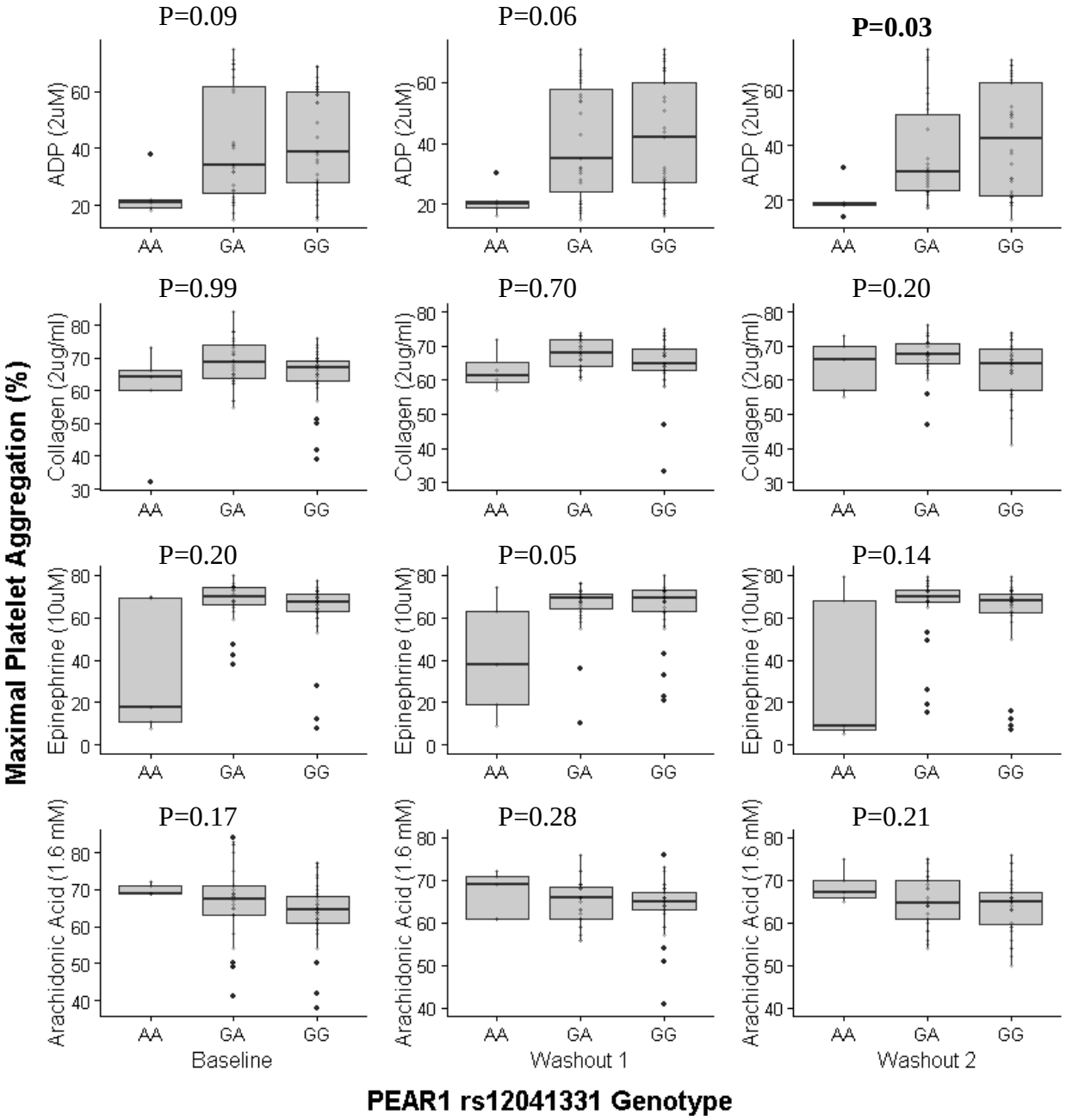

P=0.09
P=0.06
P=0.03
ADP (2uM)
P=0.99
P=0.70
P=0.20
Collagen (2ug/ml)
P=0.20
P=0.05
P=0.14
Epinephrine (10uM)
P=0.17
P=0.28
P=0.21
Arachidonic Acid (1.6 mM)
AA
GA
GG
Baseline
Washout 1
Washout 2
Maximal Platelet Aggregation (%)
PEAR1 rs12041331 Genotype

Figure 5.3. Baseline agonist-induced *ex vivo* platelet aggregation measurements were obtained in platelet-rich plasma prior to administration of the first aspirin dose and at a minimum of two weeks following the last medication taken at 3 separate visits (Baseline-prior to 81mg/day Aspirin, Washout 1-prior to 162mg/day Aspirin, and Washout 2-prior to 324mg/day Aspirin). Platelet Aggregation is expressed as the percentage of maximal aggregation using platelet-poor plasma as a referent. Adenosine diphosphate (2 µM), collagen (2 µg/ml), epinephrine (10 µM), and arachidonic acid (1.6 mM) were used to stimulate platelet aggregation.

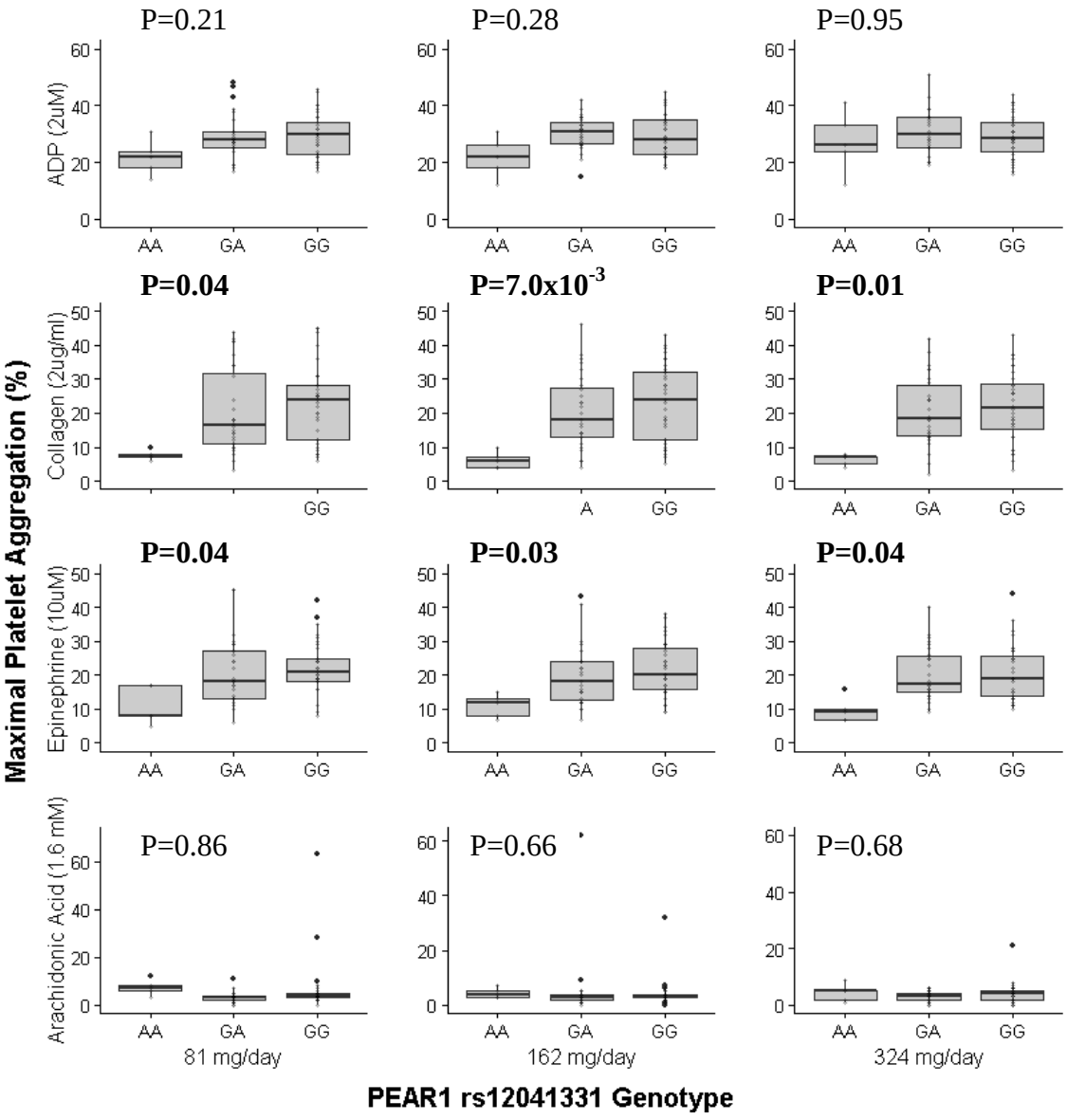

P=0.21
P=0.28
P=0.95
P=0.04
P=7.0x10-3
P=0.01
P=0.04
P=0.03
P=0.04
P=0.86
P=0.66
P=0.68
ADP (2uM)
Collagen (2ug/ml)
Epinephrine (10uM)
Arachidonic Acid (1.6 mM)
Maximal Platelet Aggregation (%)
AA
GA
GG
A
81 mg/day
162 mg/day
324 mg/day
PEAR1 rs12041331 Genotype

Figure 5.4. On-aspirin agonist-induced *ex vivo* platelet aggregation measurements were obtained in platelet-rich plasma on Day 7 of each treatment period 2 hours 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 (2 µM), collagen (2 µg/ml), epinephrine (10 µM), and arachidonic acid (1.6 mM) were used to stimulate platelet aggregation.

Table 5.4. Interaction analysis between aspirin dose and rs12041331

Agonist	Aspirin Dose (mg/day)	Maximal Platelet Aggregation (%)			Interaction P-value
		G/G homozygotes	G/A heterozygotes	A/A homozygotes	
Arachidonic Acid	81	8.3 ± 2.6	7.5 ± 3.2	7.2 ± 1.5	0.89
	162	4.2 ± 0.9	5.2 ± 2.2	4.4 ± 0.8	
	324	4.1 ± 0.7	3.3 ± 0.3	4.4 ± 1.4	
ADP	81	29.3 ± 1.3	29.3 ± 1.5	21.8 ± 2.9	0.14
	162	29.3 ± 1.3	30.3 ± 1.1	21.8 ± 3.2	
	324	29.1 ± 1.4	30.9 ± 1.5	27.2 ± 4.8	
Collagen	81	22.9 ± 2.0	20.4 ± 2.3	7.6 ± 0.7	0.76
	162	23.4 ± 1.9	20.4 ± 2.1	6.2 ± 1.1	
	324	22.0 ± 1.8	20.6 ± 2.0	6.2 ± 0.7	
Epinephrine	81	22.5 ± 1.3	21.0 ± 1.8	11.0 ± 2.5	0.54
	162	21.8 ± 1.4	19.8 ± 1.7	11.0 ± 1.5	
	324	20.6 ± 1.5	20.6 ± 1.5	9.8 ± 1.7	

Result for each genotype group reported as mean ± standard error.

Table 5.5. Urinary 11-dehyrothromboxane B_2 levels by 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*	474 ± 59	361 ± 33	365 ± 92	0.09

Urinary 11-dehydrothromboxane B_2 levels ± standard deviations *are expressed as pg/mg creatinine.*

**Measurements were recorded before and after administration of 325 mg/day aspirin for 7 days*

Discussion

While aspirin is the most frequently used antiplatelet medication globally, there is a high degree of inter-individual variation in patient response to this agent. 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 to low dose aspirin[4,101,150-153]. In past years, substantial effort has been made to identify the genetic and non-genetic determinants of aspirin response, and while 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 cyclooxygenases (COX) resulting in the inhibition of thromboxane A_2 formation from arachidonic acid, traditional tests of on-aspirin platelet function include stimulation of platelets with arachidonic acid as well as assessment of urinary 11-dehydrothromboxane B_2, a stable metabolite of thromboxane A_2. However, an increasing body of evidence suggests that evaluation of non-COX-dependent platelet pathways complement traditional aspirin response approaches and can aide in the identification of factors that lead to variation in response to this drug[4,8,101,154].

PEAR1 was identified in 2005 as a type 1 transmembrane receptor that is highly expressed in platelets and endothelial cells, among other cell types[105]. In platelets, PEAR1 acts as a contact receptor that, upon ligand binding, becomes phosphorylated in a 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 $\alpha IIb\beta_3$[1,105]. In addition, elegant work by Kauskot and colleagues using CD34(+) hematopoietic stem cells and zebrafish revealed that PEAR1 attenuates megakaryopoiesis and thrombopoiesis through PI3K and PTEN pathways[107]. 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[61,104,145-147,155] as well as after treatment with aspirin[104,156] , prasugrel[157], and dual antiplatelet therapy with aspirin and clopidogrel [8,158]. While 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[8,61,104,145,147]. rs12041331 results in G to A substitution in intron 1 and has been previously implicated in reducing PEAR1 expression [104]. In a genome-wide meta-analysis of basal platelet function in over 4,500 European and African American participants, Johnson and colleagues showed that *PEAR1* rs12041331 was the strongest determinant of basal ADP- 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[147]. 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 approximately 15% of the total phenotypic variation in platelet function, and, upon sequencing of the full *PEAR1* gene in 104 participants with extreme aspirin response, confirmed that rs12041331 accounted most strongly for the relation between *PEAR1* and platelet aggregation [145]. Surprisingly, however, a different pattern of association emerged post-aspirin administration (81 mg/day for 14 days), where significant associations were observed with collagen- and epinephrine-induced platelet aggregation but not when ADP was used as a platelet agonist[104]. To complicate matters further, in African Americans of the GeneSTAR cohort, where the minor allele frequency of this variant is substantially higher compared to Caucasian participants (0.37 vs. 0.09, respectively), rs12041331 was significantly associated with ADP-, collagen-, and epinephrine-induced platelet aggregation pre- and post-aspirin exposure. In our own GWAS of dual antiplatelet therapy response (aspirin and clopidogrel) in 565 healthy Caucasian participants of the PAPI study, *PEAR1* rs12041331 was the strongest modifier of collagen-stimulated platelet aggregation ($P = 7.7 \times 10^{-9}$)[8]. We also observed that African American and Caucasian non-emergent percutaneous coronary intervention patients who carried the minor allele of rs12041331 had reduced 1-year survival (HR = 3.97; P = 0.04 and HR = 2.62; P = 0.06, respectively) and that Caucasian stable coronary artery disease patients on aspirin experienced higher rates of myocardial infarction compared to non-carriers (OR = 2.03; P = 0.05). Interestingly, rs12041331 was not

associated with myocardial infarction in stable coronary artery disease patients who were not treated with aspirin[8].

While several retrospective studies have independently shown that *PEAR1* rs12041331 influences platelet aggregation and cardiovascular outcomes, others have not[103]. In addition, differences in participant populations (e.g. healthy vs. cardiovascular disease patients), 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 arachidonic acid). 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 non-treated patients in both the Framingham Heart Study and GeneSTAR cohorts[104,147]. 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. While 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 (MPA = 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 arachidonic acid-induced platelet aggregation or urinary 11-dehydrothromboxane B_2 levels with rs12041331 pre- 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[4,8,101,104,154,156,158].

Given the prior observation that aspirin-treated coronary artery disease patients who carried the minor allele of *PEAR1* rs12041331 experienced higher rates of myocardial infarction compared to non-treated carriers[8], 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. In fact, an aspirin dose above 81 mg did not strongly influence the degree of inhibition of platelet aggregation in our entire cohort or by rs12041331 genotype, consistent with recent U.S. guidelines that favor low-dose aspirin antiplatelet therapy [153].

While 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 [8]. While 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, 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 six-fold higher than expression in platelets[105]. Intriguing work by Vandenbriele and colleagues recently showed that PEAR1 is a negative regulator of neoangiogenesis and wound healing[108]. Furthermore, Fisch *et al*. 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 non-invasive measure of endothelial function [53]. Additional mechanistic studies both in the endothelium and in platelets will be required to clarify the role of *PEAR1* in cardiovascular disease risk both in 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. While this did not seem to inhibit our ability to observe significant changes by *PEAR1* rs12041331 genotype pre- and post-aspirin administration, the effect sizes of this variant on agonist-induced platelet aggregation may be over- or underestimated. However, the effect sizes of *PEAR1* rs12041331 on platelet aggregation in this study are similar to the effect sizes observed in previous investigations [8,104]. 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 platelet (7-10 days), we do not

believe that platelet aggregation results would differ considerably between short- 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. While our results are consistent with previously conducted retrospective analyses in Caucasian 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- and post-aspirin administration using several important platelet agonists. While no difference in platelet function was observed using traditional tests of aspirin response (i.e. arachidonic acid-induced platelet aggregation and circulating levels of urinary thromboxane B_2), *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- 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.

Chapter 6: shRNA-Mediated Knockdown of Platelet Endothelial Aggregation Receptor 1 (PEAR1) Inhibits Endothelial Cell Migration, Adhesion, and Proliferation

Abstract

Several investigations have shown that platelet endothelial aggregation receptor 1 (PEAR1) is critical in a number of physiological processes including phagocytosis, megakaryopoiesis, and platelet aggregation. However, few investigations have been conducted to elucidate the role of PEAR1 in endothelial cells, where it is most highly expressed. In this investigation, we utilized shRNA-mediated knockdown of *PEAR1* in primary human umbilical vein endothelial cells (HUVECs) to determine the impact of PEAR1 on endothelial cell migration, adhesion, and proliferation. We extended our findings by examining the impact of the well-described *PEAR1* variant, rs12041331, on HUVEC function, and evaluated the influence of this polymorphism on circulating endothelial biomarkers in 63 healthy subjects pre- and post-aspirin intervention (325 mg/day for 7 days). We observed that *PEAR1* knockdown significantly reduced HUVEC migration, adhesion, and proliferation compared to control cells (P = 0.008, 0.02, and 0.009, respectively). Aspirin exposure largely abrogated the effect of *PEAR1* knockdown on migration and adhesion (P = 0.19 and 0.24, respectively); however, proliferation remained significantly reduced compared to control cells (P = 0.01). rs12041331 did not significantly influence *PEAR1* expression, and no significant difference in migration adhesion, or proliferation was observed by *PEAR1* genotype pre- or post-aspirin administration. Furthermore, no genotypic differences in circulating endoglin, sVCAM-

1, sICAM-1, or endothelin-1 levels were observed before or after aspirin treatment. Taken together, these results suggest that PEAR1 significantly impacts endothelial cell migration, adhesion, and proliferation, although further investigation is warranted to determine the influence of genetic variation and aspirin use on these traits.

