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Degree and Date to be Conferred

Ph.D., 2015

Education

University of Maryland School of Medicine MSTP **August 2008-Present**
Ph.D., Genome Biology Track, Molecular Medicine Program
Dissertation: The Pharmacogenomics of Clopidogrel and Aspirin
Mentors: Alan Shuldiner, M.D., and Joshua Lewis, Ph.D.

M.D., first 2 years completed 2008-2010, degree anticipated May 2017

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B.S. in Physiology and Neurobiology
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Professional Publications

1. **Fisch AS**, Yerges-Armstrong LM, Parihar A, Pavlovich MA, Donnelly P, Mitchell BD, O'Connell JR, Shuldiner AR, Lewis JP. Novel association of a variant in *PEAR1* with endothelial function. [manuscript being reviewed]

2. **Fisch AS**, Perry CG, Stephens SH, Horenstein RB, Shuldiner AR. Pharmacogenomics of anti-platelet and anti-coagulation therapy. *Curr Cardiol Rep.* 2013 Jul; 15(7):381. PMCID: PMC3809070.

3. Shuldiner AR, Vesely MR, **Fisch A**. CYP2C19 genotype and cardiovascular events. *JAMA*. 2012 Apr 11;307(14):1482. PMID: 22496255.

4. Lewis JP, **Fisch AS**, O'Connell JR, Ryan K, Horenstein RB, Gibson Q, Mitchell BD, Pakzy R, Shen H, Tanner K, Tantry US, Bliden KP , Gurbel PA, Shuldiner AR. Paraoxonase 1 (*PON1*) Gene Variants are Not Associated with Clopidogrel Response. *Clin Pharmacol Ther*. 2011 Oct;90(4):568-74. PMCID: PMC3250350.

Grant Support

T32 HL072751, Cardiac And Vascular Cell Biology, PI: Steven Fisher, M.D.
Role: Trainee
August 2013 – July 2015

Oral Presentations

1. University of Maryland Graduate Research Conference, Baltimore, MD, 2015

2. University of Maryland 2nd Annual Cardiovascular Cell Biology T32 Training Grant Retreat, Baltimore, MD, 2013

3. University of Maryland Medical Scientist Training Program 4th Annual Retreat, Baltimore, MD, 2012

4. University of Maryland Medical Student Research Day, Baltimore, MD, 2009

Poster Presentations

1. **Fisch A.S.**, Lewis J.P., Yerges-Armstrong L.M., Vikas A.P., Xu H., O'Connell J.R., Ryan K.A., Horenstein R.B., Mitchell B.D., Shuldiner A.R. A Variant in *UGT2A1* is Associated with Sex-Specific Clopidogrel Response; (Abstract #3). Presented at the Spring 2015 Meeting of the Pharmacogenomics Research Network, April 28, 2015 in State College, PA.

2. **Fisch A.S.**, Bozzi L.M., Ryan K.A., Horenstein R.B., O'Connell J.R., Mitchell B.D., Shuldiner A.R., Yerges-Armstrong L.M., Lewis J.P. Novel Association Between a Genetic Variant in the *PEAR1* Gene and Platelet Count is Not Modified by Antiplatelet Treatment with Clopidogrel and Aspirin; (Abstract #2071). Presented at the 64th Annual Meeting of The American Society of Human Genetics, October 19, 2014 in San Diego, CA.

3. **Fisch A.S.**, Yerges-Armstrong L.M., O'Connell J.R., Mitchell B.D., Donnelly P., Drolet M., Newcomer S., Parihar A., Ryan K.A., Herzog W., Shuldiner A.R., Lewis J.P. Novel association of a variant in *PEAR1* with endothelial function; (Abstract #2). Presented at the 2nd Annual Cardiovascular Cell Biology T32 Training Grant Retreat, November 8, 2013 in Baltimore, MD.

4. **Fisch A.S.**, Yerges-Armstrong L.M., O'Connell J.R., Mitchell B.D., Donnelly P., Drolet M., Newcomer S., Parihar A., Ryan K.A., Herzog W., Shuldiner A.R., Lewis J.P. Novel association of a variant in *PEAR1* with endothelial function; (Abstract #2196W). Presented at the 63rd Annual Meeting of The American Society of Human Genetics, October, 23, 2013 in Boston, MA.

5. **Fisch A.S.**, Lewis J.P., Yerges-Armstrong L.M., Parihar A., O'Connell J.R., Mitchell B.D., Horenstein R.B., Ryan K.A., Gibson Q.D., Shuldiner A.R. Genetic variants in *SGCD* and *ANKS1A* are associated with platelet aggregation; (Abstract #10). Presented at the 28th Annual National MD/PhD Student Conference, July 26, 2013 in Keystone, CO.

6. **Fisch A.S.**, Liu J., Lewis J.P., Yerges-Armstrong L.M., O'Connell J.R., Mitchell B.D., Horenstein R.B, Ambulos N., Ryan K.A., Gibson Q.D., Shelton J.C., Shuldiner A.R. Novel association of functional variants in *PPARG* and *SULT1A2/3* with dual

anti-platelet therapy drug response; (Abstract #44). Presented at the Up Close and Personalized 2nd International Congress on Personalized Medicine, 2013, Paris, France.

7. **Fisch A.S.**, Liu J., Lewis J.P., Yerges-Armstrong L.M., O'Connell J.R., Mitchell B.D., Horenstein R.B, Ambulos N., Ryan K.A., Gibson Q.D., Shelton J.C., Shuldiner A.R. Novel association of functional variants in *PPARG* and *SULT1A2/3* with dual anti-platelet therapy drug response; (Abstract #602T). Presented at the 62nd Annual Meeting of the American Society of Human Genetics, November 8, 2012 in San Francisco, CA.

Positions Held

Ad-hoc reviewer for European Journal of Clinical Pharmacology, 2013-Present

MSTP Summer Rotation Symposium, Presentation Judge, University of Maryland School of Medicine, 2013

MSTP Stephen Max Lecture Selection Committee, Chair, University of Maryland School of Medicine, 2012-2013

MSTP Dual Decree Newsletter, Contributing Editor and Writer, University of Maryland School of Medicine, 2011-Present

Academic Medicine and Research Interest Group, Co-Founder and Co-President, University of Maryland School of Medicine, 2009-2010

Adolescent Empowerment Program, Co-President, University of Maryland School of Medicine, 2009-2010

Class of 2012 Council, President, University of Maryland School of Medicine, 2008-2010

Academic and Professional Honors

1. Geriatrics and Gerontology Education and Research Program and Center for Research on Aging Award for Aging Research, Biomedical Sciences Winner, University of Maryland Graduate Research Conference, 2015 – awarded to only one presenter at the entire conference

2. University of Maryland Graduate Research Conference Best Poster, 2014

3. Omicron Delta Kappa National Leadership Honor Society, 2008, University of Maryland College park

4. Phillip Merrill Presidential Scholar, 2008, University of Maryland College Park, received 1 of 2 total awards given to entire college

5. Accepted into the Phi Beta Kappa society, 2008, University of Maryland College Park

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Pharmacogenomics Research Network (PGRN), 2011-Present

Abstract

Title of Dissertation: The Pharmacogenomics of Clopidogrel and Aspirin

Adam S. Fisch, Doctor of Philosophy, 2015

Dissertation directed by:

Alan R. Shuldiner, M.D.