Introduction

Platelet Endothelial Aggregation Receptor 1 (PEAR1), first described in 2005 by Nanda and colleagues, is a type 1 transmembrane tyrosine kinase receptor involved in a number of physiological processes including megakaryopoiesis, thrombopoiesis, platelet aggregation, and vasculogenesis[53,105,107,108]. While it is most highly expressed on the plasma membrane of endothelial cells and platelets, PEAR1 is found in multiple tissue and cell types, and has been previously implicated in cardiovascular disease risk[8,105]. At this time, an increasing body of evidence has led to a better understanding of the function of PEAR1 in platelets, where it acts to stabilize growing platelet aggregates downstream of platelet activation through secondary activation of glycoprotein IIb/IIIa activation[1,105]. Interestingly, recent investigations also suggest that PEAR1 is critical in endothelial function as a negative regulator of neoangiogenesis and wound healing[53,159].

Multiple genetic studies have consistently observed that a polymorphism in intron 1 of *PEAR1* (rs12041331) significantly impacts platelet- and endothelial-related functions of PEAR1[8,53,146,147]. Indeed, recent work by Izzi and colleagues convincingly showed that genetic variability at rs12041331 results in allele-specific differences in DNA methylation and a subsequent reduction in PEAR1 expression in those who carry the minor allele of this polymorphism[160]; an observation that is consistent with previous reports[104]. Importantly, prior investigations have also shown that the influence of *PEAR1* rs12041331 on platelet-related phenotypes and cardiovascular disease risk is influenced by antiplatelet therapy with aspirin[8,156].

In this investigation, we sought to better understand the relationship between PEAR1 and endothelial function in order to gain insights regarding the mechanism(s) by

which this receptor may impact cardiovascular disease risk. Furthermore, we hypothesized that the effect of PEAR1 on endothelial-related phenotypes would be influenced by aspirin administration. To test these hypotheses, we utilized short hairpin RNA (shRNA)-mediated knockdown of *PEAR1* expression to investigate the impact of this gene on endothelial cell migration, adhesion, and proliferation pre- and post-aspirin administration in primary human umbilical vein endothelial cells (HUVECs). We extended our findings by performing experiments of endothelial cell migration, adhesion, and proliferation in the presence and absence of aspirin.in primary HUVECs stratified by *PEAR1* rs12041331 genotype. Finally, we performed a prospective genotype-directed short-term invention of aspirin (325 mg/day for 7 days) in 63 relatively healthy individuals to assess whether rs12041331 genotype was correlated with circulating biomarkers of endothelial function.

Methods

Reagents and Antibodies

Endothelial Basal Growth Medium 2 (EBM2) and all added supplements were purchased from Lonza (Walkersville, Maryland). Acetylsalicylic acid (ASA), gelatin solution (0.5%), as well as endoglin, sVCAM-1, and sICAM-1 ELISA kits were purchased from Sigma Aldrich (St. Louis, Missouri). Trypsin and Dulbecco's phosphate buffered saline (dPBS) were purchased from Mediatech, Inc. (Manassas, Virginia). Gentra Puregene Cell Kit was purchased from Qiagen (Valencia, California). RIPA lysis buffer and all added components were purchased from Teknova (Hollister, California). Gateway LR Clonase II Enzyme Mix was purchased from Invitrogen (Carlsbad, California). pENTR1A no ccDB (w48-1) and pCD/NL-BH*DDD (plasmid 17531) were purchased from Addgene (Cambridge, Massachusetts). pSMPUW Universal Lentiviral Expression Vector was purchased from Cell Biolabs (San Diego, California). CalFectin Mammalian Cell Transfection Reagent was purchased from SignaGen (Rockville, Maryland). Polybrene Transfection Reagent was purchased from EMD Millipore (Billerica, Massachusetts). cOmplete™ Protease Inhibitor Cocktail Tablets and the Rapid DNA Ligation Kit were purchased from Roche Diagnostics (Indianapolis, Indiana). TaqMan® SNP genotyping reagents, NuPAGE™ 4-12% Bis-Tris Mini Gels, XCell SureLock™ Mini-Cell Electrophoresis System, NuPAGE® MES SDS Running Buffer (20X), NuPAGE® Transfer Buffer (20X), Pierce™ BCA Protein Assay kit, and Pierce™ ECL Western Blotting Substrate were purchased from Thermo Fisher Scientific (Waltham, Massachusetts). Laemmli Sample Buffer (2X), 2-Mercaptoethanol, and nitrocellulose membranes were purchased from Bio-Rad Laboratories (Hercules,

California). Human PEAR1 antibody (antigen affinity-purified polyclonal goat-IgG) and donkey anti-goat IgG horseradish peroxidase-conjugated antibody were purchased from R&D Biosystems (Minneapolis, Minnesota). GAPDH (14C10) rabbit monoclonal antibody was purchased from Cell Signaling Technology, Inc. (Beverly, Massachusetts). Donkey anti-rabbit IgG horseradish peroxidase –conjugated antibody was purchased from Santa Cruz Biotechnology (Dallas, Texas). Luminata™ Classico and Luminata™ Cresendo Western horseradish peroxidase substrate were purchased from EMD Millipore (Billerica, Massachusetts). HEK293T cells and XTT Cell Proliferation Assay Kits were purchased from ATCC (Manassas, Virginia). Ibidi ibiTreat culture dishes with a µDish culture insert were purchased from Ibidi USA Inc. (Madison, Wisconsin). CytoSelect™ Cell Adhesion Assay Kits, polybrene transfection reagent, pVSVG envelope vector, and pSMPUW lentiviral vector were purchased from Cell Biolabs, Inc. (San Diego, California). Endothelin-1 ELISA kits were purchased from Enzo Life Sciences (Farmingdale, New York).

Cell culture

De-identified human umbilical cords were obtained from the University of Maryland Medical Center Division of Maternal and Fetal Medicine, and HUVECs were harvested as previously described[161]. Briefly, umbilical cords were cut at both ends, blood was manually drained, and the umbilical vein was washed 3 times with PSA-PBS (500ul 100X penicillin-streptomycin-amphotericin B + 50ml 1x PBS) and clamped on both ends using sterile hemostatic clips. The umbilical vein was injected with 0.2% collagenase solution and incubated for 8 minutes at 37 ^{0}C, after which the cord was

gently squeezed to dislodge and collect detached cells. Collected cells were centrifuged at 400 x g for 6 minutes and re-suspended in media for culture. Isolated HUVECs were cultured in complete EBM2 containing 2% FBS, 0.04% hydrocortisone, 0.4% hFGF, 0.1% VEGF, 0.1% R3-IGF, 0.1% ascorbic acid, 0.1% hEGF, 0.1% gentamicin-amphotericin-B [GA-1000], and 0.1% heparin and incubated under normal growth conditions (37 ^{0}C, 5% CO_2). DNA from each cell line was extracted using a Gentra Puregene Cell Kit according to the manufacturer's instructions. *PEAR1* rs12041331 genotype of each cell line was obtained using a TaqMan® SNP genotyping assay performed on an Applied Biosystems 7900HT Fast Real-Time PCR system (Applied Biosystems 7900HT Sequence Detection System) according to the manufacturer's protocol. Six primary HUVEC lines that were wild-type for *PEAR1* rs12041331 (i.e. G/G homozygotes) were randomly selected to assess the effect of *PEAR1* knockdown on endothelial migration, extracellular matrix adhesion, and proliferation. Nine additional primary HUVEC cell lines (4 G/G, 3 G/A, and 2 A/A for rs12041331) were randomly selected to assess the functional impact of *PEAR1* rs12041331 genotype on endothelial migration, extracellular matrix adhesion, and proliferation.

Aspirin dosing

The impact of *PEAR1* knockdown and rs12041331 genotype on endothelial cell migration, adhesion, and proliferation was assessed in the presence and absence of ASA, the active ingredient in aspirin. For all *in vitro* experiments, ASA was dissolved in complete EBM2 at a concentration of 1 mM, consistent with physiological plasma concentrations of salicylate after therapeutic high-dose aspirin treatment[162] and

previously published *in vitro* studies evaluating the impact of aspirin on endothelial function[163-165]. For the extracellular adhesion assay, each cell line was treated with ASA for 24 hours. After incubation under normal growth conditions, all cells were collected, counted, and re-suspended at a density of 5 x 10^5 cells/ml in serum-free EBM2. For migration and proliferation assays, untreated cells were re-suspended at a density of 5 x 10^5 cells/ml in complete EMB2 with and without freshly prepared ASA and incubated for 24 hours under normal growth conditions prior to testing.

Lentivirus-mediated shRNA knockdown of PEAR1 gene expression

A lentiviral vector for shRNA-mediated knockdown of PEAR1 was generated as previously described[166]. Briefly, a shRNA targeting *PEAR1* (shREAR1- 2058, forward strand 5'-ACCTCGACTGGACACCACTGCTTAGATCAAGAGTCTAAGCA GTGGTGTCCAGTCTT-3', reverse strand 5'- CAAAAAGACTGGACACCACTGCTTA GACTCTTGATCTAAGCAGTGGTGTCCAGTCG-3') or negative scrambled control (forward strand 5'- ACCTCGCTGGAGAACCCATTCCTAGATCAAGAGTCTAGGAA TGGGTTCTCCAGCTT-3', reverse strand 5'- CAAAAAGCTGGAGAACCCATTCCTA GACTCTTGATCTAGGAATGGGTTCTCCAGCG-3') was annealed and ligated into a modified pENTR1A entry vector using the Rapid DNA Ligation Kit and subsequently recombined into a pSMPUW lentiviral vector using the Gateway LR Clonase II Enzyme Mix. To produce constructs containing *PEAR1* shRNA, scramble shRNA, or GFP, 1.2 μg of the transfer vector (PEAR1, Scramble, or GFP), 1.2 μg of pCD/NL-BH*DDD

(plasmid 17531) and 0.2 μg of pVSVG were co-transfected into HEK293T cells using CalFectin according to manufacturer's instruction. Culture media containing the CalFectin/DNA complex was replaced with fresh complete DMEM growth medium 16 hours post-transfection. Viral medium was collected 24 and 48 hours post-transfection, pooled, and then centrifuged at 172 x g at room temperature for 5 min. The supernatant was aliquoted and stored at -80 °C. For shRNA-mediated knockdown of *PEAR1*, at approximately 50% confluence, HUVEC lines were transfected with 1 ml of either Lenti-PEAR1 shRNA or Lenti-scramble shRNA and supplemented with 0.8 μl/ml Polybrene Transfection Reagent and 4 ml complete EBM2 growth media. After incubation for 24 hours under normal growth conditions (37^0C, 5% CO_2), cells were transfected again with either Lenti-PEAR1 shRNA or Lenti-scramble shRNA as described above and incubated for an additional 24 hours, after which cells were prepared for functional testing. During experiment optimization, a lentiviral construct expressing GFP was used to assess transfection efficiency. After completion of primary experiments, HUVECs were collected to confirm PEAR1 knockdown by Western blot.

Evaluation of endothelial migration

We evaluated the impact of *PEAR1* knockdown and rs12041331 genotype on *in vitro* endothelial cell migration pre- and post-aspirin administration using methods described previously[167] with some modifications. Briefly, HUVEC cell suspensions (70 μl at 5.0 x 10^5 cells/ml) of each treatment group were seeded on each side of an Ibidi ibiTreat culture dish containing a μDish culture insert. When cells reached confluency (approximately 24 hours), culture inserts were removed, leaving an effective "wound"

width of 500 μm. Cells were photographed at 0 and 6 hours after the insert was removed using a ZEISS Axiocam MRc5 camera (Carl Zeiss Microscopy, Pleasanton, California) mounted on a Lumar.V12 microscope (Carl Zeiss Microscopy, Pleasanton, California). The area of the wound was measured using ImageJ[168] and used to calculate endothelial cell migration as described in the Statistical Analysis section. Each treatment group was measured in triplicate.

Assessment of extracellular matrix adhesion

The influence of PEAR1 on endothelial cell adhesion to extracellular matrix proteins pre- and post-aspirin administration was measured using the CytoSelect™ Cell Adhesion Assay Kit. Briefly, HUVEC cell suspensions (150 μl at 5.0 x 10^5 cells/ml) of each treatment group were added to 48-well plates pre-coated with collagen I, collagen IV, laminin I, fibronectin, and fibrinogen and incubated for 90 minutes. After unbound cells were removed through washing with PBS, adherent cells were stained with Cytoselect™ cell stain solution (Part No. 11002). After a second wash with deionized water, adherent cells were incubated with cell extract solution (Part No. 11003), transferred to a 96-well microtiter plate, and then measured colorimetrically at 560 nm using a Perkin-Elmer Victor X3 plate reader (PerkinElmer, Inc., Waltham, Massachusetts). All adhesion measurements were made in duplicate.

Measurement of endothelial cell proliferation

An XTT Cell Proliferation Assay Kit was utilized to evaluate HUVEC proliferation in each treatment group. Briefly, aspirin-treated and non-treated cells (100

µl at 5.0 x 10^{-5} cells/ml) were added to 96-well microtiter plates and proliferation was determined colorimetrically at 24 hours using a Perkin-Elmer Victor X3 plate reader (PerkinElmer, Inc., Waltham, Massachusetts). All measurements were made in triplicate.

Western Blot

HUVECs for Western Blot analysis were pelleted by centrifugation at 172 x g (room temperature) and re-suspended in 100 µl of RIPA lysis buffer containing 150 mM sodium chloride, 1% Triton X-100, 1% deoxycholic acid-sodium salt, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCL (pH 7.5), 2 mM EDTA and a cOmplete™ a Protease Inhibitor Cocktail Tablet. Cells were lysed by sonication and cell lysate protein concentration was measured using a Pierce™ BCA Protein Assay Kit. Cell lysates were subjected to SDS-PAGE under reducing conditions per the Novex NuPAGE® Tris-Glycine Mini Gel Electrophoresis protocol. Samples were denatured and separated on NuPAGE™ 4-12% Bis-Tris Mini Gels using the XCell SureLock™ Mini-Cell Electrophoresis System with NuPAGE® MES SDS Running Buffer at a constant voltage of 120 V for 4 hours. Subsequently, proteins were transferred to a nitrocellulose membrane using NuPAGE® Transfer Buffer containing 10% methanol overnight in 4 ^{0}C conditions at a constant voltage of 40V. Membranes were blocked with 5% nonfat milk in PBS supplemented with 0.02% Tween-20 (PBS-T) and blotted with PEAR1 antibody (antigen affinity-purified polyclonal goat-IgG) overnight. After incubation with HRP-conjugated donkey anti-goat secondary antibody for 1 hour, PEAR1 levels were determined using Luminata™ Cresendo Western HRP Substrate. GAPDH was used as an internal control protein for densitometry.

Prospective evaluation of circulating endothelial biomarkers

Circulating biomarkers of endothelial function were evaluated pre- and post-aspirin intervention (325 mg/day for 7 days) in 63 healthy, Old Order Amish participants selected based on known *PEAR1* rs12041331 genotype. All participants were generally healthy and agreed to withdraw from all vitamins, supplements, and medications for a minimum of 2 weeks prior to the initial clinic visit. Participants were excluded if any of the following criteria were met: platelet count $< 100{,}000$ mm^3 or $> 500{,}000$ mm^3, hematocrit < 25 or > 50, blood pressure > 140/90, TSH < 0.40 or > 5.50, creatinine > 2.0, AST or ALT > 2.0x normal, allergy to aspirin, planned warfarin or dabigatran therapy during the study period, pregnant or breast feeding, history of myocardial infarction, coronary artery bypass surgery, unstable angina, or angioplasty, history of gastrointestinal bleeding, a life-threatening bleeding event, bleeding diathesis, or coagulopathy, type 2 diabetes, surgery in the past 3 months or planned surgery in the next 12 months, any other co-existing malignancy or other condition that would place participants at unacceptable risk. Blood samples were collected after overnight fast, after which participants took 325 mg of aspirin and were instructed to take the same dose for the following 6 days. On Day 7, all participants returned to the clinic, and fasting blood samples were collected 1 hour following the final aspirin dose. Blood samples were banked at -80°C until used. Adherence to aspirin was confirmed by manual pill counts and usage logbooks. Clinic staff was blinded to participant genotype status during course of the study.

Biomarker testing

Circulating levels of endoglin, endothelin-1, sVCAM-1, and sICAM-1were measured pre- and post-aspirin administration through the use of commercially available enzyme-linked immunosorbent assays according to manufacturer's instructions. Each sample was measured in duplicate using a Perkin-Elmer Victor X3 plate reader (PerkinElmer, Inc., Waltham, Massachusetts) and concentrations of each sample were calculated using a 4-paramater logistic curve. Duplicate samples with a coefficient of variation $\geq$ 15% were re-tested.

Statistical Analysis

PEAR1 expression after shRNA knockdown was statistically evaluated using a paired t-test implemented in R. For experiments evaluating the influence of *PEAR1* knockdown on endothelial cell migration, adhesion, and proliferation, a repeated measures ANOVA implemented in R was used to determine statistical significance. Comparison groups for each assay of *PEAR1* knockdown were control vs knockdown and ASA-treated control vs ASA-treated knockdown. *PEAR1* genotype-specific *in vitro* tests of endothelial function were conducted using a one-way ANOVA implemented in R. The effect of genotype was assessed assuming an additive model (G/G = 0, G/A = 1, and A/A = 2). The impact of aspirin on endothelial cell migration, adhesion, and proliferation was assessed by repeated measures ANOVA in R.

The effect of aspirin on circulating biomarkers of endothelial function was assessed by repeated measures ANOVA in R. Given the cryptic relatedness between members of the Amish community, mixed models analyses were performed under an additive genetic effect (G/G = 0, G/A = 1 and A/A = 2) using Mixed Model Analysis for Pedigrees (MMAP; http://edn.som.umaryland.edu/mmap/) to assess the association between *PEAR1* rs12041331 and circulating biomarkers of endothelial function. MMAP conditions the genotype-phenotype correlations on the phenotypic correlations among relatives to account for relatedness between study participants. To model the polygenic component, a relationship matrix was developed from the complete Amish pedigree available through published genealogical records[119]. MMAP has been extensively used for genomic analysis in the Amish [7,8,53,91,117,118,128]. All analyses in MMAP were conducted with adjustment for age, sex, and participant relatedness. All statistical analyses were 2-sided and $P < 0.05$ was considered statistically significant.

Results

PEAR1 knockdown impacts endothelial cell migration, adhesion, and proliferation

To determine the role of *PEAR1* in endothelial cell migration, adhesion, and proliferation, primary HUVECs were transfected with lentiviral constructs expressing either a shRNA targeting *PEAR1* or a scrambled control shRNA. Western blot analysis confirmed reduction of PEAR1 protein expression by approximately 49% in HUVECs infected with *PEAR1*-targeting shRNA compared to cells infected with a scrambled control shRNA (P=0.001, Figure 1). Transfection efficiency was assessed through infection of cells with a lentiviral construct expressing GFP.

The impact of PEAR1 on endothelial cell migration was functionally evaluated *in vitro* using a modified version of the well-described scratch assay[167]. Differences in HUVEC migration between cells transfected with either *PEAR1* or control shRNA-expressing lentiviral vectors were assessed 6 hours post-insert removal. We observed that shRNA-mediated knockdown of *PEAR1* significantly reduced HUVEC migration compared to cells infected with control shRNA (P = 0.008, 95.4 ± 24.7 μm and 118.5 ± 26.4 μm, respectively, Figure 2).

Endothelial cell adhesion to extracellular matrix proteins is critical for angiogenesis, vasculogenesis, and maintenance of vascular endothelium[31]. The adhesive properties of HUVECs infected with either *PEAR1* or control shRNA-expressing lentiviral vectors to fibronectin, collagen I, collagen IV, laminin I, fibrinogen, and bovine serum albumin (BSA) were evaluated in 6 independent cell lines. We observed that *PEAR1* knockdown resulted in a strong reduction in cellular adhesion to collagen I compared to cells that were infected with viral particles expressing control shRNA (P =

0.008, Figure 3). Further, adhesion to fibronectin and fibrinogen was modestly reduced in cells infected with *PEAR1* shRNA-expressing lentiviral vectors compared to control shRNA-expressing particles (P = 0.04 and 0.04, respectively). No differences in cellular adhesion between *PEAR1* knockdown and control groups were observed when collagen IV, laminin I, or BSA was used to stimulate adhesion (P = 0.12, 0.09, and 0.06, respectively, Figure 3).

Endothelial cell proliferation in HUVECs infected with either *PEAR1* or control shRNA-expressing lentiviral vectors was assessed at 24 hours. Using 6 primary HUVEC lines, we observed that cellular proliferation was significantly reduced in HUVECs infected with *PEAR1*-targeting shRNA compared to cells infected with a scrambled control shRNA (P = 0.003, Figure 4).

Aspirin treatment influences PEAR1-mediated effects on endothelial function

Given that prior investigations have shown that the impact of genetic variation in *PEAR1* is influenced by aspirin treatment[8,104,156,158] , we also evaluated the role of *PEAR1* knockdown on endothelial cell migration, adhesion and proliferation after ASA exposure (1 mM for 24 hours). While we did observe significant differences in endothelial cell migration in untreated *PEAR1* knockdown vs. control HUVECs (see above), no difference in migration between cells infected with *PEAR1*-targeting shRNA compared to cells infected with a scrambled control shRNA was observed after ASA exposure (P = 0.13, 113.5 ± 19.4 μm and 103.7 ± 26.4 μm, respectively, Figure 2). Similarly, while differences in cellular adhesion to extracellular matrix proteins (i.e. collagen I) were observed between *PEAR1* knockdown and control groups prior to ASA treatment, no

difference in adhesion to fibronectin, collagen I, collagen IV, laminin I, fibrinogen, and BSA was observed between these groups after exposure to ASA (P = 0.29, 0.18, 0.65, 0.37, 0.16, and 0.97, respectively, Figure 3). Endothelial cell proliferation was significantly different between HUVECs infected with *PEAR1*-targeting shRNA compared to control shRNA after aspirin treatment (P = 0.003), similar to the results observed in untreated cells (Figure 4). The influence of aspirin exposure on endothelial cell migration, adhesion, and proliferation in control HUVECs and *PEAR1* knockdown HUVECs is shown in Table 6.1 and 6.2, respectively.

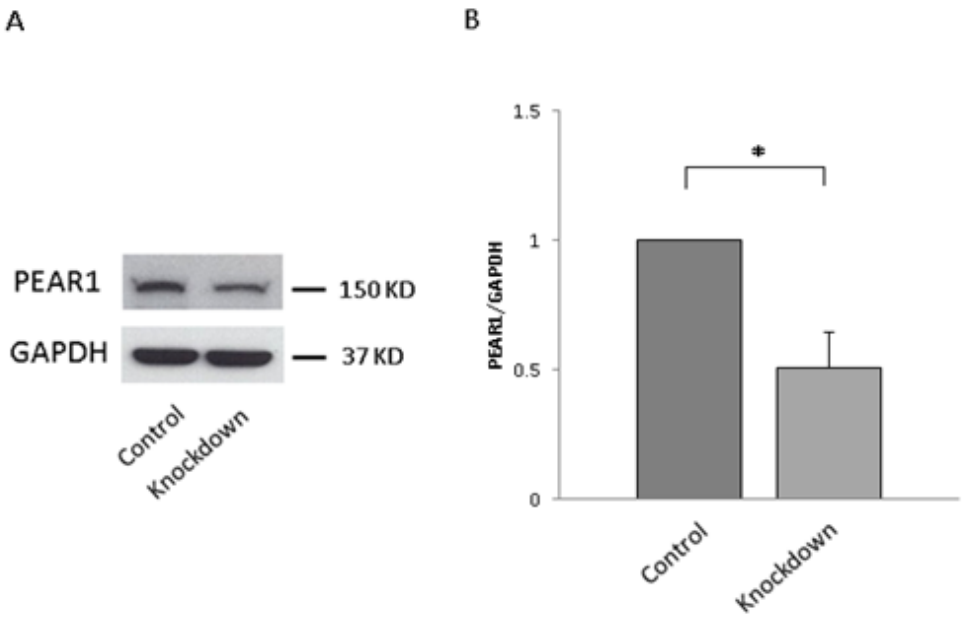
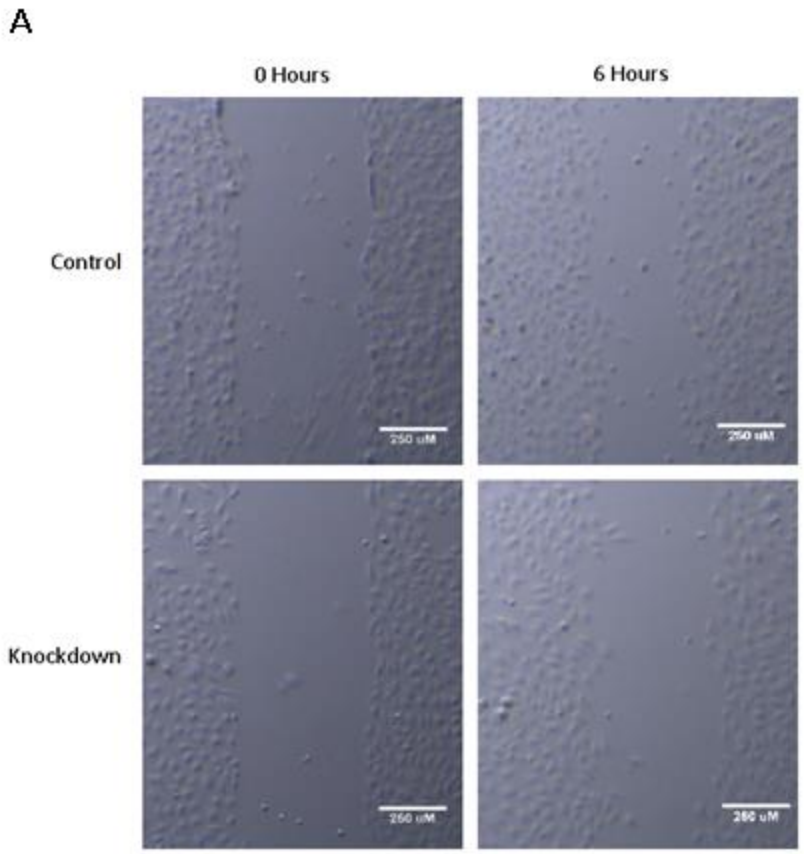


Figure 6.1. shRNA-mediated *PEAR1* knockdown. A) Western blot analysis revealed that PEAR1-targeting shRNA (shPEAR1-2058) decreased PEAR1 protein expression relative to control shRNA. A 0.1% SDS-PAGE gel was used for protein separation. **B)** PEAR1 protein levels, relative to GAPDH, was analyzed using ImageJ in 5 independent human umbilical vein endothelial cell lines. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PEAR1, platelet endothelial aggregation receptor 1. * P < 0.05.


A
0 Hours
6 Hours
Control
Knockdown
250 uM
250 uM
250 uM
250 uM

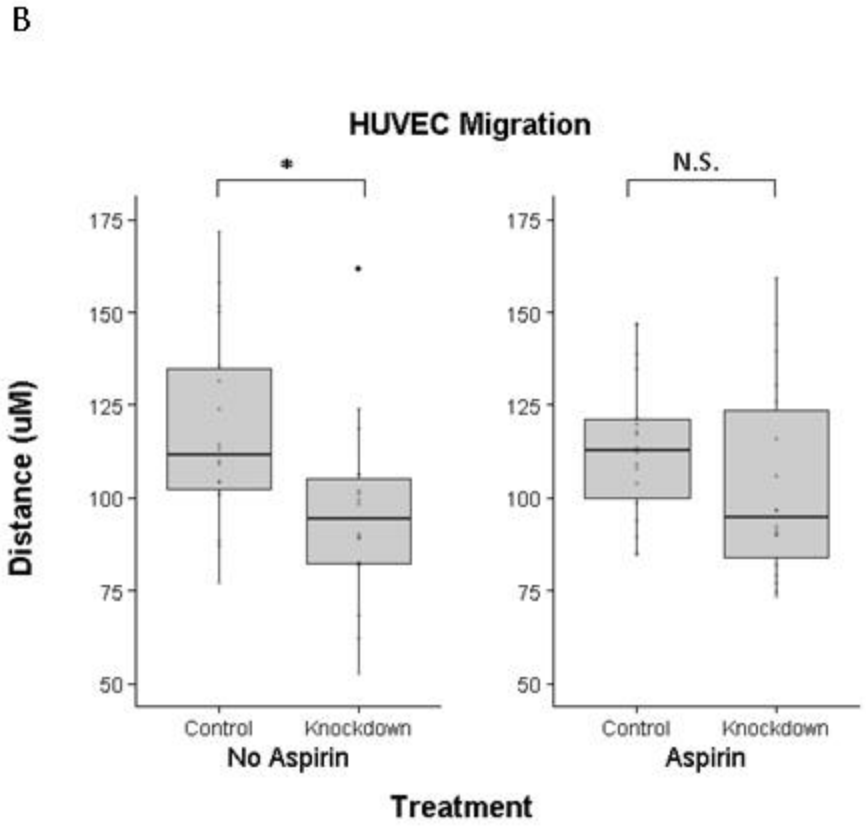

B
HUVEC Migration
*
N.S.
Distance (uM)
175
150
125
100
75
50
Control
Knockdown
No Aspirin
Aspirin
Treatment

Figure 6.2. The impact of *PEAR1* knockdown on endothelial cell migration. A) In untreated and aspirin-treated (1 mM for 24 hours) HUVECs, we evaluated the impact of shRNA-mediated knockdown of *PEAR1* on endothelial cell migration relative to cells infected with control shRNA (6 independent HUVEC lines). **B)** Phase-contrast microscopy images of human umbilical vein endothelial cells infected with lentiviral constructs expressing either *PEAR1*-targeting (shPEAR1-2058) or control shRNA in the absence or presence of aspirin. Images are shown at 20 X magnification. Abbreviations: PEAR1, platelet endothelial aggregation receptor 1; N.S., no significant difference. * $P < 0.05$

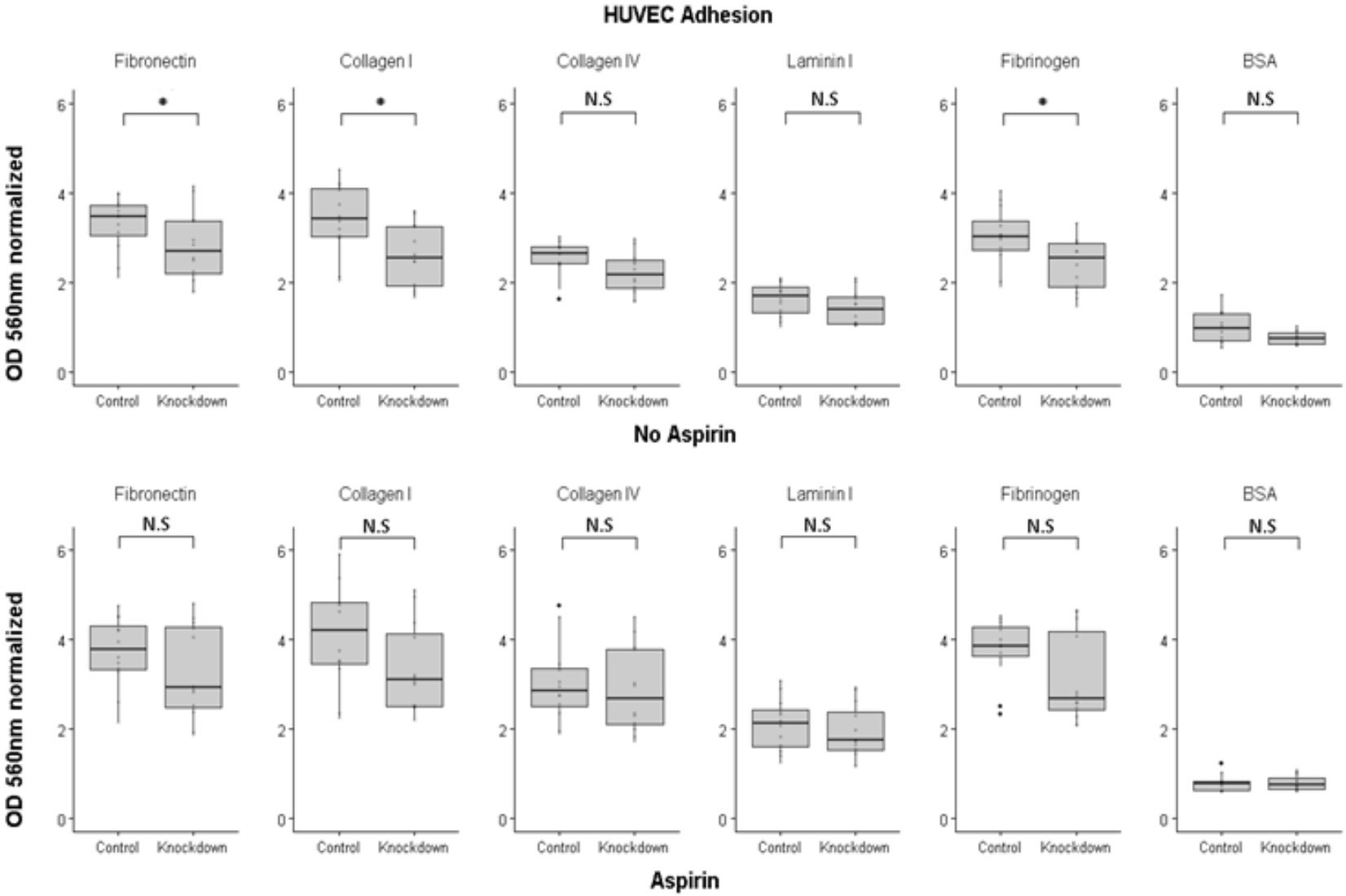

HUVEC Adhesion
Fibronectin
Collagen I
Collagen IV
Laminin I
Fibrinogen
BSA
OD 560nm normalized
N.S
Control
Knockdown
No Aspirin
Aspirin

Figure 6.3. The influence of *PEAR1* knockdown on cellular adhesion to extracellular matrix proteins. In untreated and aspirin-treated (1 mM for 24 hours) human umbilical vein endothelial cells, we tested the influence of shRNA-mediated knockdown of *PEAR1* on adhesion to the extracellular proteins fibronectin, collagen I, collagen IV, laminin I, fibrinogen, as well as BSA relative to cells infected with control shRNA (6 independent HUVEC lines). Adhesion to each extracellular matrix protein was quantified using OD560 nm as described in the Methods section and normalized to BSA binding in untreated control HUVECs. Abbreviations: BSA, bovine serum albumin; PEAR1, platelet endothelial aggregation receptor 1; N.S., no significant difference. * $P < 0.05$

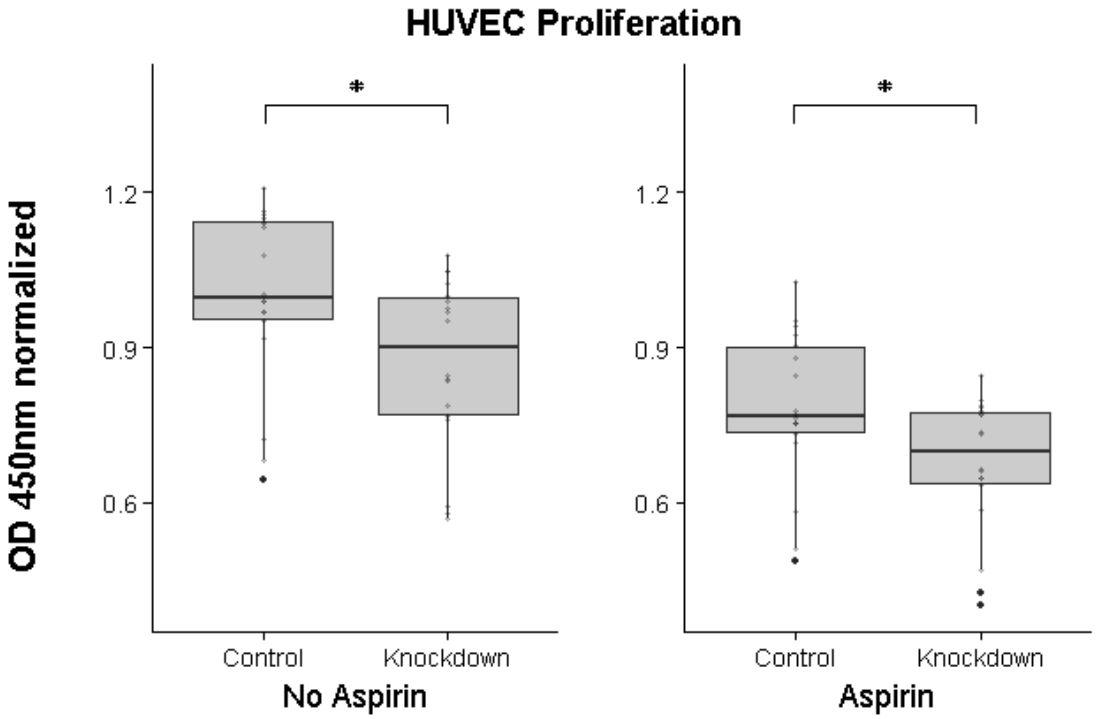


Figure 6.4. *PEAR1* knockdown results in reduced proliferation in untreated and ASA-treated endothelial cells. In untreated and aspirin-treated (1 mM for 24 hours) human umbilical vein endothelial cells, we tested the influence of shRNA-mediated knockdown of *PEAR1* on cellular proliferation in 6 independent cell lines. Proliferation was quantified using OD450 nm as described in the Methods section and normalized to the proliferation of untreated control HUVECs. Abbreviations: ASA, acetylsalicylic acid; PEAR1, platelet endothelial aggregation receptor 1; N.S., no significant difference. * $P < 0.05$

Table 6.1. Effect of ASA in control HUVECs (n=6)

	No Aspirin	Aspirin	P-value
Migration	118.47 ± 26.40	113.46 ± 19.41	0.34
Fibronectin	0.38 ± 0.07	0.42 ± 0.09	0.29
Collagen I	0.39 ± 0.09	0.46 ± 0.13	0.09
Collagen IV	0.29 ± 0.05	0.34 ± 0.10	0.25
Laminin I	0.19 ± 0.04	0.24 ± 0.07	0.02
Fibrinogen	0.34 ± 0.07	0.43 ± 0.08	0.06
BSA	0.11 ± 0.04	0.09 ± 0.02	0.08
Proliferation	1.52 ± 0.26	1.19 ± 0.22	3.52×10^{-6}

Values are listed as mean ± standard deviation. Migration distance measured as µM. Extracellular matrix adhesion measured as OD 560nM. Proliferation measured as OD 450nM.