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Coronary artery disease is a leading cause of death in the United States, and patients who develop acute coronary syndrome undergo vessel revascularization, which commonly involves percutaneous coronary intervention with dual-antiplatelet therapy, including clopidogrel and aspirin. Patient variability in both drugs is well-established. Our genome-wide association study (GWAS) in the Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study yielded significant associations between *CYP2C19**2 (rs4244285) and clopidogrel response and between *PEAR1* rs12041331 and aspirin response. Given our heritability estimates, we hypothesized that other important pharmacogenetic variants remain unidentified. To test this hypothesis, we explored the impact of 1936 variants in 231 genes on clopidogrel response using the Drug Metabolizing Enzymes and Transporters (DMET) array. In addition to confirming the

association with *CYP2C19**2 ($P = 5.34 \times 10^{-13}$), we observed a novel association between clopidogrel response and the rs11249454 variant in UDP glucuronosyltransferase 2A1 and 2A2 (*UGT2A1/2*; $P = 2.92 \times 10^{-5}$). Specifically, this variant influenced clopidogrel response in a sex-specific manner, with significance in women ($P = 8.40 \times 10^{-6}$), but not in men (P = 0.21). Additionally, given the association between *PEAR1* rs12041331 and aspirin response in the PAPI Study, we used computational methodologies to predict genes, phenotypes, and diseases related to *PEAR1* expression using data from ~75,000 publicly available microarrays. Meta-analysis of microarray data suggested that PEAR1 may significantly impact endothelial cell biology. To confirm these results, we functionally validated the impact of *PEAR1* rs12041331 on endothelial cell migration distance in primary human umbilical vein endothelial cells using an *ex vivo* migration assay (P = 0.04). Finally, we confirmed the impact of this variant on endothelial function clinically through assessment of *in vivo* flow-mediated dilation of the brachial artery in 641 participants of the Heredity and Phenotype Intervention (HAPI) Heart Study (P = 0.02). Taken together, the results of these studies highlight a novel variant in clopidogrel response and a novel biological mechanism of an aspirin response gene. These studies provide the framework for future investigations to better understand how *UGT2A1/2* and endothelial *PEAR1* affect clopidogrel and aspirin response, respectively.

The Pharmacogenomics of Clopidogrel and Aspirin

by
Adam S. Fisch

Dissertation submitted to the Faculty of the Graduate School of the
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There are too many people to name who contributed in some way to my completion of this doctorate, with several that clearly deserve the bulk of my thanks. To my mentors, Dr. Alan Shuldiner and Dr. Josh Lewis, thank you for your unwavering devotion to my growth as a student and a scientist. Your guidance was invaluable, and the skills I gained in the last 5 years, including wet lab experimentation, computational analysis, reading scientific papers with a discerning eye, writing scientifically, and much more will undoubtedly serve me for the rest of my career. The examples you have both set for me through the milestones you have achieved in your respective careers are inspiring, and a bit daunting, and I can only hope to experience the success you both have. Beyond the scientific pearls you passed to me over the years, your combined tenacity for me to succeed is irreplaceable, and I will always appreciate it. I look forward to staying in touch both personally and professionally as we move forward in our careers, and cannot wait to see what the future has in store for us.

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every single day, and that is my wife Jessica Fernandes. For the last 5 years she softened the sting of failures and increased the joy of success, always pushing me forward in the face of whatever graduate school threw my way. Coming home to her has been a major source of calm for me during this process, and I am forever grateful for that. With all of the exciting steps that will follow the defense of this thesis, I cannot imagine any better way to experience them than with her.

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List of Abbreviations:

ACS: Acute coronary syndrome
ADP: Adenosine diphosphate
ARS: Aspirin response signature
BMI: Body mass index
CAD: Coronary artery disease
CPIC: Clinical Pharmacogenomics Implementation Consortium
CVD: Cardiovascular disease
CYP: Cytochrome P450
DAPT: Dual-antiplatelet therapy
D_{base}: Baseline brachial artery width
DBP: Diastolic blood pressure
DMET: Drug Metabolizing Enzymes and Transporters
FDA: Food and Drug Administration
FMD: Flow-mediated dilation
GAMMA: Global Microarray Meta-Analysis
GP: Glycoprotein
GWAS: Genome-wide association study
HAPI: Heredity and Phenotype Intervention
HDL: High-density lipoprotein
HR: Heart rate
HTPR: High on-treatment platelet reactivity
HUVEC: Human umbilical vein endothelial cell
LDL: Low-density lipoprotein
LOF: Loss-of-function
MAF: Minor allele frequency
MAP: Mean arterial pressure
MMAP: Mixed model program for analysis of pedigree
OOA: Old Order Amish
PAPI: Pharmacogenomics of Anti-Platelet Intervention
PCI: Percutaneous Coronary Intervention
PGRN: Pharmacogenomics Research Network
POC: Point-of-care
PPP: Platelet-poor plasma
PRP: Platelet-rich plasma
SBP: systolic blood pressure
SNP: single nucleotide polymorphism
TF: Tissue factor
TXA_2: Thromboxane

I. Introduction

Cardiovascular disease (CVD) includes a variety of conditions that lead to pathologies of the heart and blood vessels. Such conditions include myocardial infarction (MI), ischemic stroke, hemorrhagic stroke, heart failure, arrhythmia, and heart valve problems.[1] MI and ischemic stroke arise due to atherosclerosis, a condition caused by the development of plaque in blood vessels. This plaque forms from progressive chronic inflammation in the blood vessel wall, leading to stiffening of the arteries and poor blood flow. As plaque formation increases over time, it ultimately impedes blood flow in the artery. This can be particularly damaging in coronary arteries supplying blood to the cardiac muscle, a condition termed coronary artery disease (CAD). Progression of atherosclerosis can ultimately lead to acute coronary syndrome (ACS), a condition characterized by sudden loss of blood flow to the heart and, ultimately, myocardial infarction and potentially cardiac arrest.[1-3]

CVD is a widespread condition in the United States, affecting millions of people. There are multiple risk factors for CVD, most notably hypertension, elevated low-density lipoprotein (LDL) cholesterol levels, and smoking, with roughly half of all Americans possessing at least one of these risk factors.[4] Other medical conditions can contribute to the development of CVD including poor diet, physical inactivity, and obesity, all of which contribute to type II diabetes mellitus, another independent risk factor for CVD. In addition, excessive alcohol intake is highly correlated with the development of CVD.[4] According to the Center for Disease Control, approximately one quarter of all deaths in the United States are caused by CVD, amounting to roughly 600,000 people annually, making it the number one cause of mortality of Americans. The health-related burdens of

CVD is apparent when the American population is broken down into constituent ethnicities, being the number one cause of death in Caucasians, African Americans, and people of Hispanic descent. Interestingly, however, cardiovascular death does trend by location, with the peak incidence occurring in the southeastern United States, and the lowest incidence in the western United States.[4] Of all CVD-related deaths, coronary artery disease (CAD) accounts for roughly 380,000 per year. In addition to its effects on morbidity and mortality, the effect of CAD is significant from an economic perspective, where the costs in drugs, health care management, and reduced work productivity cost the United States $108.9 billion in 2010, a figure projected to double by 2030.[5]