Table 6.2. Effect of ASA in *PEAR1* knockdown HUVECs (n=6)

	No Aspirin	Aspirin	P-value
Migration	95.36 ± 24.67	103.74 ± 26.41	0.29
Fibronectin	0.32 ± 0.09	0.37 ± 0.12	0.42
Collagen I	0.30 ± 0.08	0.38 ± 0.11	0.11
Collagen IV	0.25 ± 0.05	0.33 ± 0.11	0.12
Laminin I	0.16 ± 0.04	0.22 ± 0.07	0.03
Fibrinogen	0.27 ± 0.07	0.35 ± 0.11	0.13
BSA	0.09 ± 0.02	0.09 ± 0.02	0.48
Proliferation	1.32 ± 0.25	1.02 ± 0.20	1.53×10^{-5}

Values are listed as mean ± standard deviation. Migration distance measured as µM. Extracellular matrix adhesion measured as OD 560nM. Proliferation measured as OD 450nM.

Evaluation of PEAR1 rs12041331 on endothelial function

In addition to investigating the effect of *PEAR1* knockdown on endothelial cell migration, adhesion, and proliferation, we also assessed whether the well-described *PEAR1* rs12041331 variant significantly influenced these phenotypes as well as protein expression. In 9 primary HUVEC lines that were selected based on rs12041331 genotype (4 G/G, 3 G/A, and 2 A/A), PEAR1 protein expression was moderately reduced in A-allele carriers, though this difference was not significant (P=0.45, Figure 6.5).

Genotypic differences in endothelial cell migration were assessed at 6 hours post-insert removal. In contrast to the results obtained after shRNA-mediated knockdown of *PEAR1*, no difference in endothelial migration was observed between genotype groups prior to ASA treatment (P = 0.28, 138.3 ± 47.5 μm for the 4 G/G cell lines, 120.5 ± 20.9 μm for the 3 G/A cell lines, and 121.343 ± 19.3 μm for the 2 A/A cell lines). Similarly, no difference in endothelial cell migration was observed by *PEAR1* rs12041331 genotype after ASA exposure (P = 0.99, 118.1 ± 40.3 μm for the 4 G/G cell lines, 115.7 ± 34.9 μm for the 3 G/A cell lines, and 118.7 ± 10.5 μm for the 2 A/A cell lines).

Differences in cellular adhesion to fibronectin, collagen I, collagen IV, laminin I, fibrinogen, and BSA were assessed in the same HUVEC lines by *PEAR1* rs12041331 genotype. In contrast to the results obtained after shRNA-mediated knockdown of *PEAR1*, the A-allele of rs12041331 did not strongly influence adhesion to any extracellular matrix protein investigated in the absence of ASA treatment. Additionally, no difference in extracellular matrix adhesion was observed by *PEAR1* rs12041331 genotype after ASA exposure (Figure 5).

In contrast to the results obtained after shRNA-mediated knockdown of *PEAR1*, no difference in endothelial cell proliferation was observed between rs12041331 genotype groups prior to ASA treatment ($P = 0.34$). Although HUVEC proliferation was generally reduced after ASA treatment ($P = 4.9 \times 10^{-6}$), no difference was observed between rs12041331 genotype groups after ASA treatment ($P = 0.61$).

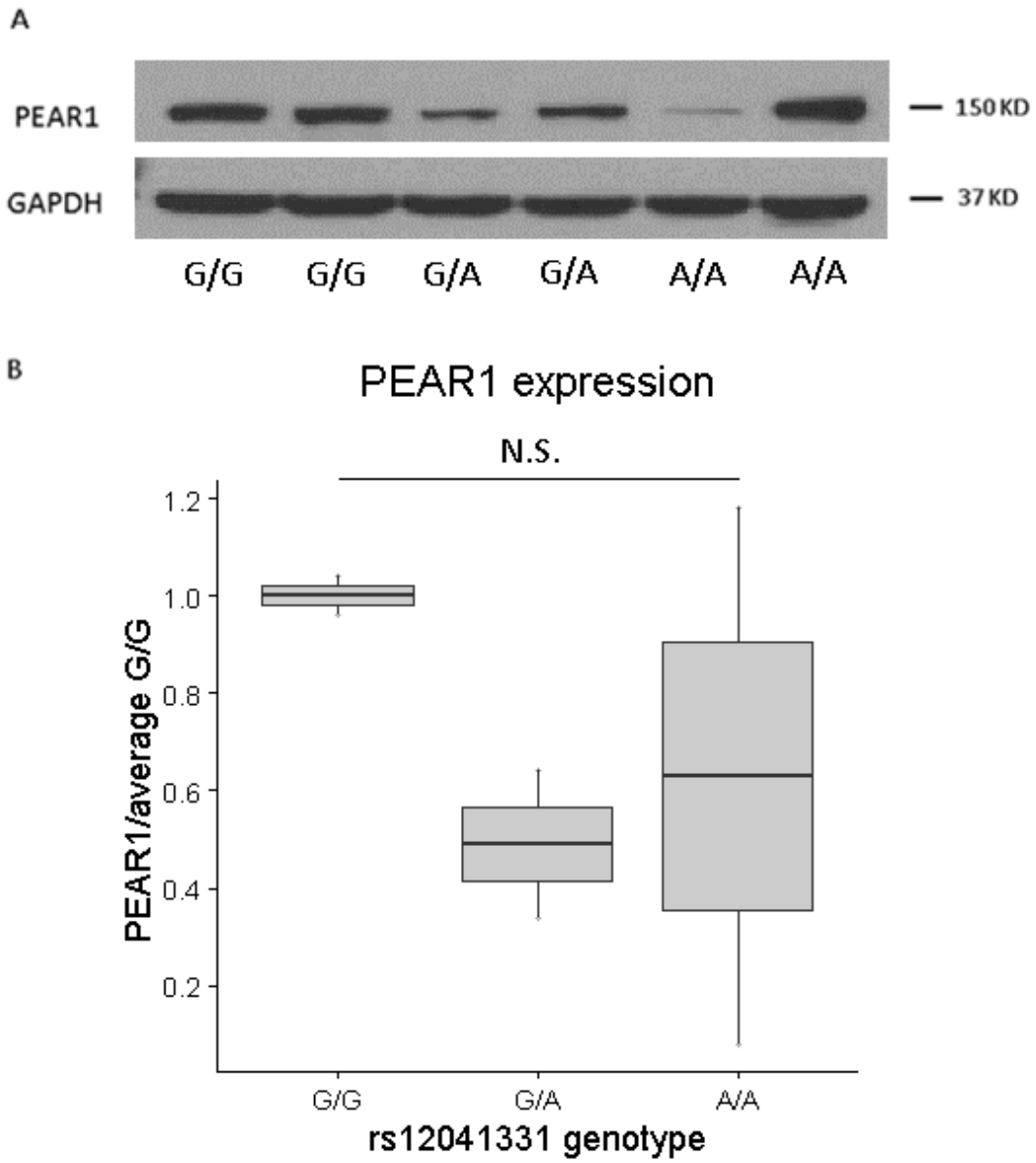


Figure 6.5. PEAR1 expression by rs12041331 A) Western blot analysis revealed that the A-allele of rs12041331 decreased PEAR1 protein expression relative to control shRNA. A 12% SDS-PAGE gel was used for protein separation. **B)** PEAR1 protein levels, normalized to the average PEAR1 expression in G/G homozygotes, were analyzed using ImageJ in 6 (2 G/G, 2 G/A, and 2 A/A)dependent human umbilical vein endothelial cell lines. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PEAR1, platelet endothelial aggregation receptor 1; N.S., no significant difference.

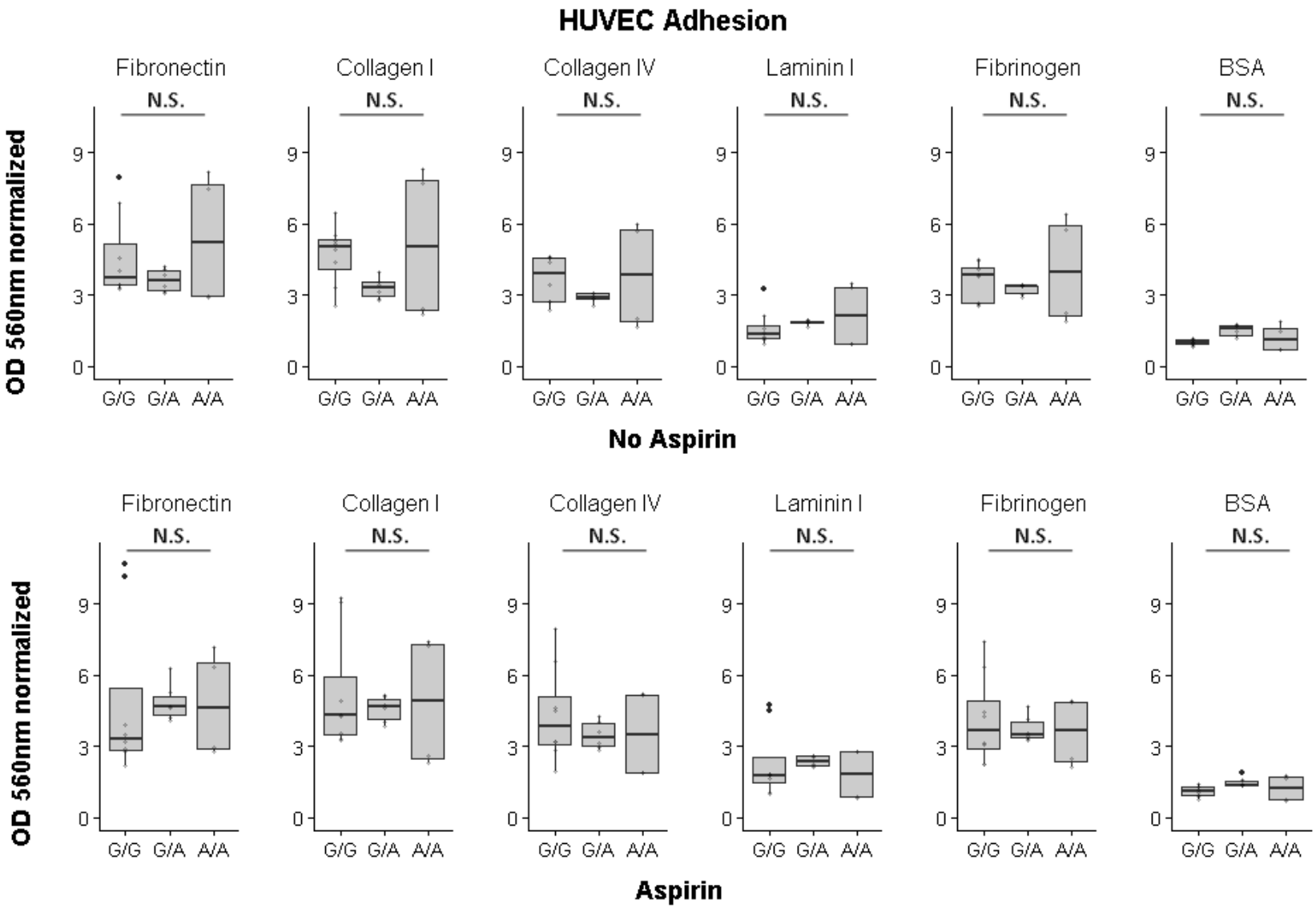

HUVEC Adhesion
Fibronectin
Collagen I
Collagen IV
Laminin I
Fibrinogen
BSA
N.S.
OD 560nm normalized
0
3
6
9
G/G
G/A
A/A
No Aspirin
Aspirin

Figure 6.6. The impact of *PEAR1* rs12041331 genotype on cellular adhesion to extracellular matrix proteins. In untreated and aspirin-treated (1 mM for 24 hours) HUVECs, we tested the influence of *PEAR1* rs12041331 on adhesion to the extracellular proteins fibronectin, collagen I, collagen IV, laminin I, fibrinogen, as well as BSA. Comparisons were based on 4 HUVEC lines that were homozygous for the rs12041331 major allele (G), 3 that were heterozygotes (G/A), and 2 that were homozygous for the minor allele (A). Adhesion to each extracellular matrix protein was quantified using OD560 nm as described in the Methods section and normalized to BSA binding in untreated G/G HUVECs. Abbreviations: BSA, bovine serum albumin; HUVEC, human umbilical vein endothelial cell; PEAR1, platelet endothelial aggregation receptor 1; NS, no significant difference

Impact of PEAR1 rs12041331 on circulating biomarkers of endothelial function

To assess the potential relationship between *PEAR1* and aspirin use on circulating biomarkers of endothelial function, 63 relatively healthy individuals were recruited by rs12041331 genotype (32 participants were G/G homozygotes, 26 were heterozygotes [G/A], and 5 were A/A homozygotes) and underwent a short-term aspirin intervention (325 mg/day for 7 days). Clinical characteristics of these participants are shown in Table 6.3. Pre- and post-aspirin levels of endoglin, endothelin-1, VCAM-1, and ICAM-1 in the entire cohort (N = 63) were not significantly different from each other (Table 6.4). Circulating levels of each of these biomarkers were assessed pre- and post- aspirin administration by rs12041331 genotype (Table 6.5). No evidence of association was observed between rs12041331 genotype and levels of endoglin pre- (P = 0.51) or post-aspirin (P = 0.67) administration. Similarly, circulating levels of endothelin-1 did not differ by *PEAR1* genotype before or after aspirin treatment (P = 0.93 and 0.82, respectively). Furthermore, no genotypic differences in VCAM-1 and ICAM-1 levels were observed before (P = 0.34 and 0.62, respectively) or after (P = 0.15 and 0.68, respectively) aspirin administration (Table 6.5).

Table 6.3. Characteristics of PEAR1 Study participants

Trait (units)	Total	*PEAR1* rs12041331 Genotype			P-Value[‡]
		G/G	G/A	A/A	
Number (n)	63	32	26	5	
Age ± SD (yr)	50.0 ± 9.7	49.9 ± 9.8	50.8 ± 9.6	45.4 ± 10.4	0.65
Sex (n=Female) (%)	27 (43)	13 (41)	12 (46)	3 (60)	0.96
BMI ± SD (kg/m^2)	28.4 ± 4.7	27.9 ± 4.7	29.2 ± 4.8	27.4 ± 4.9	0.39
Hypertension[*] (%)	4 (6)	2 (6)	2 (8)	0 (0)	0.82
Hypercholesterolemia[†] (%)	17 (27)	9 (28)	8 (31)	0 (0)	0.45
Self-reported diabetes (%)	0	0	0	0	N/A
Current smoker (%)	6 (10)	3 (9)	3 (12)	0 (0)	0.86
Hematocrit ± SD (%)	40.2 ± 2.9	40.1 ± 3.0	40.5 ± 3.1	39.9 ± 2.2	0.91
White blood count ± SD (n x 1000/μl)	5.8 ± 1.2	5.5 ± 1.0	6.1 ± 1.4	5.8 ± 1.3	0.12
Platelet count ± SD (n x 100,000/μl)	224.0 ± 47.9	206.5 ± 43.8	246.5 ± 45.2	219.2±46.6	**0.03**

Abbreviations: BMI, body mass index; N/A, not applicable; PEAR1, platelet endothelial aggregation receptor 1; SD, standard deviation

** Defined 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*

[†] Defined as LDL-cholesterol greater than 160 mg/dl or taking prescription medication for previously diagnosed hypercholesterolemia

[‡] P-values represent statistical difference of each trait by PEAR1 rs12041331 genotype

Table 6.4. Mean biomarker concentration pre- and post- aspirin

Biomarker	Pre-Aspirin	Post-Aspirin	P-value
Endoglin (ng)*	6.58 ± 1.96	6.79 ± 1.77	0.13
Endothelin-1 (pg) †	0.76 ± 0.56	0.62 ± 0.41	0.12
VCAM-1 (ng)*	100.43 ± 23.37	100.30 ± 28.68	0.97
ICAM-1(ng)*	145.03 ± 40.54	153.14 ± 60.73	0.23

Values are listed as mean ± standard deviation.
**Endoglin, VCAM-1, and ICAM-1 were assayed in 63 individuals (32 G/G, 26 G/A, 5 A/A).*
†Endothelin-1 was assayed in 41 individuals (20 G/G, 18 G/A, 3 A/A).

Table 6.5. Biomarkers of endothelial function stratified by *PEAR1* rs12041331 genotype

Biomarker	Treatment	*PEAR1* rs12041331 Genotype			Beta	P-value
		G/G	G/A	A/A		
Endoglin (ng)*	Pre-aspirin	6.46 ± 0.39	6.79 ± 0.35	6.21 ± 0.67	-0.25 ± 0.39	0.52
	Post-aspirin	6.73 ± 0.36	6.97 ± 0.30	6.26 ± 0.52	-0.14 ± 0.36	0.71
Endothelin-1 (pg)†	Pre-aspirin	0.79 ± 0.14	0.74 ± 0.10	0.68 ± 0.13	-0.01 ± 0.13	0.93
	Post-aspirin	0.60 ± 0.10	0.64 ± 0.09	0.59 ± 0.17	0.03 ± 0.11	0.82
VCAM-1 (ng)*	Pre-aspirin	104.61 ± 4.18	96.20 ± 4.71	95.74 ± 7.04	-5.59 ± 4.67	0.24
	Post-aspirin	106.08 ± 5.29	95.41 ± 5.26	88.76 ± 12.04	-8.20 ± 5.58	0.15
ICAM-1 (ng)*	Pre-aspirin	145.16 ± 6.04	150.40± 9.08	116.17 ± 18.93	-4.05 ± 8.12	0.62
	Post-aspirin	144.43 ± 6.90	168.57 ± 15.93	128.66 ± 15.59	5.07 ± 12.32	0.68

Values are listed as mean ± standard error.
**Endoglin, VCAM-1, and ICAM-1 were assayed in 63 individuals (32 G/G, 26 G/A, 5 A/A).*
†Endothelin-1 was assayed in 41 individuals (20 G/G, 18 G/A, 3 A/A).

Discussion

The endothelial monolayer that lines the interior of blood vessels serves as a protective barrier between the blood and surrounding tissue and is critical for maintaining cardiovascular health[20]. The major functions of the endothelium include regulating hemostatic balance, vascular tone, wound healing, angiogenesis, and inflammation[20]. During wound repair and angiogenesis, endothelial cells must be able to migrate from the vasculature, adhere to the extracellular matrix, and proliferate to form new vasculature[31]. Risk factors such as high lipid levels and tobacco use[20,26], as well as genetic variation in genes involved in essential endothelial processes compromise the integrity of the endothelium and result in increased risk of cardiovascular diseases. Given the results of previous investigations by our group and others[53,108,160], we hypothesized that PEAR1 was an important determinant of endothelial cell function and that changes in *PEAR1* expression would influence endothelial-related processes important in cardiovascular-related health.

PEAR1 is a recently identified 150 kDa transmembrane protein involved in a number of diverse biological processes including phagocytosis, thrombosis, and vasculogenesis[1,105,107,108,169] and has been previously implicated in risk of myocardial infarction[8]. While PEAR1 is well-characterized as an important determinant of growing platelet aggregates through induction of secondary glycoprotein IIb/IIIa activation[1], the function of PEAR1 in the endothelium, where expression is 6-fold higher than in platelets[105], is less understood, though recent investigations suggest a significant role in angiogenesis and wound healing[53,108]. In this investigation, we extend on previous studies

by showing that PEAR1 knockdown significantly impacts endothelial cell migration, adhesion, and proliferation.

Through the use of shRNA-mediated knockdown of *PEAR1* in primary HUVECs, which resulted in a 50% reduction in protein expression, we observed an approximate 20% reduction in endothelial cell migration compared to cells treated with a control shRNA. In addition, given the known role of PEAR1 as a contact-contact receptor that propagates thrombus formation through secondary stabilization of aggregation-based pathways, we also evaluated the influence of PEAR1 knockdown on endothelial cell binding capacity to several extracellular matrix proteins critical in cellular adhesion. We observed that reduction in PEAR1 expression impacted the ability of endothelial cells to bind to collagen I by approximately 25%. Interestingly, we also observed that HUVEC binding capacity to fibrinogen and fibronectin was reduced upon *PEAR1* knockdown ($P = 0.04$ and 0.04, respectively). No difference in cellular adhesion to collagen IV or laminin I was observed upon *PEAR1* knockdown. Finally, we evaluated the influence of PEAR1 on the ability of HUVECs to proliferate and observed an approximate 15% reduction in proliferation upon *PEAR1* knockdown compared to control cells.

While gene knockdown is a useful method to elucidate novel functions of genes with unknown or poorly understood features, the impact of naturally occurring genetic variation in those genes can better define the importance of these functions *in vivo* and is often more clinically relevant. Therefore, we complemented our *PEAR1* knockdown experiments by evaluating the effect of rs12041331, the most widely investigated polymorphism in *PEAR1*, on HUVEC migration, adhesion and proliferation. Previous investigations have consistently shown that rs12041331 genotype significantly influences

platelet and endothelial-related functions of PEAR1[8,53,61,104,145,156] and is associated with cardiovascular disease risk[8]. Moreover, rs12041331 was recently shown to modify *PEAR1* expression through disruption of a DNA methylation site, presumably leading to differences in transcription factor binding capacity[160]. Though there was a trend for reduced PEAR1 protein expression with the minor allele of rs12041331, this difference was not statistically significant. We did not find increasing copies of the rs12041331 minor allele to be significantly associated with reduced adhesion to collagen I, collagen IV, fibrinogen, laminin I, or fibronectin. This stands in contrast to the results obtained after shRNA-mediated knockdown of *PEAR1*, and is likely due to the high variability in PEAR1 expression observed in A/A cell lines. Further, no difference in endothelial cell migration or proliferation was observed by rs12041331 genotype.

Prior investigations suggest that platelet-related functions of *PEAR1* are substantially influenced by aspirin use[8,104,156]. Given the known effects of aspirin on endothelial functions including cell migration, adhesion, and proliferation[3,100,163-165,170], we tested whether exposure to aspirin modified the impact of *PEAR1* on these phenotypes. Indeed, while differences in endothelial cell migration were observed between HUVECs infected with lentivirus expressing PEAR1-targeting vs. control shRNA prior to ASA exposure, no difference in endothelial cell migration was observed between these groups after ASA treatment. Furthermore, *PEAR1* knockdown reduced HUVEC adhesion to extracellular matrix proteins including collagen I prior ASA administration; however, no difference in cellular adhesion to these proteins was noted upon *PEAR1* knockdown after exposure to ASA. In contrast, HUVEC proliferation was significantly reduced by *PEAR1* knockdown both pre- and post-aspirin treatment.

In addition to assessing the effect of *PEAR1* on endothelial-related processes *in vitro*, we sought to determine whether *PEAR1* also influenced circulating biomarkers of endothelial function. To test this, we performed a prospective, rs12041331 genotype-stratified, short-term aspirin invention in 63 relatively healthy Old Order Amish individuals and measured several endothelial-related biomarkers (VCAM-1, ICAM-1, endoglin, and endothelin) pre- and post-aspirin administration. While a trend towards decreased VCAM-1 was observed in those that carried the minor allele of rs12041331, no statistical difference in the level of any measured biomarker was observed by *PEAR1* genotype pre- or post-aspirin administration. Given the sample size of our cohort (N = 63), it is possible that lack of genotypic differences in biomarker level could be the consequence of modest statistical power. Alternatively, these results may suggest that while *PEAR1* significantly impacts basic endothelial cell function, genetic variation in this gene may not affect commonly measured biomarkers of endothelial function in circulation.

In the last year, two investigations have been published, one by our group and one by Vandenbriele and colleagues, that were the first to assess the potential impact of PEAR1 on endothelial function[53,108]. Previously, we performed an agnostic meta-analysis of publically available microarray data in order to predict, in silico, novel biological processes that are influenced by *PEAR1*. The results of this analysis suggested that the most likely processes to be influenced by changes in *PEAR1* expression included angiogenesis, cell adhesion, cell migration, and cell proliferation. We extended these findings by showing that rs12041331 significantly impacted *in vitro* endothelial migration and was associated with flow-mediated dilation of the brachial artery, the most

commonly used, non-invasive assessment of endothelial health. In the current investigation, we show that *PEAR1* knockdown substantially reduced endothelial cell migration, adhesion, and proliferation, consistent with the results of our meta-analysis of microarray data. However, we failed to replicate the association between rs12041331 genotype and endothelial cell migration. While differences in methodologies used between the respective studies may contribute to discrepant findings, other factors including differences in genetic backgrounds of tested primary HUVEC lines may have also influenced these results.

More recently, a study by Vandenbriele and colleagues showed that *PEAR1* knockdown resulted in increased matrigel tube formation, an *in vitro* model of angiogenesis, as well as increased endothelial cell migration and proliferation[108]. Furthermore, they evaluated PEAR1 function in vivo by testing hind limb ischemia and skin wound healing of a *PEAR1* knockout mouse. Compared to control mice, Vandenbriele and colleagues observed increased ischemia-induced angiogenesis and two-fold increase in wound closure after dermal biopsy punch in *PEAR1* knockout mice[108]. It is important to note that the effects of *PEAR1* knockdown on endothelial cell migration and proliferation reported herein are in the opposite direction of the results observed by Vandenbriele et al. However, a number of factors such as cell confluency, different experimental design and the different patient populations from which primary cells were derived (Vandenbriele et al collected HUVECs in Belgium, whereas we collected HUVECs in Baltimore, Maryland) are likely to contribute to variation in results. Furthermore, the mechanisms by which PEAR1 functions in the endothelium are not fully understood and could potentially explain the difference in observations.

There are limitations in this investigation that should be taken into consideration when evaluating our results. *In vivo*, the endothelium is constantly exposed to shear stress because of high laminar blood flow, while the *in vitro* assays conducted in the current study were under static conditions. While the assays used in this study are well established and are useful in determining the role of PEAR1 in endothelial cell migration, adhesion, and proliferation, examining the effects of PEAR1 under flow conditions, such as with the Bioflux System[171], would be an informative future approach. In addition, while HUVECs are the most commonly used cellular model to investigate vascular endothelial function, differences between microvascular and macrovascular endothelial cells are likely to influence the results of these assays. Indeed, *PEAR1* expression varies widely between endothelial cell types[108].

It is difficult to model chronic or prolonged aspirin exposure *in vitro.* Though we used concentrations and durations of ASA exposure consistent with many previous experiments and within physiological levels of salicylate in serum[152,162-165], the difference between acute ASA exposure *in vitro* and chronic aspirin treatment *in vivo* is likely to be significant. However, this is the first study to evaluate the influence of aspirin exposure on PEAR1 function in endothelial cells. While we attempted to strengthen our results by evaluating circulating biomarkers *in vivo* in participants treated with aspirin, we were likely under-powered due to a limited sample size; however, participants of the PEAR1 Study were specifically recruited by rs12041331 genotype, and confounding factors were minimized through the strict study design and exclusion criteria.

In this study, we show that *PEAR1* knockdown modifies basic processes of endothelial function. *PEAR1* expression was strongly correlated with HUVEC migration,

adhesion, and proliferation prior to aspirin exposure. However, after ASA treatment, only HUVEC proliferation was impacted by *PEAR1* knockdown. Endothelial dysfunction is characterized by an increased adhesive capacity of the endothelium, as well as a decreased repair capacity for wound healing, both of which contribute to increased thrombotic risk[65,172,173]. While the discordance in the scant published studies on PEAR1 endothelial function makes it difficult to speculate how deleterious genetic variation in *PEAR1* might impact endothelial health *in vivo*, the observed association with extracellular matrix adhesion after *PEAR1* knockdown makes biological sense in consideration of the known role for PEAR1 as a platelet contact receptor. Future investigations of PEAR1 signaling as well as evaluations of endothelial biomarkers in at-risk patients with known *PEAR1* genotype will provide further clarity to the mechanisms by which PEAR1 functions and offer insight into the influence of PEAR1 in the context of cardiovascular disease. Additionally, variation in *PEAR1* may augment the effectiveness of aspirin, which has implications for at-risk patients in need of antiplatelet therapy. Further research is needed to characterize PEAR1 in the endothelium, as well as further clarify a potential interaction between *PEAR1* variation and aspirin response.

Chapter 7: Discussion

Summary:

The overall objective of this investigation was to further elucidate the mechanisms that alter patient response to clopidogrel and aspirin, and to gain insight into the heritable variation that contributes to platelet aggregation and endothelial dysfunction. Despite the widespread use of DAPT, a subset of patients still do not achieve sufficient risk reduction, and aside from the well-described *CYP2C19**2 variant and a few others, traditional *ex vivo* platelet aggregation studies have been insufficient in identifying reproducible genetic factors that significantly influence antiplatelet resistance. The primary strategy for these situations in genetic epidemiology is to exponentially increase sample size; however such efforts have come up short, and most well-characterized DAPT studies with genetic data have already been thoroughly explored. Further, this approach often lacks the nuance required for translatable discoveries because generating larger sample size often comes at the expense of a specific case or intervention definition. With this in mind, we hypothesized that expanding our focus beyond traditional endophenotypes (i.e. platelet aggregation) through examination of measures such as clopidogrel active metabolite concentration and endothelial function, we would not only be able to identify novel genetic variants that influence DAPT response but also improve our understanding of currently established variants that significantly impact clinical outcomes. We addressed this hypothesis through three different approaches: a genome-wide analysis of circulating clopidogrel active metabolite levels, a prospective clinical evaluation of a newly identified aspirin response gene (*PEAR1*), and functional

characterization of *PEAR1* in multiple cell types relevant to cardiovascular risk. These three research endeavors are summarized below.

Genome-wide analysis of serum clopidogrel active metabolite

As described in Chapter 4, we performed a genome-wide analysis of an under-utilized phenotype, serum clopidogrel active metabolite, to identify novel variants that influence clopidogrel pharmacokinetics. Our hypothesis was that this novel endophenotype would facilitate the discovery of unknown effectors of clopidogrel response that would otherwise be missed in studies exclusively examining *ex vivo* platelet reactivity. This is the first study to directly evaluate clopidogrel pharmacokinetics using a genome-wide approach. Consistent with previous investigations, we identified the strongest determinant of clopidogrel active metabolite concentration to be within the *CYP2C19* locus on chromosome 10q24 corresponding to the well-known clopidogrel response effector *CYP2C19**2. Additionally, the GWAS of serum clopidogrel active metabolite identified 2 novel loci on chromosomes 3p25 and 17q11 with significant associations ($P < 5x10^{-8}$) and 6 additional loci with suggestive significance ($P \leq 1 \times 10^{-6}$) for variable clopidogrel metabolism. Consistent with these data, 4 of these 8 loci (chromosomes 17q11, 1q25, 5q32, and 7q21) also exhibited nominal ($P < 0.05$) associations with on-clopidogrel platelet aggregation. In agreement with our hypothesis, none of these loci exhibited a genome-wide level of significance for on-clopidogrel platelet aggregation, and these variants would not have been identified if we only evaluated platelet aggregation. In addition to the aforementioned association signals, a rare variant on chromosome 19p25 identified through exome-wide analysis was strongly associated with clopidogrel active metabolite concentration, though no corresponding

effect was observed on on-clopidogrel platelet reactivity. Additional analytical models, including SKAT and a bivariate analysis with canonical correlation association provided further support for the novel loci identified on chromosomes 3p25 and 17q11.

Since no true replication cohort was available that had clopidogrel active metabolite data, we extended our findings by conducting follow-up investigations in patients from the International Clopidogrel Pharmacogenomics Consortium (ICPC) to determine if any identified variants that influenced clopidogrel active metabolite levels also associated with on-clopidogrel platelet aggregation or ischemic event risk. While no associations were observed for on-clopidogrel platelet aggregation or MACE after correction for multiple testing, a number of trending associations were identified proximal to intriguing candidate genes. On chromosome 1p25, several mutations near *RNF2*, a gene that codes for a protein known to regulate the intestinal clopidogrel transporter P-glycoprotein, displayed trends towards increased on-treatment platelet reactivity and increased MACE. Knockdown of *RNF2* has previously been shown to down-regulate P-glycoprotein[141], and may suggest that genetic variation that influences *RNF2* could decrease intestinal absorption of clopidogrel, leading to decreased clopidogrel active metabolite levels, as observed in the PAPI Study, and increased on-clopidogrel platelet reactivity, as nominally seen in participants of both the PAPI Study and ICPC. On chromosome 3p25, a cluster of variants were identified in *ATP2B2*, which encodes a calcium transporter previously associated with clopidogrel response in an study by Price et al[135]. While no strong evidence was found in the ICPC for variants near this gene, further investigations of these signals is warranted. Additional variants on chromosome 17q11 in *ASIC2* were strongly associated with clopidogrel response in the

PAPI Study and nominally associated with antiplatelet response in ICPC, although the direction of effect is inconsistent between populations. While little is known regarding the function of *ASIC2*, members of this gene family have a role in acid sensing in the gastro-intestinal tract[138], and could influence drug absorption.

By conducting the first GWAS of serum clopidogrel active metabolite, a direct measure of clopidogrel pharmacokinetics, we identified several novel variants on chromosomes 1p25, 3p25, and 17q11 that influence the metabolism of clopidogrel. Though further follow-up is required, this investigation demonstrates the utility of alternative measures of clopidogrel response in addition to platelet reactivity assays, and significantly contributes new information pertinent to clopidogrel pharmacokinetics, and has potential to inform next-generation drug development and clinical decision making.

Clinical evaluation of *PEAR1* rs12041331

In Chapter 5, we recruited individuals by known *PEAR1* rs12401331 genotype to prospectively evaluate the impact of this variant on platelet aggregation pre- and post-aspirin treatment. rs12041331 is an intronic variant that has recently been correlated with variation in on-aspirin platelet reactivity. To gain further insight into the relationship between PEAR1 genotype and aspirin response, we used three different therapeutic aspirin doses (81mg/day, 162 mg/day, and 325 mg/day for 7 days) and multiple agonists (i.e. ADP, collagen, epinephrine, and arachidonic acid) to induce platelet reactivity *ex vivo* in a fixed-sequence, crossover study design. Interestingly, the effect of rs12041331 on *ex vivo* platelet aggregation was pathway-specific and strongly impacted by the presence or absence of aspirin, but not aspirin-dose. Specifically, in the absence of aspirin, A-allele carriers of rs12041331 had significantly lower ADP-mediated platelet

aggregation, similar to data that has been previously reported[104,147]. No observable impact on collagen or arachidonic acid-mediated platelet aggregation pathways was observed prior to aspirin administration, while there was a trend towards reduced epinephrine-mediated platelet aggregation in those who carried the minor allele of this variant. The observed associations between on-aspirin collagen- and epinephrine- mediated platelet aggregation and rs12041331 were consistent with our previously reported retrospective analysis of DAPT-treated patients in the PAPI Study[8]. Surprisingly, no difference in ADP-mediated platelet aggregation was noted with any aspirin dose tested. The relationship between aspirin dose and degree of antiplatelet response by *PEAR1* genotype had not previously been evaluated because of the lack of available data for multiple aspirin doses. No interaction between aspirin dose and *PEAR1* genotype was observed in our prospective evaluation, indicating that low-dose aspirin, which is recommended by the American Heart Association and the American College of Cardiology Foundation[153], may be appropriate regardless of rs12041331 genotype. However, an evaluation of genotype-dose interactions and clinical endpoints would be required to draw any firm conclusions. Additionally, while aspirin directly acetylates COX-1, the COX1-dependent pathway as assessed by arachidonic acid-mediated platelet aggregation and urinary 11-dehydrothromboxane B_2 was near fully inhibited regardless of genotype or aspirin dose, whereas response to the non-COX1-dependent pathways varied by genotype. This highlights the importance of incorporating multiple agonist-induced platelet aggregation pathways in defining aspirin resistance.