A. Pathophysiology of Coronary Artery Disease

CAD typically begins with the development of atherosclerosis. The pathophysiology of this condition begins with damage to the endothelium, the key component of the tunica intima, typically from a combination of high lipid levels in the bloodstream, high blood pressure, and smoking.[2] Because of the loss of elastin confluency and the introduction of proteoglycans to the intimal-bloodstream interface, the atherosclerotic lesion attracts and provides a point of attachment for LDL, causing its accumulation in the subendothelial layer as a fatty streak. With the accumulation and oxidation of LDL, endothelial cells begin to secrete chemokines and present adhesion molecules on their surfaces, attracting immune cells such as monocyte-derived macrophage-like cells to the region. These cells attach to adhesion molecules presented on the endothelium resulting in cell rolling and extravasation into the subendothelial region in an attempt to phagocytose the lipids. The accumulation of lipids in macrophages results in the formation of "foam cells," ultimately leading to apoptosis and

necrosis of the lesion's core. This is followed by smooth muscle cell migration and proliferation on the luminal side of the lesion, as well as the deposition of collagen, forming a fibrous cap.[6,7] As the lesion accumulates more material and grows over time, it is termed an atheroma, or atheromatous plaque, which is a structural formation within the vessel. As CAD worsens, the walls of the coronary artery itself become hardened and the lumen of the artery becomes increasingly occluded. Typically, however, the vessel lumen remains patent enough to continue delivering blood to the tissues it supplies due to dynamic expansion of the overall artery.[8] The occurrence of a plaque rupture is the primary occlusive event that overpowers arterial compensation mechanisms and can lead to a dangerous, potentially fatal, ACS. Studies show that as the number of inflammatory cells infiltrating the plaque increases, the lipid core grows, and the fibrous cap thins, particularly under 55 μm thickness,[9] the odds of plaque rupture increases.[10] This relationship is causative, since inflammation initiated by the presence of T cells causes the dysregulation of collagen production, which significantly reduces the structural integrity of the fibrous cap. This effect is specifically mediated by the T cells' secretion of interferon-γ and expression of CD154 on their surfaces, leading to decreased production of collagen by smooth muscle cells and increased collagenase-mediated breakdown of collagen by macrophages, respectively.[9]

Once the fibrous cap of the atheromatous plaque is compromised and a rupture occurs, the patient will experience a transition from a chronic, somewhat manageable CAD to a painful, life-threatening ACS. The dramatic increase in risk of experiencing an emergent coronary event following plaque rupture is caused by multiple factors, most notably thrombosis. With damage to the plaque's structure, core thrombogenic elements

of the plaque that are typically inaccessible are now exposed to mediators of thrombosis in circulation. One such mediator is tissue factor (TF), also known as thromboplastin, which is a stimulator of the extrinsic pathway of coagulation.[9] The expression of TF ultimately leads to thrombin formation, which then causes recruitment, activation, and aggregation of platelets.[11] The rapid development of a thrombus can lead to total or near-total coronary occlusion and severely restrict blood supply to the myocardium. Given the central role platelets have throughout this process, platelet physiology and function are key elements in understanding the life-threatening nature of ACS.

B. Platelet Biology

Platelets are small, anucleate cellular fragments, ranging from 1-3 μm in diameter, that form from much larger megakaryocytes, a lineage-specific descendant of the pluripotent hematopoietic stem cells (HSCs) in the bone marrow.[12] Thrombopoietin produced in the kidneys and liver binds to the cellular myeloproliferative leukemia (c-Mpl) receptor on the surface of HSCs, causing differentiation to megakaryocytes and the initiation of megakaryocyte maturation to platelets.[12] Megakaryocyte maturation is a two-step process with the first step including growth in size, an increase in the amount of cytoskeletal proteins in the cytoplasm, the formation of platelet-specific granules, and the invagination of their membranes. During this step, megakaryocytes also replicate their DNA internally without performing cell division in a process called endomitosis, increasing their chromosomal content to 4n, or even 8n, unlike the typical haploid (n) and diploid (2n) content found in gametes and the rest of the body, respectively. The second step of megakaryocyte maturation includes the formation of long cytoplasmic processes, protruding from the cellular surface, called proplatelets. These protrusions ultimately bud

off to become platelets lasting typically 7-10 days in the circulation, fulfilling critical roles in hemostasis and innate immunity.[12]

The control of hemostasis, probably the role platelets are best known for, is critical in response to traumatic injury where blood vessels are damaged and quick action is required to prevent life-threatening blood loss. Platelets perform this function through three phases, namely the initiation phase, the extension phase, and the perpetuation phase. The initiation phase is marked by platelet adhesion caused by the exposure of subendothelial collagen and TF after vascular injury, and is mediated by the platelet glycoprotein (GP) Ib-IX-V complex interacting with the exposed sites via von Willebrand Factor, as well as GP VI, which interacts directly with the exposed collagen. With the initial platelets firmly adhered to the site of injury, they begin to release soluble messengers to recruit and activate more platelets as part of the extension phase. These messengers serve as agonists for a variety of receptors on the platelet surfaces and include adenosine diphosphate (ADP), thromboxane (TXA_2), epinephrine, serotonin, collagen, and thrombin. As more platelets are recruited and activated, the increased activation of GP IIb/IIIa on the platelet surfaces creates platelet-platelet connections via fibrinogen, expanding the size of the clot. The formation of the clot is completed with the perpetuation phase, where platelets and coagulation factors reciprocally activate each other in a feed-forward manner, thus strengthening the clot. In contrast to physiological situations where hemostasis is necessary, the same process can become pathophysiological when in the context of an atheromatous plaque rupture as it can lead to the rapid occlusion of the artery. Medical treatments aim to prevent this rapid thrombotic formation, specifically through the prescription of antiplatelet agents such as

clopidogrel and aspirin, which target the platelet $P2Y_{12}$ ADP receptor and the platelet cyclooxygenase 1 (COX-1) enzyme, respectively.[13]

C. Clopidogrel

Individuals receiving percutaneous coronary intervention (PCI) to unblock an occluded artery usually receive dual-antiplatelet therapy (DAPT), comprised of clopidogrel and aspirin.[14] Clopidogrel was first released in 1997 under the brand name Plavix as an antiplatelet agent for the treatment of ACS and prevention of recurrent coronary events.[15] Clopidogrel continues to be widely prescribed due to its efficacy in the majority of patients as well as its relatively low price since its patent expired.[16]