Functional characterization of *PEAR1* in the endothelium

As described in Chapter 6, we aimed to define the impact of *PEAR1* in the endothelium by conducting a suite of *in vitro* functional experiments in HUVECs in the presence and absence of aspirin. We first evaluated the impact of *PEAR1* knockdown on HUVEC adhesion, migration, and proliferation, and then extended our results by evaluating endothelial function by *PEAR1* rs12041331 genotype. Notably, we observed a significant reduction in endothelial cell migration, proliferation, and adhesion to collagen I, fibrinogen, and fibronectin with PEAR1 knockdown as compared to control. Interestingly, after treatment with aspirin, no significant difference in migration or extra-cellular matrix adhesion was observed between treatment groups. However, differences in cell proliferation between the *PEAR1* knockdown and control groups remained significant after aspirin treatment. In our previous investigation, we observed increased HUVEC migration in *PEAR1* rs12041331 genotype[53]. No association with endothelial cell migration was observed in this current study, which may be due to differences in methodology, or the different genetic backgrounds of selected primary cell lines. We also evaluated cell proliferation and extra-cellular matrix adhesion in the context of rs12041331 genotype. In contrast to the results after shRNA knockdown of *PEAR1*, we did not observe a notable decrease in adhesive capacity in A-allele carriers in the absence of aspirin to any extra-cellular matrix protein tested. Further, no genotypic differences in adherence were apparent for any extra-cellular matrix protein tested after aspirin treatment. In contrast to the major proliferative differences observed after *PEAR1* knockdown, no difference was observed by *PEAR1* genotype. We anticipated that shRNA knockdown of *PEAR1* would have a larger effect on endothelial function than

rs12041331. *PEAR1* knockdown resulted in an approximate 50% decrease in PEAR1 expression in our study; however, while expression appears reduced in A-allele carriers, we did not observe a statistically significant difference in PEAR1 protein levels by genotype. While Faraday *et al*. previously noted a significant decrease in platelet PEAR1 expression by rs12041331 genotype[104], differences in PEAR1 abundance may explain the discrepancy between the observed results for knockdown and genotype-stratified assays. In addition to the *in vitro* functional work, we aimed to determine the effect *PEAR1* on circulating biomarkers of endothelial function in healthy participants pre- and post-aspirin intervention (7-day 325 mg/day). No difference was noted between rs12041331 A-allele carriers and G/G homozygote participants for circulating Endothelin-1, Endoglin, VCAM-1, or ICAM-1 pre- or post- aspirin. This suggests that rs12041331 genotype does not strongly influence endothelial activation in cardiovascularly healthy individuals, though rs12041331 may influence the endothelium of unhealthy individuals differently. Previous investigations have implicated PEAR1 in angiogenesis, wound healing, and flow-mediated dilation of the brachial artery[53,159]. The characterization of PEAR1 in this project establishes a role for PEAR1 in endothelial cell adhesion to the extra-cellular matrix and re-affirms a role for PEAR1 in endothelial migration and proliferation, although it remains to be seen whether PEAR1 is a major modifier of endothelial function, and further, a logical pharmacological target.

Strengths and Limitations:

Many of the aspects of this study design were novel, and there are several strengths and limitations that should be considered when interpreting data. All participants of the PAPI Study, described in chapter 4, and all participants of the PEAR1

Study, described in chapters 5 and 6, were members of the Old Order Amish community of Lancaster, Pennsylvania. The Amish are a closed, founder population comprised, to date, of approximately 30,000 individuals[174]. Nearly every member of the community can trace their ancestry back to the original group of settlers who emigrated from Switzerland in the early 18th century. Due to the cultural beliefs of the Old Order Amish and limited to no in-migration, the Old Order Amish of Lancaster County have become a genetic isolate with a much more homogeneous genetic pool than outbred populations of European-descent. While this has benefits for discovery of rare variation that may have propagated to high enough frequencies for detection in the Amish after several generations of consanguinity, this may also limit generalizability to outbred populations. While there are numerous instances of genetic associations in the Amish translating to more diverse European ancestry groups[7,117,175], follow-up investigation in the ICPC, an outbred cohort, mitigates this generalizability issue. We utilized imputed data to increase genomic coverage and increase our ability to fine-map association results. Since inaccuracies in imputation can lead to false positive associations, we Taqman genotyped our two top associations to confirm genotyping quality and found concordance to be greater than 98%. One more limitation of imputation is that it is less accurate for rare variants, which hindered our ability to identify variants below 1% minor allele frequency. We therefore supplemented our GWAS results with an exWAS and SKAT. The exome chip used for the exWAS and SKAT is enriched for rare, potentially deleterious variants, and allowed us to analyze variants with a minor allele frequency below 1% in the Amish. While one method to improve novel variant discovery is to increase sample size, an alternative approach, which we utilized, is the development and analysis of more refined

phenotypes. Serum clopidogrel active metabolite is a novel endophenotype that is a proximal measure of clopidogrel metabolism. However, few, if any, other cohorts have genotype data and serum clopidogrel active metabolite phenotypes available in a large enough sample size for a replication analysis. With no true replication, it is therefore difficult to definitively link the novel variants identified in this study with altered clopidogrel metabolism. However, the ICPC is the largest cohort of on-clopidogrel cardiovascular disease patients with genome-wide genotyping and comprehensive phenotyping data available, which provided us the best opportunity to validate these findings. Further, extension to the ICPC enabled us to assess the potential clinical impact of the newly discovered variants. In addition, while analysis of clopidogrel active metabolite indeed facilitated the discovery of novel variants, the derivatization process required to stabilize the clopidogrel active metabolite does not differentiate between the H3 (inactive) and H4 (active) enantiomers[130]. Though H3 and H4 are highly correlated[142], we cannot rule out associations driven by variants that impact H3, but not H4. New methods to isolate and quantify H4 would be beneficial.

In our clinical study, we utilized genotype-directed recruitment to conduct the first prospective evaluation of the effect of *PEAR1* rs12041331 genotype on platelet reactivity. By design this enabled us to evaluate platelet aggregation both pre- and post-multiple therapeutic aspirin doses with minimal confounding variables. While we are limited by a relatively modest sample size, we are able to conduct analyses that would otherwise not be possible in retrospective analyses or evaluation of electronic medical record data. Although significant associations were detected with some platelet aggregation pathways, we may have been underpowered to detect smaller effect sizes or

measures with significant phenotypic variation. However, our findings are in agreement with previously published retrospective analyses of rs12041331[8,104,147]. While no aspirin dose-specific effect was observed, we cannot yet extend the results of this work to cardiovascular event risk, as all participants were generally healthy. Further, *ex vivo* platelet aggregation, while widely used for assessing platelet reactivity, may not be a true representation of platelet aggregation *in vivo*. Additional experiments, such as studies replicating arterial flow conditions or utilizing animal models, should be considered.

In the functional work highlighted in chapter 6, all experiments were carried out in primary HUVECs harvested from de-identified umbilical cords. Though the sample size used is modest, power calculations indicated sufficient ability to detect associations with moderate effect sizes. Additionally, the total number of cell lines and replicates assessed per assay is comparable to similar evaluations of endothelial function *in vitro.* In this study we also evaluated the impact of aspirin on endothelial function by incubating HUVECs with 1 mM acetylsalicylic acid for 24 hours prior to each functional experiment. While it is difficult to adequately represent the physiological impact of prolonged aspirin use *in vivo,* the ASA concentration used in our study is similar to levels used in other published *in vitro* studies, and conforms to expected physiological salicylate levels. We also utilized shRNA knockdown to reduce PEAR1 levels for experimentation. Though we used a scrambled control for comparison, off-target effects of the shRNA could potentially impact our findings. However, the average migration, adhesion, and proliferation of HUVECs that received scrambled shRNA (all *PEAR1* rs12041331 G/G) are similar to that of the *PEAR1* rs12041331 G/G HUVECs. While *in vitro* static assays are commonly used, they test isolated cells under very controlled conditions, and are not

truly representative of *in vivo* function. Evaluation of HUVECs under flow conditions such as through use of the Bioflux System[171]is an alternative approach.

Future Directions

In this study we have identified several new variants that may influence clopidogrel metabolism and further characterized the functional role of *PEAR1* in platelets and endothelial cells. In addition to the work conducted here, many other aspects of these research topics, listed below, are worth investigation.

1) While we completed the largest GWAS of clopidogrel active metabolite to date using state-of the art genotyping approaches, analysis using whole genome sequencing or exome sequencing could potentially identify novel variants not captured in the current genotyping data. Though we would anticipate having many variants in linkage disequilibrium with causal alleles, it is possible that there are Amish-specific variants not well captured by the current genotyping and imputation approaches. This re-analysis would also facilitate fine-mapping of associated or suggestive signals that were identified in this work.
2) The utilization of a novel measure of clopidogrel response, serum clopidogrel active metabolite, facilitated the identification of novel pharmacokinetic clopidogrel variants that would otherwise not have been detected by evaluation of platelet aggregation or other pharmacodynamic phenotypes. However, this measure did not differentiate between H3 (inactive) and H4 (active) clopidogrel metabolites. While levels of these metabolites are highly concordant[142], there is some evidence that slight differences exist in the metabolic pathways of H3 and H4[143]. Isolation and measurement of the H4 metabolite may improve our ability to detect novel variants.

3) In the functional study of *PEAR1* in HUVECs, three major pathways of endothelial function were assessed. While we acquired great insight into *PEAR1* biology, additional tests should be conducted, such as a matrigel tube formation assay, an *in* vitro method for assessing angiogenesis. In addition, the exact signaling pathway of PEAR1 in the endothelium has not been clarified. In platelets and megakaryocytes, PEAR1 is known to stimulate phosphorylation of AKT, modulate PTEN expression, and induce activation of integrin IIb/IIIa[1,107]. Activity of these proteins in endothelial cells, in addition to key endothelial and cardiovascular biomarkers such as eNOS, VEGF, and Ang-2, would be extremely informative.
4) Laminar blood flow in arteries exposes endothelial cells and platelets to high amounts of shear stress. An evaluation of platelet aggregation under flow conditions, such as with the Bioflux System[171], would be more consistent with in vivo thrombus formation and endothelial dysfunction than static assays. Further, complex models can be tested, such as the evaluation of endothelial-leukocyte and endothelial-platelet interactions under a variety of conditions.
5) While genomic studies provide excellent insight into the heritable variation that contributes to DAPT response, the causal gene or causal pathway is often not clear, as observed in the GWAS of clopidogrel active metabolite. Gene expression profiling, such as by RNAseq, can offer substantial insight into the mechanisms by which genes, and potentially genetic variants, interfere with antiplatelet response.
6) In this study, we investigated PEAR1 function through several *ex vivo* and *in vitro* evaluations of human platelets, endothelial cells, and blood and urine biomarkers. Though we gained valuable new insights, additional studies with animal models

would complement our findings. For instance, Vandenbriele et al. generated a knockout mouse for *PEAR1*, and described reduced wound healing and neo-angiogenesis in PEAR1 knockout animals[108]. Further characterization of the knockout mouse, such as in the presence of aspirin treatment, would be quite informative. In addition, knock-in of *PEAR1* rs12041331 with CRISPR/CAS9 in a mouse model would provide new opportunity to evaluate the impact of this mutation on cardiovascular risk.

Potential Impact

The global health burden of coronary heart disease is significant, with more than 7.4 million deaths each year due to ischemic events[16]. Coronary heart disease also carries a substantial economic burden, costing billions of dollars each year in healthcare payments and lost productivity[176]. DAPT with clopidogrel and aspirin is the most widely used antiplatelet regimen prescribed for the secondary prevention of ischemic events. However, up to 40% of individuals are estimated to have some degree of clopidogrel resistance, with heritability of clopidogrel responsiveness estimated at 63% via *ex vivo* platelet aggregation[109,110]. Further, aspirin resistance, though not clearly defined, ranges anywhere from 5% to 60% in the general population[14], with up to 77% of this variation estimated to be due to genetic variation [101]. Therefore, the development of novel therapeutics or better treatment strategies is desperately needed to improve the future outlook of cardiovascular care.

The evaluation of *ex vivo* platelet reactivity has facilitated the identification of a number of *bona fide* clopidogrel response candidate variants, such as *CYP2C19**2, *CES1* G143E, and *ABCB1* C3435T[7,91,111]. However, much of the estimated heritable variation

remains unaccounted for and new methods are required to clarify the mechanisms that impact clopidogrel response. By utilizing serum clopidogrel active metabolite concentration, a proximal measure of clopidogrel pharmacokinetics, we were able to identify several novel variants in genes such as *RNF2*, *ASIC2*, and *ATP2B2* that potentially influence clopidogrel metabolism that otherwise would not have been identified solely by evaluation of *ex vivo* platelet aggregation. In the Amish cohort, 10% of the heritable variation in clopidogrel active metabolite concentration is explained by *CYP2C19**2. Inclusion of rs80343429 and rs111985173, the two most strongly associated variants in our study, increased the variance explained to 21%. This demonstrates the utility of serum clopidogrel active metabolite concentration for the discovery of novel clopidogrel response variants, and refinement of the clopidogrel pharmacokinetic phenotype is likely to improve novel variant discovery even further. Although an independent replication is necessary, this investigation significantly contributes to the clarification of the clopidogrel pharmacokinetic pathway, which has potential to inform next-generation drug development and clinical decision making.

PEAR1 is a type 1 transmembrane protein highly expressed in endothelial cells and platelets [105]. While a number of retrospective studies have found an intronic variant in *PEAR1*, rs12041331, to significantly influence platelet reactivity both in drug-naïve and on-aspirin individuals[8,61,104,147], no prospective evaluation had yet been conducted. Further, prior to this study it was unknown how different therapeutic doses of aspirin might influence platelet reactivity by rs12041331 genotype. This is significant, as ischemic event risk in aspirin-treated patients appears to be modified by rs12041331 genotype[8]. By utilizing genotype-directed recruitment in our prospective evaluation of

rs12041331, we are able to specifically assess association with a number of phenotypes in a controlled setting. We noted a clear difference in response by rs12041331 in the non-COX1 dependent platelet aggregation pathways, whereas no differences were noted by *PEAR1* genotype for the COX1-dependent pathway, highlighting the importance of testing multiple pathways for the evaluation of anti-thrombotic response to aspirin. Further, our results clearly demonstrate no difference in benefit for platelet reactivity with differing therapeutic doses of aspirin. While an evaluation of ischemic event risk in carriers of rs12041331 stratified by aspirin dose would be informative, it appears that higher doses of aspirin would not be beneficial, conforming to recommendations by the American Heart Association and American College of Cardiology Foundation[153].

As previously mentioned, in addition to lowering on-aspirin platelet aggregation, the A-allele of rs12041331 has also paradoxically been correlated with increasing risk of on-aspirin cardiovascular events. While one potential avenue by which alterations in PEAR1 function might cause this increased ischemic risk is via unstable thrombus formation, the differences might also be due to the non-platelet functions of PEAR1 (e.g. the endothelium). Recent functional investigations in endothelium cells predict PEAR1 to have a role in angiogenesis and wound healing, processes critical to normal maintenance of endothelial health and patient recovery after ischemic events or percutaneous coronary intervention. In our *in vitro* study, we not only evaluated the impact of *PEAR1* knockdown of endothelial function, but also evaluated the impact of *PEAR1* rs12041331 genotype status on endothelial function, which enabled us to clarify the mechanisms by which rs12041331 might influence endothelial function and therefore ischemic event risk. Our *PEAR1* knockdown results indeed suggest a role for PEAR1 in endothelial cell

migration, adhesion, and proliferation, although functional assessment of rs12041331 and evaluation of clinical biomarkers for endothelial adhesive capacity and angiogenesis were inconclusive. It merits note, however, that we did not observe a significant difference in PEAR1 expression in A-allele HUVECs in our study, while Faraday *et al*. previously reported decreased PEAR1 expression in platelets of rs12041331 A-allele carriers. However, given that different cell types were assessed it is possible that rs12041331, an intronic variant, may have different regulatory effects on PEAR1 expression. Our results also contradict the reports of Vandenbriele et al, who demonstrated increased HUVEC migration and proliferation with PEAR1 knockdown, though the different experimental conditions or different genetic backgrounds of the primary HUVECs utilized in our respective studies (our samples were collected in Baltimore, while Vandenbriele et al collected samples in Belgium) may have contributed to this difference. Further work is required to define the exact role of PEAR1 in endothelial function.

Overall, in addition to novel biological insight, which can be exploited for next-generation drug design, our results broaden our knowledge of the effects of clopidogrel and aspirin non-responsiveness. While confirmation and characterization of the novel variants identified would be required, increasing our understanding of variation in response to antiplatelet therapy could impact genotype-directed clinical programs. In addition, the characterization of PEAR1 in endothelial cells with and without aspirin therapy and the clinical measures of aspirin response strongly contribute to our collective understanding of the complexity of cardiovascular disease pharmacogenomics, though it remains to be seen whether PEAR1 may serve as a plausible drug target. Ultimately, these developments provide groundwork for future investigations and may hold promise

for contributing to improved healthcare and a reduction in recurrent cardiovascular event rates in patients in the future.

References

1. Kauskot A, Di Michele M, Loyen S, Freson K, Verhamme P & Hoylaerts MF. A novel mechanism of sustained platelet alphaIIbbeta3 activation via PEAR1. Blood, 2012. **119**:4056-65.

2. Spiel AO, Gilbert JC & Jilma B. von Willebrand factor in cardiovascular disease: focus on acute coronary syndromes. Circulation, 2008. **117**:1449-59.

3. Chiang N, Bermudez EA, Ridker PM, Hurwitz S & Serhan CN. Aspirin triggers antiinflammatory 15-epi-lipoxin A4 and inhibits thromboxane in a randomized human trial. Proc Natl Acad Sci U S A, 2004. **101**:15178-83.

4. Mathias RA, Kim Y, Sung H, Yanek LR, Mantese VJ, Hererra-Galeano JE*et al.* A combined genome-wide linkage and association approach to find susceptibility loci for platelet function phenotypes in European American and African American families with coronary artery disease. BMC Med Genomics, 2010. **3**:22.

5. Yusuf S, Zhao F, Mehta SR, Chrolavicius S, Tognoni G, Fox KK*et al.* Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med, 2001. **345**:494-502.

6. Gladding P, Webster M, Zeng I, Farrell H, Stewart J, Ruygrok P*et al.* The pharmacogenetics and pharmacodynamics of clopidogrel response: an analysis from the PRINC (Plavix Response in Coronary Intervention) trial. JACC Cardiovasc Interv, 2008. **1**:620-7.