Clopidogrel is an oral thienopyridine that inhibits platelet activation and aggregation by irreversibly binding the $P2Y_{12}$ ADP receptors on the platelet surfaces.[17] Upon ingestion, clopidogrel is absorbed in the duodenum, where some of the drug is immediately effluxed into the lumen by the ABCB1 transporter, and the rest passes to the bloodstream.[18] Once in circulation, approximately 85% of the clopidogrel is inactivated via hydrolysis by carboxylesterases, primarily carboxylesterase 1 (CES1), in the liver.[19] The rest of the drug is biotransformed from a pro-drug into the active metabolite. This process requires two steps involving several CYP enzymes in the liver. One key CYP enzyme involved in this activation step is CYP2C19, while some of the others that take part in this pathway include CYP1A2, CYP2B6, CYP2C9, CYP3A4/5, and PON1.[20-22] The active metabolite then performs its action by oxidizing cysteine residues and irreversibly blocking $P2Y_{12}$ ADP receptors on the platelet surfaces. The inhibition of $P2Y_{12}$ causes their associated G_i proteins to stop inhibiting adenylyl cyclase. The resultant increase in cAMP is followed by decreased phosphoinositide 3-kinase (PI3K)

activation and GP IIb/IIIa activation. Ultimately, this leads to the effective blockade of the slow-starting, long-term activation and aggregation of platelets.[23]

Clopidogrel response variability among patients is well established.[24-29] Patients treated with clopidogrel who demonstrate higher *ex vivo* on-clopidogrel platelet aggregation, termed high on-treatment platelet reactivity (HTPR), are at increased risk of ischemic events.[24,30] In a study of the Old Order Amish of Lancaster, Pennsylvania, called the Amish Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study, our group explored the genetic basis of clopidogrel response variability.[30] First, we estimated the heritability of clopidogrel response, as measured by post-drug ADP-stimulated platelet aggregation, at 70% in a healthy population of 429 Amish individuals. Our group then performed a genome-wide association study (GWAS) of on-clopidogrel ADP-stimulated platelet aggregation traits. The main finding of the study was that the *CYP2C19**2 (rs4244285) allele was significantly associated with lower clopidogrel response as evidenced by HTPR in the Amish as well as HTPR and increased cardiovascular event rates in 227 non-emergent PCI patients from the Sinai Hospital of Baltimore.[30] Furthermore, these results showed that the *CYP2C19**2 variant accounts for 12% of the variability observed in on-clopidogrel ADP-stimulated platelet aggregation. To confirm our findings, and in search of other genetic variants that influence clopidogrel response, a number of candidate gene studies have been published. Some of the most prominent variants found to affect clopidogrel response in those studies are in the *CYP2C19*, *ABCB1*, and *CES1* genes, while there is some debate regarding the effect of genetic variation in *PON1* on clopidogrel response.

1. CYP2C19

Loss-of-function (LOF) variants in *CYP2C19* have the strongest evidence in the literature as genetic determinants of clopidogrel responsiveness. The most common LOF variant is *2 (rs4244285), with allele frequencies of 31% in Asians, and 15-17% in Caucasians and Africans.[31] Other LOF alleles include *3-*8, which are mostly considered rare besides *3 in Asian populations. Carriers of one *CYP2C19* LOF allele have been termed intermediate metabolizers, signifying the decreased function of the CYP2C19 enzyme in comparison to wild-type individuals, termed extensive metabolizers. Furthermore, individuals carrying 2 LOF alleles for *CYP2C19* are called poor metabolizers as they have an even greater decrease in CYP2C19 function. Studies show that LOF variants associate with lower clopidogrel active metabolite concentrations, greater on-treatment residual platelet function, and poorer cardiovascular outcomes in PCI patients treated with clopidogrel.[30,32-41] Meta-analyses further support these studies, and suggest a clinically important role of *CYP2C19* LOF variants, with consistent effects across ethnic populations.[42-46]

In March 2010, in response to the overwhelming evidence regarding *CYP2C19* LOF variants and clopidogrel response, the Food and Drug Administration (FDA) added a boxed warning to clopidogrel's label, signifying that *CYP2C19* LOF allele carriers may not respond fully to clopidogrel, and that *CYP2C19**2 genotyping tests are available to assess metabolizer status. Furthermore, the warning stated that alternative drugs or doses are recommended in poor metabolizers.[47] Shortly thereafter, the American College of Cardiology Foundation Task Force on Clinical Expert Consensus Documents and the American Heart Association published a report in June 2010 recommending that

CYP2C19 LOF variants not be routinely tested, stating that *CYP2C19**2 accounting for only 12% of the variation in clopidogrel response signifies low positive predictive value, which further highlights the need for novel clopidogrel response variant discovery.[48] The Pharmacogenomics Research Network (PGRN) Clinical Pharmacogenomics Implementation Consortium (CPIC) published guidelines and an algorithm that incorporate *CYP2C19* genotyping, interpretation, and treatment.[49] While these guidelines will have utility in some patients, the results of properly designed and powered randomized clinical trials are necessary for sweeping changes in therapeutic recommendations.

In addition to LOF variants in *CYP2C19*, the gain of function variant *CYP2C19**17 (rs12248560) is in the 5' regulatory region of the gene. This variant's minor allele frequencies of 21% and 16% in individuals of European and African ancestry, respectively, and it is associated with greater transcription of the *CYP2C19* gene.[30,50,51] The mechanism by which transcription is increased has been suggested to involve altered hepatocyte nuclear binding as evidenced by electrophoretic mobility shift assays.[51]

So far, the results of association analyses exploring the effect of *CYP2C19**17 on clopidogrel response traits have been inconsistent. While some studies have observed a decrease in cardiovascular event rates in *CYP2C19**17 carriers receiving clopidogrel, others have failed to replicate this observation.[18,30,39,52-60] Similarly, while some found that carriers of the minor allele had increased rates of bleeding while on clopidogrel therapy, this was not universally replicated.[18,32,52,55-58,61,62] Meta-analyses investigating the effect of *CYP2C19**17 on clopidogrel response have demonstrated an association of

the minor allele with lower cardiovascular event rates and higher incidence of bleeding, but more recent work has highlighted a confounding issue in this association, namely that *CYP2C19**2 and *CYP2C19**17 genotypes are in linkage disequilibrium and thus not independent of one another.[55,56] Carriers of the *CYP2C19**17 variant alleles are less unlikely to carry a copy of the *CYP2C19**2 allele, while non-carriers of the *CYP2C19**17 variant are more likely to carry *CYP2C19**2 variant alleles. Because these variants are linked to each other, it has been suggested that any improvement in clopidogrel response in carriers of *CYP2C19**17 could really be an observation of individuals responding well to the drug due to their lack of *CYP2C19**2 alleles.[63] Future *CYP2C19**17 associations with clopidogrel response should include *CYP2C19**2 adjustment in the model to account for their linkage disequilibrium with one another.