7. Shuldiner AR, O'Connell JR, Bliden KP, Gandhi A, Ryan K, Horenstein RB*et al.* Association of cytochrome P450 2C19 genotype with the antiplatelet effect and clinical efficacy of clopidogrel therapy. JAMA, 2009. **302**:849-57.

8. Lewis JP, Ryan K, O'Connell JR, Horenstein RB, Damcott CM, Gibson Q*et al.* Genetic variation in PEAR1 is associated with platelet aggregation and cardiovascular outcomes. Circ Cardiovasc Genet, 2013. **6**:184-92.

9. Fritsma GA. Laboratory Evaluation of Hemostasis. Vol. 2016 (2015).

10. Mayo Clinic Staff. Causes of myocardial ischemia. (2015).

11. Sangkuhl K, Klein TE & Altman RB. "Clopidogrel pathway". Pharmacogenet Genomics, 2010.

12. Sangkuhl K, Shuldine AR, Klein TE & Altman RB. "Platelet aggregation pathway". Pharmacogenet Genomics, 2010.

13. Libby P, Ridker PM & Hansson GK. Progress and challenges in translating the biology of atherosclerosis. Nature, 2011. **473**:317-25.

14. Goodman T, Ferro A & Sharma P. Pharmacogenetics of aspirin resistance: a comprehensive systematic review. Br J Clin Pharmacol, 2008. **66**:222-32.

15. American Heart Association. Heart and Stroke Statistics. (2015).

16. Cardiovascular Diseases (CVD). Vol. 2016 (World Health Organization).

17. Tousoulis D, Kampoli AM, Tentolouris C, Papageorgiou N & Stefanadis C. The role of nitric oxide on endothelial function. Curr Vasc Pharmacol, 2012. **10**:4-18.

18. Weber C & Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nat Med, 2011. **17**:1410-22.

19. Bentzon JF, Otsuka F, Virmani R & Falk E. Mechanisms of plaque formation and rupture. Circ Res, 2014. **114**:1852-66.

20. Widmer RJ & Lerman A. Endothelial dysfunction and cardiovascular disease. Glob Cardiol Sci Pract, 2014. **2014**:291-308.

21. Walczak M, Suraj J, Kus K, Kij A, Zakrzewska A & Chlopicki S. Towards a comprehensive endothelial biomarkers profiling and endothelium-guided pharmacotherapy. Pharmacol Rep, 2015. **67**:771-7.

22. Flammer AJ, Anderson T, Celermajer DS, Creager MA, Deanfield J, Ganz P*et al.* The assessment of endothelial function: from research into clinical practice. Circulation, 2012. **126**:753-67.

23. Rajendran P, Rengarajan T, Thangavel J, Nishigaki Y, Sakthisekaran D, Sethi G*et al.* The vascular endothelium and human diseases. Int J Biol Sci, 2013. **9**:1057-69.

24. Tsai YC, Lee CS, Chiu YW, Kuo HT, Lee SC, Hwang SJ*et al.* Angiopoietin-2 as a Prognostic Biomarker of Major Adverse Cardiovascular Events and All-Cause Mortality in Chronic Kidney Disease. PLoS One, 2015. **10**:e0135181. doi:10.1371/journal.pone.0135181.

25. BlÃ¡zquez-Medela AM, GarcÃ-a-Ortiz L, GÃ³mez-Marcos MA, Recio-RodrÃ-guez JI, SÃ¡nchez-RodrÃ-guez A, LÃ³pez-Novoa JM*et al.* Increased plasma soluble endoglin levels as an indicator of cardiovascular alterations in hypertensive and diabetic patients. BMC Med, 2010. **8**:86-7015-8-86.

26. Messner B & Bernhard D. Smoking and cardiovascular disease: mechanisms of endothelial dysfunction and early atherogenesis. Arterioscler Thromb Vasc Biol, 2014. **34**:509-15.

27. Sibal L, Agarwal SC, Home PD & Boger RH. The Role of Asymmetric Dimethylarginine (ADMA) in Endothelial Dysfunction and Cardiovascular Disease. Curr Cardiol Rev, 2010. **6**:82-90.

28. Ganz P & Hsue PY. Endothelial dysfunction in coronary heart disease is more than a systemic process. Eur Heart J, 2013. **34**:2025-2027.

29. Cai H & Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. Circ Res, 2000. **87**:840-4.

30. Davignon J & Ganz P. Role of endothelial dysfunction in atherosclerosis. Circulation, 2004. **109**:III27-32.

31. Reinhart-King CA. Endothelial cell adhesion and migration. Methods Enzymol, 2008. **443**:45-64.

32. Ras RT, Streppel MT, Draijer R & Zock PL. Flow-mediated dilation and cardiovascular risk prediction: a systematic review with meta-analysis. Int J Cardiol, 2013. **168**:344-51.

33. Xu Y, Arora RC, Hiebert BM, Lerner B, Szwajcer A, McDonald K*et al.* Non-invasive endothelial function testing and the risk of adverse outcomes: a systematic review and meta-analysis. Eur Heart J Cardiovasc Imaging, 2014. **15**:736-46.

34. Nguyen A, Thorin-Trescases N & Thorin E. Working under pressure: coronary arteries and the endothelin system. Am J Physiol Regul Integr Comp Physiol, 2010. **298**:R1188-94.

35. Gossl M & Lerman A. Endothelin: beyond a vasoconstrictor. Circulation, 2006. **113**:1156-8.

36. Naseem KM. The role of nitric oxide in cardiovascular diseases. Mol Aspects Med, 2005. **26**:33-65.

37. Loscalzo J. Nitric oxide insufficiency, platelet activation, and arterial thrombosis. Circ Res, 2001. **88**:756-62.

38. Jeremy JY, Rowe D, Emsley AM & Newby AC. Nitric oxide and the proliferation of vascular smooth muscle cells. Cardiovasc Res, 1999. **43**:580-94.

39. Patel JV, Lim HS, Varughese GI, Hughes EA & Lip GYH. Angiopoietin-2 levels as a biomarker of cardiovascular risk in patients with hypertension. Ann Med, 2008. **40**:215-222.

40. Libby P. Inflammation and cardiovascular disease mechanisms. Am J Clin Nutr, 2006. **83**:456S-460S.

41. Sader MA & Celermajer DS. Endothelial function, vascular reactivity and gender differences in the cardiovascular system. Cardiovasc Res, 2002. **53**:597-604.

42. Chowienczyk PJ, Watts GF, Cockcroft JR, Brett SE & Ritter JM. Sex differences in endothelial function in normal and hypercholesterolaemic subjects. Lancet, 1994. **344**:305-6.

43. Green DJ, Hopkins ND, Jones H, Thijssen DH, Eijsvogels TM & Yeap BB. Sex differences in vascular endothelial function and health in humans: impacts of exercise. Exp Physiol, 2016. **101**:230-42.

44. Mosca L, Barrett-Connor E & Wenger NK. Sex/Gender Differences in Cardiovascular Disease Prevention What a Difference a Decade Makes. Circulation, 2011. **124**:2145-2154.

45. Mizia-Stec K, Gasior Z, Mizia M, Haberka M, Holecki M, Zwolinska W*et al.* Flow-mediated dilation and gender in patients with coronary artery disease: arterial size influences gender differences in flow-mediated dilation. Echocardiography, 2007. **24**:1051-7.

46. Yang PT, Yuan H, Wang YQ, Cao X, Wu LX & Chen ZH. Correlations between brachial endothelial function and cardiovascular risk factors: a survey of 2,511 Chinese subjects. J Thorac Dis, 2014. **6**:1441-51.

47. Benjamin EJ, Larson MG, Keyes MJ, Mitchell GF, Vasan RS, Keaney JF, Jr.*et al.* Clinical correlates and heritability of flow-mediated dilation in the community: the Framingham Heart Study. Circulation, 2004. **109**:613-9.

48. Loehr LR, Espeland MA, Sutton-Tyrrell K, Burke GL, Crouse JR, 3rd & Herrington DM. Racial differences in endothelial function in postmenopausal women. Am Heart J, 2004. **148**:606-11.

49. Mata-Greenwood E & Chen DB. Racial differences in nitric oxide-dependent vasorelaxation. Reprod Sci, 2008. **15**:9-25.

50. Tao J, Jin YF, Yang Z, Wang LC, Gao XR, Liu L*et al.* Reduced arterial elasticity is associated with endothelial dysfunction in persons of advancing age - Comparative study of noninvasive pulse wave analysis and laser Doppler blood flow measurement. Am J Hypertens, 2004. **17**:654-659.

51. Mauricio MD, Aldasoro M, Ortega J & Vila JM. Endothelial dysfunction in morbid obesity. Curr Pharm Des, 2013. **19**:5718-29.

52. Beckman JA & Creager MA. The nonlipid effects of statins on endothelial function. Trends Cardiovasc Med, 2006. **16**:156-62.

53. Fisch AS, Yerges-Armstrong LM, Backman JD, Wang H, Donnelly P, Ryan KA*et al.* Genetic Variation in the Platelet Endothelial Aggregation Receptor 1 Gene Results in Endothelial Dysfunction. PLoS One, 2015. **10**:e0138795.

54. Chatsuriyawong S, Gozal D, Kheirandish-Gozal L, Bhattacharjee R, Khalyfa AA, Wang Y*et al.* Genetic variance in Nitric Oxide Synthase and Endothelin Genes among children with and without Endothelial Dysfunction. J Transl Med, 2013. **11**.

55. Kathiresan S, Larson MG, Vasan RS, Guo CY, Vita JA, Mitchell GF*et al.* Common genetic variation at the endothelial nitric oxide synthase locus and relations to brachial artery vasodilator function in the community. Circulation, 2005. **112**:1419-1427.

56. Jones LC & Hingorani AD. Genetic regulation of endothelial function. Heart, 2005. **91**:1275-7.

57. Ferguson AD, Dokainish H & Lakkis N. Aspirin and clopidogrel response variability: review of the published literature. Tex Heart Inst J, 2008. **35**:313-20.

58. Patel SR, Hartwig JH & Italiano JE, Jr. The biogenesis of platelets from megakaryocyte proplatelets. J Clin Invest, 2005. **115**:3348-54.

59. Cimmino G & Golino P. Platelet biology and receptor pathways. J Cardiovasc Transl Res, 2013. **6**:299-309.

60. Angiolillo DJ, Ueno M & Goto S. Basic principles of platelet biology and clinical implications. Circ J, 2010. **74**:597-607.

61. Qayyum R, Becker LC, Becker DM, Faraday N, Yanek LR, Leal SM*et al.* Genome-wide association study of platelet aggregation in African Americans. BMC Genet, 2015. **16**:58.

62. Paniccia R, Priora R, Liotta AA & Abbate R. Platelet function tests: a comparative review. Vasc Health Risk Manag, 2015. **11**:133-48.

63. Renda G, Zurro M, Malatesta G, Ruggieri B & De Caterina R. Inconsistency of different methods for assessing ex vivo platelet function: relevance for the detection of aspirin resistance. Haematologica, 2010. **95**:2095-101.

64. Wang TY, Henry TD, McCoy LA, Berger PB, Cohen DJ, Effron MB*et al.* Contemporary use of platelet function and pharmacogenomic testing among patients with acute myocardial infarction undergoing percutaneous coronary intervention in the United States. Am Heart J, 2015. **170**:706-14.

65. Ferroni P, Riondino S, Vazzana N, Santoro N, Guadagni F & Davi G. Biomarkers of platelet activation in acute coronary syndromes. Thromb Haemost, 2012. **108**:1109-23.

66. Mumaw MM & Nieman MT. Race differences in platelet reactivity: is protease activated receptor 4 a predictor of response to therapy? Arterioscler Thromb Vasc Biol, 2014. **34**:2524-6.

67. Becker DM, Segal J, Vaidya D, Yanek LR, Herrera-Galeano JE, Bray PF*et al.* Sex differences in platelet reactivity and response to low-dose aspirin therapy. JAMA, 2006. **295**:1420-7.

68. Marcucci R, Cioni G, Giusti B, Fatini C, Rossi L, Pazzi M*et al.* Gender and anti-thrombotic therapy: from biology to clinical implications. J Cardiovasc Transl Res, 2014. **7**:72-81.

69. O'Donnell CJ, Larson MG, Feng D, Sutherland PA, Lindpaintner K, Myers RH*et al.* Genetic and environmental contributions to platelet aggregation: the Framingham heart study. Circulation, 2001. **103**:3051-6.

70. Terres W, Weber K, Kupper W & Bleifeld W. Age, Cardiovascular Risk-Factors and Coronary Heart-Disease as Determinants of Platelet-Function in Men - a Multivariate Approach. Thromb Res, 1991. **62**:649-661.

71. Schneider DJ. Factors contributing to increased platelet reactivity in people with diabetes. Diabetes Care, 2009. **32**:525-7.

72. Johnson AD. The genetics of common variation affecting platelet development, function and pharmaceutical targeting. J Thromb Haemost, 2011. **9 Suppl 1**:246-57.

73. Morrell CN, Aggrey AA, Chapman LM & Modjeski KL. Emerging roles for platelets as immune and inflammatory cells. Blood, 2014. **123**:2759-2767.

74. Collaboration AT. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. The Lancet, 2009. **373**:1849-1860.

75. Eidelman RS, Hebert PR, Weisman SM & Hennekens CH. An update on aspirin in the primary prevention of cardiovascular disease. Arch Intern Med, 2003. **163**:2006-10.

76. Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S*et al.* Prasugrel versus clopidogrel in patients with acute coronary syndromes. N Engl J Med, 2007. **357**:2001-15.

77. Wallentin L, Becker RC, Budaj A, Cannon CP, Emanuelsson H, Held C*et al.* Ticagrelor versus clopidogrel in patients with acute coronary syndromes. N Engl J Med, 2009. **361**:1045-57.

78. Garabedian T & Alam S. High residual platelet reactivity on clopidogrel: its significance and therapeutic challenges overcoming clopidogrel resistance. Cardiovasc Diagn Ther, 2013. **3**:23-37.

79. Franchi F, Rollini F & Angiolillo DJ. Defining the Link Between Chronic Kidney Disease, High Platelet Reactivity, and Clinical Outcomes in Clopidogrel-Treated

Patients Undergoing Percutaneous Coronary Intervention. Circulation-Cardiovascular Interventions, 2015. **8**:e002760.

80. Whirl-Carrillo M, McDonagh EM, Hebert JM, Gong L, Sangkuhl K, Thorn CF*et al.* Pharmacogenomics knowledge for personalized medicine. Clin Pharmacol Ther, 2012. **92**:414-7.

81. Plavix Prescribing Information. Vol. 2016 (2015).

82. Dupont AG, Gabriel DA & Cohen MG. Antiplatelet therapies and the role of antiplatelet resistance in acute coronary syndrome. Thromb Res, 2009. **124**:6-13.

83. Breet NJ, Sluman MA, van Berkel MA, van Werkum JW, Bouman HJ, Harmsze AM*et al.* Effect of gender difference on platelet reactivity. Neth Heart J, 2011. **19**:451-7.

84. Berger JS, Bhatt DL, Cannon CP, Chen Z, Jiang L, Jones JB*et al.* The relative efficacy and safety of clopidogrel in women and men a sex-specific collaborative meta-analysis. J Am Coll Cardiol, 2009. **54**:1935-45.

85. Patti G, De Caterina R, Abbate R, Andreotti F, Biasucci LM, Calabrò P*et al.* Platelet function and long-term antiplatelet therapy in women: is there a gender-specificity? A 'state-of-the-art' paper. Eur Heart J, 2014. **35**:2213-2223.

86. Motovska Z, Widimsky P, Petr R, Bilkova D, Marinov I, Simek S*et al.* Factors influencing clopidogrel efficacy in patients with stable coronary artery disease undergoing elective percutaneous coronary intervention: statin's advantage and the smoking "paradox". J Cardiovasc Pharmacol, 2009. **53**:368-72.

87. Pankert M, Quilici J, Loundou AD, Verdier V, Lambert M, Deharo P*et al.* Impact of obesity and the metabolic syndrome on response to clopidogrel or prasugrel and bleeding risk in patients treated after coronary stenting. Am J Cardiol, 2014. **113**:54-9.

88. Geisler T, Anders N, Paterok M, Langer H, Stellos K, Lindemann S*et al.* Platelet response to clopidogrel is attenuated in diabetic patients undergoing coronary stent implantation. Diabetes Care, 2007. **30**:372-374.

89. Gurbel PA, Bliden KP, Logan DK, Kereiakes DJ, Lasseter KC, White A*et al.* The influence of smoking status on the pharmacokinetics and pharmacodynamics of clopidogrel and prasugrel: the PARADOX study. J Am Coll Cardiol, 2013. **62**:505-12.

90. Mega JL, Close SL, Wiviott SD, Shen L, Hockett RD, Brandt JT*et al.* Cytochrome p-450 polymorphisms and response to clopidogrel. N Engl J Med, 2009. **360**:354-62.

91. Lewis JP, Horenstein RB, Ryan K, O'Connell JR, Gibson Q, Mitchell BD*et al.* The functional G143E variant of carboxylesterase 1 is associated with increased clopidogrel active metabolite levels and greater clopidogrel response. Pharmacogenet Genomics, 2013. **23**:1-8.

92. Price MJ, Murray SS, Angiolillo DJ, Lillie E, Smith EN, Tisch RL*et al.* Influence of genetic polymorphisms on the effect of high- and standard-dose clopidogrel after percutaneous coronary intervention: the GIFT (Genotype Information and Functional Testing) study. J Am Coll Cardiol, 2012. **59**:1928-37.

93. Horenstein RB, Madabushi R, Zineh I, Yerges-Armstrong LM, Peer CJ, Schuck RN*et al.* Effectiveness of clopidogrel dose escalation to normalize active metabolite exposure and antiplatelet effects in CYP2C19 poor metabolizers. J Clin Pharmacol, 2014. **54**:865-73.

94. Society for Cardiovascular Angiography Interventions, Society of Thoracic Surgeons, Writing Committee Members. ACCF/AHA Clopidogrel clinical alert: approaches to the FDA "boxed warning": a report of the American College of Cardiology Foundation Task Force on Clinical Expert Consensus Documents and the American Heart Association. Circulation, 2010. **122**:537-57.

95. Johnson JA & Cavallari LH. Pharmacogenetics and cardiovascular disease--implications for personalized medicine. Pharmacol Rev, 2013. **65**:987-1009.

96. Wells QS, Delaney JT & Roden DM. Genetic determinants of response to cardiovascular drugs. Curr Opin Cardiol, 2012. **27**:253-61.

97. Heitzer T, Rudolph V, Schwedhelm E, Karstens M, Sydow K, Ortak M*et al.* Clopidogrel improves systemic endothelial nitric oxide bioavailability in patients with coronary artery disease: evidence for antioxidant and antiinflammatory effects. Arterioscler Thromb Vasc Biol, 2006. **26**:1648-52.