2. ABCB1

Upon absorption, clopidogrel travels from the duodenal lumen through enterocytes to the circulation. During this passage, some of the drug is effluxed back into the lumen by the P-glycoprotein ATP-dependent efflux pump, coded by *ABCB1*, also known as multidrug resistant 1 (MDR1). The most widely studied variant in *ABCB1*, C3435T (rs1045642), has been shown to affect gene transcription. The T allele is associated with increased expression, suggesting it would be associated decreased clopidogrel response due to lower net absorption.[18] The T allele's frequency is 52% in Caucasian individuals, 40% in Asian individuals (Chinese), and 15% in individuals of African descent.[31]

The modest association between the *ABCB1* 3435T allele and decreased clopidogrel active metabolite, increased on-treatment platelet reactivity, and increased

cardiovascular events has been demonstrated in several studies.[64-66] In one study, a group found a significant association between *ABCB1* genotype and platelet reactivity as well as cardiovascular event risk while investigating genes for inclusion in a clopidogrel resistance risk score.[58]

Other studies have not been able to replicate the association observed between *ABCB1* C3435T and clopidogrel response. This could potentially be due to inadequate power to observe low effect sizes (false negative results), specific characteristics of the patient populations, or false positive results of other studies. Recently, a meta-analysis reviewing 12 studies investigating *ABCB1* C3435T found no association between *ABCB1* C3435T and on-treatment platelet reactivity, MI, ischemic stroke, all-cause mortality, stent thrombosis, or long-term major cardiovascular events. When they stratified the cohort by clopidogrel loading dose, they found a significant association between the C3435T variant and cardiovascular events and bleeding in the 300 mg loading dose group, but failed to see these association in the 600 mg group.[65] These observations may hint to an increased clopidogrel dose being able to overcome higher efflux rates seen in T allele carriers.

3. CES1

CES1 hydrolyzes clopidogrel into an inactive carboxylic acid metabolite from its prodrug and intermediate states.[19] A rare variant (rs71647871; G143E) in the gene causes a LOF substitution that causes a glycine at position 143 of the protein to become a glutamine.[67] The frequency of the decreased function 143Gln allele is estimated to be 3.7% in Caucasians, 4.3% in individuals of African descent, and 2% in individuals of Hispanic descent.[68] In 566 healthy participants of the PAPI study, the 143Gln allele

significantly associated with higher active metabolite levels and greater clopidogrel response, as demonstrated by greater inhibition of ADP-simulated platelet aggregation.[69] Though the variant was uncommon in the PAPI study population (minor allele frequency, MAF = 0.008), the effect size was approximately double that of *CYP2C19**2. In 330 PCI patients receiving clopidogrel, 143Gln carriers replicated the PAPI Study findings with lower levels of on-treatment platelet reactivity. While a trend toward lower cardiovascular event rates in 143Gln carriers was observed, it was not statistically significant; this could be due to small sample size and lack of statistical power. These observations require larger study replication, but the data so far suggest that this rare variant with a large effect on clopidogrel response may become clinically important for individuals who carry the minor allele.[69]

4. PON1

PON1, expressed in the liver, is typically found in the bloodstream associating with HDL-cholesterol. Two *PON1* variants that are commonly investigated are A575G (rs662; Gln192Arg) and T163A (rs854560; Leu55Met), with the Gln and Met variants both associating with lower paraoxonase activity.[70,71] A recent study observed a significant association between genotype of *PON1* rs662 and key clopidogrel response phenotypes, including active clopidogrel metabolite concentration, level of platelet inhibition, and stent thrombosis.[72] These observed effect size of *PON1* rs662 and the fact that this enzyme was not previously recognized to be involved in clopidogrel bioactivation were remarkable. Curiously, the investigators in this study failed to replicate the association between *CYP2C19**2 genotype and on-clopidogrel platelet reactivity or stent thrombosis. Since this study's publication, multiple groups have failed

to replicate their findings, specifically exploring the association between *PON1* rs662 and multiple phenotypes including clopidogrel active metabolite levels, platelet function, cardiovascular outcomes, and stent thrombosis.[41,70,73-76] It is unclear why these discrepancies are occurring. There was one study where a significant association was observed between *PON1* rs662 and on-treatment platelet reactivity at 1 and 6 months post-PCI in 300 PCI patients being treated for ischemic heart disease. The effect sizes observed, however, in this study were far less substantial than *CYP2C19**2, *17, and *ABCB1* genotypes.[77] These data suggest that the original group reporting the large effect of *PON1* rs662 on clopidogrel response may have experienced "the winner's curse" where their initial report will not be able to be replicated in other typical cohorts due to the potentially smaller effect size of *PON1* genotype on clopidogrel response than initial reports. In contrast, it is possible that negative studies regarding this variant did not have statistical power to observe its effects, particularly for the stent thrombosis phenotype. Another study suggested that PON1's action may not form clopidogrel's active metabolite, and may instead form a different, albeit similar, metabolite that does not play a role in antiplatelet response.[78] Recently, a significant association was observed between *PON1* rs662 genotype and on-treatment platelet reactivity in *CYP2C19**1 homozygotes, but not in carriers of the *CYP2C19**2 allele, from a cohort of 424 Chinese patients with acute coronary syndrome, suggesting a potential interaction between different pathways of clopidogrel metabolism.[79]

Several studies have demonstrated that the observed effects regarding *PON1* genotype and clopidogrel response may have more to do with underlying cardiovascular disease risk. A substudy of the CURE trial demonstrated a significant association

between *PON1* genotype and cardiovascular event rates in the placebo group of a controlled study.[80] The context of placebo therapy suggests a non-pharmacogenomic role for *PON1* genotype, and the observed effect on cardiovascular event risk fits well with data regarding PON1 associating with HDL particles. Furthermore, *PON1* genotype is associated with enzymatic activity and the ability of HDL to prevent oxidation of LDL particles.[81] Overall, the burden of evidence does not support a role for *PON1* genotype in clopidogrel response at this time.[82,83]

D. Aspirin

In addition to clopidogrel, aspirin is commonly prescribed as part of DAPT. Aspirin therapy has multiple effects, including antiplatelet and anti-inflammatory, and is taken by many for the primary and secondary prevention of ACS.[84] The drug is taken orally and its mechanism of action is through the inhibition of COX-1, which catalyzes the conversion of arachidonic acid to PGH_2, a precursor to TXA_2, which potently activates platelets.[85] In addition, it weakly inhibits cyclooxygenase 2 (COX-2), platelet factors 3 and 4, and coagulation factors II, VII, IX, and X, requiring higher doses to inhibit these targets ($\geq$ 200 mg per day).[86] Aspirin inhibits COX-1 and COX-2 by causing the acetylation of Ser529 and Ser516, respectively, thus irreversibly blocking the COX channel where catalytic activity occurs.[85] Aspirin is removed from circulation via hydrolysis in plasma and red blood cells.[87,88]

Because the use of aspirin has been shown to reduce cardiovascular event rates, including death, in ACS patients when given acutely and chronically, it is often prescribed for the primary and secondary prevention of cardiovascular events.[89,90] With proper adherence to the typical drug regimen of under 100 mg per day, it is estimated that

patients' aspirin intake can inhibit greater than 99% of COX-1 activity, diminishing the formation of TXA_2 significantly.[91,92] However, investigators have observed significant inter-individual variability in response to aspirin, with resistance reaching incidence of 27%, prompting the search for genetic mediators of aspirin resistance. While exploring genetic variation in candidate genes pertaining to aspirin's canonical COX-1-mediated mechanism of action, some investigations suggest that patient noncompliance was the cause of COX-1-mediated "aspirin resistance."[86,93] In fact, the frequency of aspirin non-responders seems to be significantly lower in clinical situations where aspirin intake is physically observed by someone with the patient.[94]

Despite the near-total inhibition of the COX-1 pathway by aspirin in compliant patients, platelet activation can be stimulated by multiple other agonists, including ADP, epinephrine, serotonin, collagen, and thrombin. Where these pathways, collectively termed non-COX-1-dependent platelet function, have been studied in the context of aspirin treatment, individuals have shown wide variability in pre- and post-aspirin platelet aggregation, and these traits have been shown to be significantly heritable (H^2 = 27-76%).[95,96] Furthermore, individuals with HTPR, as measured by ADP-stimulated platelet aggregation, receiving aspirin therapy are at greater risk for cardiovascular events while on aspirin. Given the widespread use of aspirin, the variability of non-COX-1-dependent platelet function in the context of aspirin therapy is an extremely important clinical question that needs to be addressed.[97,98]

Because of the heritable nature of non-COX-1-dependent platelet function-mediated aspirin resistance, many have looked to genomic methods, including candidate gene studies and GWAS, to discover variants that may have a pharmacogenomic effect.