98. Willoughby SR, Luu LJ, Cameron JD, Nelson AJ, Schultz CD, Worthley SG*et al.* Clopidogrel improves microvascular endothelial function in subjects with stable coronary artery disease. Heart Lung Circ, 2014. **23**:534-41.

99. Paul BZS, Jin J & Kunapuli SP. Molecular Mechanism of Thromboxane A2-induced Platelet Aggregation: ESSENTIAL ROLE FOR P2T AC and 2ARECEPTORS. J Biol Chem, 1999. **274**:29108-29114.

100. Husain S, Andrews NP, Mulcahy D, Panza JA & Quyyumi AA. Aspirin improves endothelial dysfunction in atherosclerosis. Circulation, 1998. **97**:716-20.

101. Faraday N, Yanek LR, Mathias R, Herrera-Galeano JE, Vaidya D, Moy TF*et al.* Heritability of platelet responsiveness to aspirin in activation pathways directly and indirectly related to cyclooxygenase-1. Circulation, 2007. **115**:2490-6.

102. Lanham KJ, Oestreich JH, Dunn SP & Steinhubl SR. Impact of genetic polymorphisms on clinical response to antithrombotics. Pharmgenomics Pers Med, 2010. **3**:87-99.

103. Voora D, Shah SH, Horton JR, Shaw LK & Newby LK. Polymorphisms Associated With in vitro Aspirin Resistance Are Not Associated With Clinical Outcomes During Regular Aspirin Use. Circulation, 2009. **120**:S599-S599.

104. Faraday N, Yanek LR, Yang XP, Mathias R, Herrera-Galeano JE, Suktitipat B*et al.* Identification of a specific intronic PEAR1 gene variant associated with greater platelet aggregability and protein expression. Blood, 2011. **118**:3367-75.

105. Nanda N, Bao M, Lin H, Clauser K, Komuves L, Quertermous T*et al.* Platelet endothelial aggregation receptor 1 (PEAR1), a novel epidermal growth factor repeat-containing transmembrane receptor, participates in platelet contact-induced activation. J Biol Chem, 2005. **280**:24680-9.

106. Sun Y, Vandenbriele C, Kauskot A, Verhamme P, Hoylaerts MF & Wright GJ. A Human Platelet Receptor Protein Microarray Identifies the High Affinity Immunoglobulin E Receptor Subunit alpha (FcepsilonR1alpha) as an Activating Platelet Endothelium Aggregation Receptor 1 (PEAR1) Ligand. Molecular & cellular proteomics : MCP, 2015. **14**:1265-1274.

107. Kauskot A, Vandenbriele C, Louwette S, Gijsbers R, Tousseyn T, Freson K*et al.* PEAR1 attenuates megakaryopoiesis via control of the PI3K/PTEN pathway. Blood, 2013. **121**:5208-17.

108. Vandenbriele C, Kauskot A, Vandersmissen I, Criel M, Geenens R, Craps S*et al.* Platelet endothelial aggregation receptor-1: a novel modifier of neoangiogenesis. Cardiovasc Res, 2015. **108**:124-38.

109. Nguyen TA, Diodati JG & Pharand C. Resistance to clopidogrel: a review of the evidence. J Am Coll Cardiol, 2005. **45**:1157-64.

110. Kolandaivelu K & Bhatt DL. Overcoming 'resistance' to antiplatelet therapy: targeting the issue of nonadherence. Nat Rev Cardiol, 2010. **7**:461-7.

111. Su J, Xu J, Li X, Zhang H, Hu J, Fang R*et al.* ABCB1 C3435T polymorphism and response to clopidogrel treatment in coronary artery disease (CAD) patients: a meta-analysis. PLoS One, 2012. **7**:e46366.

112. Calderon-Cruz B, Rodriguez-Galvan K, Manzo-Francisco LA, Vargas-Alarcon G, Fragoso JM, Pena-Duque MA*et al.* C3435T polymorphism of the ABCB1 gene is associated with poor clopidogrel responsiveness in a Mexican population undergoing percutaneous coronary intervention. Thromb Res, 2015. **136**:894-8.

113. Bozzi LM, Mitchell BD, Lewis JP, Ryan KA, Herzog WR, O'Connell JR*et al.* The Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study: Variation in Platelet Response to Clopidogrel and Aspirin. Curr Vasc Pharmacol, 2015.

114. Howie BN, Donnelly P & Marchini J. A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. PLoS Genet, 2009. **5**:e1000529.

115. Delaneau O, Marchini J, Genomes Project C & Genomes Project C. Integrating sequence and array data to create an improved 1000 Genomes Project haplotype reference panel. Nat Commun, 2014. **5**:3934.

116. Exome Chip Design. Vol. 2016 (Center for Statistical Genetics, University of Michigan).

117. Pollin TI, Damcott CM, Shen H, Ott SH, Shelton J, Horenstein RB*et al.* A null mutation in human APOC3 confers a favorable plasma lipid profile and apparent cardioprotection. Science, 2008. **322**:1702-5.

118. Albert JS, Yerges-Armstrong LM, Horenstein RB, Pollin TI, Sreenivasan UT, Chai S*et al.* Null mutation in hormone-sensitive lipase gene and risk of type 2 diabetes. N Engl J Med, 2014. **370**:2307-15.

119. Agarwala R, Biesecker LG, Hopkins KA, Francomano CA & Schaffer AA. Software for constructing and verifying pedigrees within large genealogies and an application to the old order Amish of Lancaster County. Genome Res, 1998. **8**:211-221.

120. Wu MC, Lee S, Cai T, Li Y, Boehnke M & Lin X. Rare-variant association testing for sequencing data with the sequence kernel association test. Am J Hum Genet, 2011. **89**:82-93.

121. Purcell S NBT-BKTLFMARBDMJSPdBPIWD & Sham PC. PLINK: a toolset for whole-genome association and population-based linkage analysis. Am J Hum Genet, 2007. **81**.

122. Yang J, Lee SH, Goddard ME & Visscher PM. GCTA: a tool for genome-wide complex trait analysis. Am J Hum Genet, 2011. **88**:76-82.

123. U.S. Department of Health & Human Services. Health, United States, 2015. CDC/National Center for Health Statistics, 2016.

124. Jamal A, Homa DM, O'Connor E, Babb SD, Caraballo RS, Singh T*et al.* Current cigarette smoking Among Adults - United States, 2005-2014. Mmwr-Morbidity and Mortality Weekly Report, 2015. **64**:1233-1240.

125. Nugent KL, Million-Mrkva A, Backman J, Stephens SH, Reed RM, Kochunov P*et al.* Familial Aggregation of Tobacco Use Behaviors Among Amish Men. Nicotine & Tobacco Research, 2014. **16**:923-930.

126. Cooke GE, Liu-Stratton Y, Ferketich AK, Moeschberger ML, Frid DJ, Magorien RD*et al.* Effect of platelet antigen polymorphism on platelet inhibition by aspirin, clopidogrel, or their combination. J Am Coll Cardiol, 2006. **47**:541-6.

127. Angiolillo DJ, Fernandez-Ortiz A, Bernardo E, Ramirez C, Cavallari U, Trabetti E*et al.* Contribution of gene sequence variations of the hepatic cytochrome P450 3A4 enzyme to variability in individual responsiveness to clopidogrel. Arterioscler Thromb Vasc Biol, 2006. **26**:1895-900.

128. Lewis JP, Stephens SH, Horenstein RB, O'Connell JR, Ryan K, Peer CJ*et al.* The CYP2C19*17 variant is not independently associated with clopidogrel response. Journal of thrombosis and haemostasis : JTH, 2013. **11**:1640-1646.

129. Plavix® prescribing information Vol. 2016 (Bristol-Myers Squibb/Sanofi Pharmaceuticals).

130. Peer CJ, Spencer SD, VanDenBerg DA, Pacanowski MA, Horenstein RB & Figg WD. A sensitive and rapid ultra HPLC-MS/MS method for the simultaneous detection of clopidogrel and its derivatized active thiol metabolite in human plasma. J Chromatogr B Analyt Technol Biomed Life Sci, 2012. **880**:132-9.

131. Ferreira MA & Purcell SM. A multivariate test of association. Bioinformatics, 2009. **25**:132-3.

132. Sasatani M, Xu YB, Kawai H, Cao LL, Tateishi S, Shimura T*et al.* RAD18 Activates the G2/M Checkpoint through DNA Damage Signaling to Maintain Genome Integrity after Ionizing Radiation Exposure. PLoS One, 2015. **10**.

133. Pruitt KD, Brown GR, Hiatt SM, Thibaud-Nissen F, Astashyn A, Ermolaeva O*et al.* RefSeq: an update on mammalian reference sequences. Nucleic Acids Res, 2014. **42**:D756-63.

134. Tempel BL & Shilling DJ. The plasma membrane calcium ATPase and disease. Subcell Biochem, 2007. **45**:365-83.

135. Price MJ, Carson AR, Murray SS, Phillips T, Janel L, Tisch R*et al.* First Pharmacogenomic Analysis Using Whole Exome Sequencing to Identify Novel Genetic Determinants of Clopidogrel Response Variability: Results of the Genotype Information and Functional Testing (Gift) Exome Study. J Am Coll Cardiol, 2012. **59**:E9-E9.

136. Lonsdale J, Thomas J, Salvatore M, Phillips R, Lo E, Shad S*et al.* The Genotype-Tissue Expression (GTEx) project. Nat Genet, 2013. **45**:580-585.

137. Jancin B. Two More Clopidogrel Resistance Genes Found. (Frontline Medical Communications LLC, 2012).

138. Holzer P. Acid-sensing ion channels in gastrointestinal function. Neuropharmacology, 2015. **94**:72-9.

139. Gubbins PO & Bertch KE. Drug absorption in gastrointestinal disease and surgery. Clinical pharmacokinetic and therapeutic implications. Clin Pharmacokinet, 1991. **21**:431-47.

140. Vidal M. Role of polycomb proteins Ring1A and Ring1B in the epigenetic regulation of gene expression. Int J Dev Biol, 2009. **53**:355-70.

141. Rao PS, Mallya KB, Srivenugopal KS, Balaji KC & Rao US. RNF2 interacts with the linker region of the human P-glycoprotein. Int J Oncol, 2006. **29**:1413-9.

142. Karazniewicz-Lada M, Danielak D, Burchardt P, Kruszyna L, Komosa A, Lesiak M*et al.* Clinical pharmacokinetics of clopidogrel and its metabolites in patients with cardiovascular diseases. Clin Pharmacokinet, 2014. **53**:155-64.

143. Liu C, Chen Z, Zhong K, Li L, Zhu W, Chen X*et al.* Human Liver Cytochrome P450 Enzymes and Microsomal Thiol Methyltransferase Are Involved in the Stereoselective Formation and Methylation of the Pharmacologically Active Metabolite of Clopidogrel. Drug Metab Dispos, 2015. **43**:1632-41.

144. Serebruany VL, Steinhubl SR, Berger PB, Malinin AI, Bhatt DL & Topol EJ. Variability in platelet responsiveness to clopidogrel among 544 individuals. J Am Coll Cardiol, 2005. **45**:246-51.

145. Kim Y, Suktitipat B, Yanek LR, Faraday N, Wilson AF, Becker DM*et al.* Targeted Deep Resequencing Identifies Coding Variants in the PEAR1 Gene That Play a Role in Platelet Aggregation. PLoS One, 2013. **8**.

146. Herrera-Galeano JE, Becker DM, Wilson AF, Yanek LR, Bray P, Vaidya D*et al.* A novel variant in the platelet endothelial aggregation receptor-1 gene is associated with increased platelet aggregability. Arteriosclerosis Thrombosis and Vascular Biology, 2008. **28**:1484-1490.

147. Johnson AD, Yanek LR, Chen MH, Faraday N, Larson MG, Tofler G*et al.* Genome-wide meta-analyses identifies seven loci associated with platelet aggregation in response to agonists. Nat Genet, 2010. **42**:608-13.

148. Mitchell BD, McArdle PF, Shen H, Rampersaud E, Pollin TI, Bielak LF*et al.* The genetic response to short-term interventions affecting cardiovascular function: rationale and design of the Heredity and Phenotype Intervention (HAPI) Heart Study. Am Heart J, 2008. **155**:823-8.

149. Machlus KR & Italiano JE, Jr. The incredible journey: From megakaryocyte development to platelet formation. J Cell Biol, 2013. **201**:785-96.

150. Hall HM, de Lemos JA, Enriquez JR, McGuire DK, Peng SA, Alexander KP*et al.* Contemporary patterns of discharge aspirin dosing after acute myocardial infarction in the United States: results from the National Cardiovascular Data Registry (NCDR). Circ Cardiovasc Qual Outcomes, 2014. **7**:701-7.

151. Peters RJ, Mehta SR, Fox KA, Zhao F, Lewis BS, Kopecky SL*et al.* Effects of aspirin dose when used alone or in combination with clopidogrel in patients with acute coronary syndromes: observations from the Clopidogrel in Unstable angina to prevent Recurrent Events (CURE) study. Circulation, 2003. **108**:1682-7.

152. Yu J, Mehran R, Dangas GD, Claessen BE, Baber U, Xu K*et al.* Safety and efficacy of high- versus low-dose aspirin after primary percutaneous coronary intervention in ST-segment elevation myocardial infarction: the HORIZONS-AMI (Harmonizing Outcomes With Revascularization and Stents in Acute Myocardial Infarction) trial. JACC Cardiovasc Interv, 2012. **5**:1231-8.

153. O'Gara PT, Kushner FG, Ascheim DD, Casey DE, Jr., Chung MK, de Lemos JA*et al.* 2013 ACCF/AHA guideline for the management of ST-elevation myocardial infarction: executive summary: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Circulation, 2013. **127**:529-55.

154. Voora D, Cyr D, Lucas J, Chi JT, Dungan J, McCaffrey TA*et al.* Aspirin exposure reveals novel genes associated with platelet function and cardiovascular events. J Am Coll Cardiol, 2013. **62**:1267-76.

155. Eicher JD, Xue L, Ben-Shlomo Y, Beswick AD & Johnson AD. Replication and hematological characterization of human platelet reactivity genetic associations in men from the Caerphilly Prospective Study (CaPS). J Thromb Thrombolysis, 2016. **41**:343-50.

156. Wurtz M, Nissen PH, Grove EL, Kristensen SD & Hvas AM. Genetic determinants of on-aspirin platelet reactivity: focus on the influence of PEAR1. PLoS One, 2014. **9**:e111816.

157. Xiang Q, Cui YM, Zhao X & Zhao N. Identification of PEAR1 SNPs and their influences on the variation in prasugrel pharmacodynamics. Pharmacogenomics, 2013. **14**:1179-1189.

158. Yao Y, Tang XF, Zhang JH, He C, Ma YL, Xu JJ*et al.* Association of PEAR1 genetic variants with platelet reactivity in response to dual antiplatelet therapy with aspirin and clopidogrel in the Chinese patient population after percutaneous coronary intervention. Thromb Res, 2016. **141**:28-34.

159. Vandenbriele C, Sun Y, Criel M, Cludts K, Van Kerckhoven S, Izzi B*et al.* Dextran sulfate triggers platelet aggregation via direct activation of PEAR1. Platelets, 2016. **27**:365-72.

160. Izzi BA-Ohoo, Pistoni M, Cludts K, Akkor P, Lambrechts D, Verfaillie CA-Ohoo*et al.* Allele-specific DNA methylation capacitates PEAR1 enhancer activity. LID - blood-2015-11-682153 [pii].

161. Cheung AL. Isolation and culture of human umbilical vein endothelial cells (HUVEC). Curr Protoc Microbiol, 2007. **Appendix 4**:Appendix 4B.

162. Aspirin Comprehensive Prescribing Information. (Bayer Corporation).

163. Ou HC, Lee WJ, Wu CM, Chen JF & Sheu WH. Aspirin prevents resistin-induced endothelial dysfunction by modulating AMPK, ROS, and Akt/eNOS signaling. J Vasc Surg, 2012. **55**:1104-15.

164. Hu Z, Zhang F, Yang Z, Zhang J, Zhang D, Yang N*et al.* Low-dose aspirin promotes endothelial progenitor cell migration and adhesion and prevents senescence. Cell Biol Int, 2008. **32**:761-8.

165. Borthwick GM, Johnson AS, Partington M, Burn J, Wilson R & Arthur HM. Therapeutic levels of aspirin and salicylate directly inhibit a model of angiogenesis through a Cox-independent mechanism. FASEB J, 2006. **20**:2009-16.

166. Song H & Yang PC. Construction of shRNA lentiviral vector. N Am J Med Sci, 2010. **2**:598-601.

167. Liang CC, Park AY & Guan JL. In vitro scratch assay: a convenient and inexpensive method for analysis of cell migration in vitro. Nat Protoc, 2007. **2**:329-33.

168. Schneider CA, Rasband WS & Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods, 2012. **9**:671-5.

169. Scheib JL, Sullivan CS & Carter BD. Jedi-1 and MEGF10 signal engulfment of apoptotic neurons through the tyrosine kinase Syk. J Neurosci, 2012. **32**:13022-31.

170. Steinhubl SR, Badimon JJ, Bhatt DL, Herbert JM & Luscher TF. Clinical evidence for anti-inflammatory effects of antiplatelet therapy in patients with atherothrombotic disease. Vasc Med, 2007. **12**:113-22.

171. BioFlux Systems. Vol. 2016 (Fluxion Biosciences, Inc.).

172. Hwang SJ, Ballantyne CM, Sharrett AR, Smith LC, Davis CE, Gotto AM, Jr.*et al.* Circulating adhesion molecules VCAM-1, ICAM-1, and E-selectin in carotid

atherosclerosis and incident coronary heart disease cases: the Atherosclerosis Risk In Communities (ARIC) study. Circulation, 1997. **96**:4219-25.

173. Deanfield JE, Halcox JP & Rabelink TJ. Endothelial function and dysfunction: testing and clinical relevance. Circulation, 2007. **115**:1285-95.

174. Lee WJ, Pollin TI, O'Connell JR, Agarwala R & Schaffer AA. PedHunter 2.0 and its usage to characterize the founder structure of the Old Order Amish of Lancaster County. BMC Med Genet, 2010. **11**:68.

175. Speliotes EK, Willer CJ, Berndt SI, Monda KL, Thorleifsson G, Jackson AU*et al.* Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index. Nat Genet, 2010. **42**:937-48.

176. Heidenreich PA, Trogdon JG, Khavjou OA, Butler J, Dracup K, Ezekowitz MD*et al.* Forecasting the future of cardiovascular disease in the United States: a policy statement from the American Heart Association. Circulation, 2011. **123**:933-44.