Unfortunately, conflicting results, small sample sizes, and the failure by the cardiovascular field to systematically define what criteria constitute aspirin resistance, most studies were unable to observe significant, reliable variants that significantly impacted on-aspirin platelet aggregation.[99] Still, the results of some studies, including our own, have led to the identification of variants in the following promising candidate genes that affect aspirin response.

1. PEAR1

One gene that has recently been evaluated in the context of aspirin pharmacogenetics is the platelet endothelial aggregation receptor 1 (*PEAR1*) gene, also known as *MEGF12* and *JEDI*. PEAR1 is a recently identified type I transmembrane receptor composed of 15 extracellular epidermal growth factor-like repeats, a transmembrane domain, and a cytoplasmic domain with 5 proline-rich domains. It is mostly prominently expressed in megakaryocytes and the endothelium.[100] While it has also been identified as a receptor for engulfment-dependent apoptotic neuron clearance in embryonic dorsal root ganglia, most of the published work about PEAR1 in recent years has been in relation to its function in platelets, particularly in platelet aggregation.[101,102] In terms of mechanism, it has been shown that PEAR1 functions as a platelet-platelet contact receptor that ultimately leads to the activation of the GP IIb/IIIa surface integrin, the key step to platelet aggregation, as well as a regulator of megakaryopoiesis and thrombopoiesis.[102,103] Its ligand was only recently identified to be FcεR1α, a high affinity IgE receptor subunit.[104]

Several studies have explored the impact of genetic variation in *PEAR1* from a clinical standpoint using *ex vivo* platelet aggregation studies and *in vivo* clinical data. The

first group, Herrera-Galeano et al, found that genetic variation in *PEAR1* was significantly associated with altered baseline platelet aggregation, an association that was only strengthened when the phenotype was replaced by post-aspirin platelet aggregation. In that study, the investigators focused on the rs2768759 SNP, finding carriers of the minor C-allele to have increased aggregation over AA homozygotes.[105] The next study regarding *PEAR1* was performed by Johnson et al, where they used a 2.5 million SNP array to explore associations with platelet aggregation, and found the minor allele of variants in the *PEAR1* locus to be significantly associated with decreased baseline platelet aggregation after stimulation by ADP and epinephrine.[106] Shortly thereafter, Faraday et al fine-mapped the *PEAR1* gene via sequencing, and identified a SNP, rs12041331, in intron 1 of the gene that accounts for 15% of the total phenotypic variation in platelet function. The group also observed significant associations between other SNPs in *PEAR1*, including rs12566888 and rs3737224, and both baseline and post-aspirin platelet aggregation traits stimulated by multiple agonists. However, ultimately they found that the rs12041331 SNP accounted most strongly for platelet function, with the rs12041331 minor A-allele associated with decreased pre- and post-aspirin platelet aggregation compared to GG homozygotes.[107]In members of the PAPI study, we observed, paradoxically, that the minor A-allele of rs12041331 was significantly associated with decreased on-aspirin collagen-stimulated platelet aggregation in the Amish, as well as increased on-aspirin cardiovascular event rate in a PCI patient population from the Sinai Hospital of Baltimore and an aspirin-treated cohort from the INVEST-GENES study.[108]

2. Other Genes Affecting Aspirin Response

There have been several other genetic candidates to explain some of the variability observed in aspirin response. Due to aspirin's clearance via plasma-mediated hydrolysis, the butyrylcholinesterase (*BCHE*) gene was investigated, specifically at its rs6445035 SNP, and the minor allele was found to be significantly associated with a 1.2 nmol/ml/min decrease in aspirin hydrolysis, accounting for a small 3% of overall aspirin response variability.[87] Others have explored the effect of genetic variation in *ABCC4*, a transporter on the surface of platelets that effluxes acetylsalicylate, the main derivative of aspirin, and found that there was no significant association with aspirin response.[109] Some of the other genes that have been studied include *ITGB3, VAV3, GP6, SHH, F2R, GP1BA,* and *LPA*.[105,110-112] The rs5918 variant in *ITGB3*, the gene that codes for the β3 integrin that forms part of the αIIbβ3 integrin, also known as the GP IIb/IIIa, has been linked to increased platelet reactivity in individuals receiving aspirin therapy.[113-115] Specifically, individuals carrying the A2-minor allele of the PIA1/A2 variant (Leu33Pro), have been shown to have reduced aspirin response, as measured by post-aspirin collagen-induced platelet aggregation compared to A1/A1 homozygotes.[114] In addition, rs869244 in *ADRA2A*, rs2893923 in *JMJD1C*, rs6943029 in *SHH*, rs1671152 in *GP6*, and rs1874445 in *MRVI1* have all been significantly associated with baseline platelet function, though no reproducibly replicated.[106] Carriers of the minor allele of the rs3798220 variant in *LPA* have been shown to have a decrease in cardiovascular event rate while receiving aspirin as compared to major allele homozygotes.[116] A more recent concept that has been used in some investigations of the effects on aspirin response is the "aspirin response signature" (ARS) based on gene expression profiling. The ARS is a

group of genes involved in platelet biology that are co-expressed or co-repressed in response to aspirin therapy, and by correlating these genes with each other we can more easily discover genetic candidates whose variants may be affecting aspirin response. To date, the ARS includes 62 genes whose co-expression has been reliably associated with platelet aggregation following aspirin therapy.[117]

E. Next-Generation Antiplatelet Medications

The discovery of patient resistance to clopidogrel and aspirin has prompted pharmaceutical companies and healthcare providers to steer towards newer drugs that are less likely to be impacted by pharmacogenomic variants. Prasugrel, a thienopyridine like clopidogrel, functions by inhibiting the $P2Y_{12}$ ADP receptor on platelet surfaces just as clopidogrel does. The major advantage of prasugrel over clopidogrel is its increased bioavailability of active metabolite following activation.[118,119] Another major antiplatelet drug that has been released in recent years is ticagrelor. Unlike clopidogrel and prasugrel, ticagrelor is not a thienopyridine but still targets the same $P2Y_{12}$ ADP receptor by functioning as a reversible antagonist. Unlike the aforementioned two thienopyridine antiplatelets, ticagrelor requires no biotransformation, reducing the number of genes it is dependent on to exert its action, which reduces the number of variants that can cause dysfunction of that action.[120] In terms of the pharmacogenomics of these drugs, not nearly as many studies have been carried out focusing on them as there have been focusing on clopidogrel and aspirin. However, early studies show that on-prasugrel platelet function may be associated with genetic variants in *CYP2C19* and *PEAR1*, while ticagrelor active metabolite levels have been significantly associated with genetic variants in *SLCO1B1* and *UGT2B7*.[121-124]

F. Overall Hypothesis

Through a GWAS in participants of the PAPI Study, our group recently uncovered novel associations between clopidogrel and aspirin on-treatment platelet function and genetic variants in critical genes that provide insights into antiplatelet function and platelet biology. Consistent with other investigations, our data suggest that *CYP2C19**2 is an important variant in predicting an individual's ability to respond to clopidogrel, with the minor allele carriers having worse response, and accounts for 12% of the variability in the trait.[30] These data led to our hypothesis, that despite the large effect of the *CYP2C19**2 variant, most of the genetic variation that influences clopidogrel response remains unidentified, and that variants known to affect patient response to other drugs are likely to play some role in the pharmacogenomics of clopidogrel. To this end, as detailed in Chapter 2, we genotyped the entire PAPI cohort using the Drug Metabolizing Enzymes and Transporters (DMET) array (Affymetrix, Santa Clara, California), to evaluate rare, high-effect variants previously shown to affect drug response as a complement to the more common, low-effect variants captured by the genome-wide SNP array methodology.

In addition to the PAPI Study GWAS findings concerning clopidogrel, the study also showed a striking association between the *PEAR1* rs12041331 variant and aspirin response, as measured by both platelet aggregation and cardiovascular event rates. Specifically, the minor A-allele of the rs12041331 SNP was associated with decreased on-aspirin collagen-stimulated platelet aggregation, and increased on-aspirin cardiovascular event rates. This combination of trends is atypical in the cardiovascular medicine field, where *in vivo* thrombosis represented by *ex vivo* platelet function

measurements is typically predictive of and directly correlated with *in vivo* cardiovascular event risk. The paradoxical effect of rs12041331 genotype in the context of aspirin therapy is intriguing, and led us to explore alternative pathways by which genetic variation of the *PEAR1* gene could affect the cardiovascular system and risk for events. It has been observed that *PEAR1* is expressed roughly seven-fold higher in endothelial cells than in megakaryocytes, leading to our hypothesis that the observed paradoxical effect of rs12041331 genotype on platelet aggregation and cardiovascular risk in the context of aspirin therapy is mediated by PEAR1's functional role in the endothelium. In Chapter 3 we go into further detail concerning how we measured that function, and whether the rs12041331 variant significantly affects endothelial function.

At the end of this study, the data derived will be substantial for understanding the pharmacogenomics of clopidogrel and aspirin. The results of the analyses in Chapter 2 will shed light on whether genetic variants known to affect patient response to multiple other drugs are also impactful in clopidogrel response. Should we find a significant association between variants on the DMET array and clopidogrel response, the novel variant will become a part of the clopidogrel pharmacogenomics conversation, where it will be evaluated through replication by other studies for its potential implications in the clinical setting.

By completing the experiments of Chapter 3, we anticipate to identify a novel role for *PEAR1* in cardiovascular biology. This would provide clarity for *PEAR1*'s effect as an aspirin pharmacogene as well as stimulate future studies in aspirin pharmacogenomics focusing on the endothelium. By impacting our understanding of the efficacy and pitfalls

of these regularly used antiplatelet drugs, the findings of this study become another element in the implementation and advancement of personalized medicine.

II. Variant in *UGT2A1/2* is Associated with Sex-Specific Clopidogrel Response

The data presented in this chapter is currently being prepared for submission for publication. Thanks to the EDN PPGM Bioinformatics WebKit by James Perry and Tushar Dave, I was able to formulate the Manhattan plot seen in Figure 2. Also, Mary Pavlovich assisted with the development of Table 1.

A. Introduction

1. Coronary Artery Disease

Each year, heart disease results in the deaths of over 600,000 individuals in the United States and approximately 60% of these mortalities can be attributed to the development of coronary artery disease (CAD).[4,125] CAD is a chronic condition caused by atherosclerosis of the coronary artery, a multi-factorial pathology that develops, in part, from chronic inflammation and cellular deposition of plaque in response to long-term vessel damage, ultimately leading to acute coronary syndrome (ACS). Development and progression of CAD is influenced by several comorbidities including hypertension, hyperlipidemia, hypercholesterolemia, and diabetes as well as non-clinical variables including genetic predisposition and lifestyle habits (e.g. smoking).[8] There have also been observations of associations between rate of cardiovascular disease rate as well as its risk factors and biological sex of the patient, affecting myocardial infarction and left ventricular hypertrophy among other phenotypes.[126] The approximate costs related to CAD in the United States $108.9 billion per year, including hospitalizations, treatments, and loss of productivity.[4,125]

2. Acute Coronary Syndrome Treatment

The mainstay treatment for ACS is vessel revascularization, which often involves the use of percutaneous coronary intervention to physically unblock the obstructed artery, as well as medical treatment using clopidogrel as part of dual-antiplatelet therapy with aspirin.[127] Given its widespread use in the medical management of ACS patients, clopidogrel was the second-most sold therapeutic worldwide in 2011 with sales of over $9 billion reported in 2010.[16] Clopidogrel is a second-generation oral thienopyridine that effectively prevents platelet activation and aggregation.[17] After ingestion, the clopidogrel prodrug is transported to the liver and, in a two-step process mediated by several cytochrome P450 (CYP) enzymes, undergoes biotransformation into its active thiol metabolite. In circulation, the active metabolite irreversibly inhibits platelet $P2Y_{12}$ ADP receptors, causing decreased platelet activation and reduced thrombus formation, ultimately reducing adverse cardiovascular event risk.[19-23]

Despite its general effectiveness, there is wide inter-individual response to clopidogrel therapy. Prior studies have shown that approximately 30% of patients treated with clopidogrel do not respond properly to it resulting in high on-treatment platelet reactivity (HTPR) and a greater risk of experiencing an ischemic event. Furthermore, previous heritability estimates suggest that on-clopidogrel platelet aggregation response is primarily determined by genetic factors ($H^2 = 0.73$).[24,30] Additional factors that influence variation in platelet function in response to clopidogrel include use of statins, calcium channel blockers, proton pump inhibitors, St. John's Wort, and smoking. However, these factors account for only a small fraction of the variation in drug

response.[128-130] Additionally, the sex of the patient has been observed as a factor affecting clopidogrel response, with women shown to have lower response than men.[131,132]

3. Clopidogrel Pharmacogenomics

Given the impact of genetic variation on clopidogrel response, multiple groups including our own have conducted genetic investigations including candidate gene studies and genome-wide association studies (GWAS) in order to identify polymorphisms that significantly influence clopidogrel efficacy and safety. The most consistent result from these investigations is that a loss-of-function variant in the *CYP2C19* gene (*CYP2C19**2, rs4244285) significantly reduces formation of the clopidogrel active metabolite resulting in HTPR and an increase in recurrent cardiovascular events. Given the impact of this variant, the clopidogrel label was updated in 2010 by the United States Food and Drug Administration (FDA) warning clinicians that poor metabolizers of clopidogrel are at increased risk of experiencing a cardiovascular event, that genetic variation in CYP2C19 influences clopidogrel metabolism, and that genetic testing can be used as an aid in determining therapeutic strategy. It is important to note, however, that despite its significant effect size the *CYP2C19**2 variant accounts for 12% of the heritability in clopidogrel response indicating that more variants that significantly impact clopidogrel response remain unidentified.[30]

While the use of GWAS is effective at identifying common genetic variants that influence a particular phenotype, this agnostic methodology is prone to missing high effect size variants with low minor allele frequencies. In this investigation, we extend on our prior work and pursue our hypothesis that other impactful variants affecting clopidogrel response are left to be discovered, and that those variants are likely to be

known pharmacokinetic and pharmacodynamic variants for other drugs, by evaluating candidate variants that on the Drug Metabolizing Enzyme and Transporter (DMET) chip (Affymetrix, Santa Clara, California). The DMET chip is a genotyping panel comprised of 1936 variants in 231 genes critical to drug metabolism and transport, we evaluated the genetic determinants of clopidogrel response in 688 participants of the Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study in order to identify novel genetic variants that influence drug efficacy.

B. Materials and Methods

1. Study Population

The Amish Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study (NCT0079936) included 688 subjects recruited from the Old Order Amish (OOA) community of Lancaster, Pennsylvania. Population characteristics, recruitment, and study details have been described previously.[30,69,108] Briefly, all participants were Caucasian and age 20 years or older. Data obtained included medical and family histories, anthropometric measures, physical examinations, and blood samples following overnight fasts. Quest Diagnostics (Horsham, Pennsylvania) was used for measurements of platelet count and serum lipids, including total cholesterol, high-density lipoprotein cholesterol (HDL), and triglycerides. Low-density lipoprotein cholesterol (LDL) was calculated using the Friedewald equation.[133] LDL-cholesterol levels greater than 160 mg/dL and/or use of prescription cholesterol-lowering medications was used to define hyperlipidemia. Subjects were designated as hypertensive if SBP > 140 mm Hg, and/or DBP > 90 mm Hg, and/or they were using prescription blood pressure lowering medications. Diabetes and current smoking status (cigarette, cigar, or pipe) was obtained by self-report.

Following discontinuation of all drugs and supplements for 7 days, baseline platelet aggregation measurements were recorded for each participant. Following these measurements, participants received an oral loading dose of 300 mg clopidogrel, and were instructed to take a maintenance dose of 75 mg clopidogrel per day for the following 7 days. On day 8, blood samples were drawn approximately 2 hours after the last clopidogrel dose for the assessment of on-clopidogrel platelet aggregation.

2. Platelet Aggregation Testing

Platelet aggregation was evaluated in platelet-rich plasma (PRP) (200,000 platelets/μl) isolated from blood samples by optical aggregometry using a PAP8E Aggregometer (Bio/Data Corporation, Horsham, Pennsylvania). All platelet aggregation measurements were expressed as a percent maximum aggregation via light transmittance aggregometry of PRP stimulated by 20 μmol/L ADP, with platelet-poor plasma (PPP) as a referent. Further details regarding this methodology have been described previously.[30]

3. Genotyping

All participants of the PAPI study were genotyped using the Affymetrix Drug Metabolizing Enzyme and Transporters (DMET) array according to the manufacturer's instructions (Affymetrix Inc, Santa Clara, California). Genotype calls were determined using the DMET Console software which is based on the Bayesian Robust Linear Model with Mahalanobis distance classifier (BRLMM) algorithm. Samples with a high missing rate (N = 55 samples) were removed and the final data set had an average call rate per sample of 99.68%. The mean genotype across all single nucleotide polymorphisms (SNPs) was 99.3%.

4. Statistical Analysis

Summary statistics for the OOA population were generated using SAS 9.2 (SAS Institute Inc, Cary, North Carolina). The heritability of post-clopidogrel platelet aggregation as well as its correlation with other clinical measurements, including age, sex, body mass index (BMI), smoking, diabetes, lipid levels (total cholesterol, HDL, LDL), log-transformed triglycerides, systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), heart rate (HR), and hypertension were

assessed using Mixed Model program for Analysis of Pedigree (MMAP) as previously reported in the original cohort of 429 individuals. [30,134] All correlations were age- and sex-adjusted except in analyses specifically estimating the effect sizes of these variables. Analyses of heritability and clinical correlates were considered significant if P-values were less than 0.05. All statistical tests were two-sided.

The primary outcome of this investigation was change in ADP-stimulated platelet aggregation after clopidogrel administration (i.e. baseline platelet aggregation minus post-clopidogrel platelet aggregation). Association analyses between SNPs on the DMET array and clopidogrel response were performed under an additive variance component model that examines the impact of genotype on ADP-stimulated platelet aggregation. This method also estimates the effects of covariates in the model, including age, sex, other traits included in post-hoc analysis due to significant correlation with clopidogrel response, and relatedness among study participants via a polygenic component as a random effect. The polygenic component was determined using a relationship matrix derived from full OOA pedigree available in published genealogical records maintained by the OOA church.[135] Our analysis included only SNPs that were polymorphic in the OOA and relatively common (minor allele frequency [MAF] $\geq$ 0.01), resulting in a total of 673 SNPs being analyzed in this investigation. A Bonferroni-corrected P-value threshold of 7.43×10^{-5} was used to define significance (0.05 / 673 SNPs).

Power calculations were performed using QUANTO and estimated that this study population (N = 688) has 80% power to detect SNPs with MAFs of 0.025 to 0.25 and effect sizes of 4.2 and 11.8 percent maximum aggregation, which would account for 3% of the variance in clopidogrel response.[136,137]

C. Results

1. Population Descriptors

The participants of the PAPI Study were healthy, normotensive, middle-aged individuals with, on average, slight hypercholesterolemia and overweight BMIs (Table 1). Clopidogrel response had wide inter-individual variability and was normally distributed (Figure 1). The even distribution of this trait did not allow for a clear cutoff to define clopidogrel resistance. The heritability of clopidogrel response after stimulation with 20 μmol/L was estimated at 39.4% ($P = 1.15 \times 10^{-6}$). After examining the correlations between clopidogrel response and common clinical measurements, greater clopidogrel response was significantly associated with lower age (0.44% of the variance, $P = 0.04$), lower BMI (2.35% of the variance, $P = 4.60 \times 10^{-4}$), lower self-reported diabetes (1.24% of the variance, $P = 0.01$), lower LDL (0.66% of the variance, $P = 0.01$), lower total cholesterol (0.58% of the variance, $P = 3.44 \times 10^{-3}$), lower triglycerides (3.06% of the variance, $P= 3.26 \times 10^{-6}$), lower DBP (1.24% of the variance, $P = 0.01$), lower SBP (2.24% of the variance, $P = 5.39 \times 10^{-4}$), and lower MAP (1.93% of the variance, $P = 1.00 \times 10^{-3}$) (Table 2).

Table 1: Characteristics of PAPI Study Participants

Characteristic (Units)	Men	Women
Number (n)	341	346
Age ± SD (years)	43.50 ± 12.98	46.46 ± 13.79
BMI ± SD (kg/m^2)	25.95 ± 3.63	28.22 ± 5.41
Systolic blood pressure ± SD (mm Hg)	116.89 ± 11.6	117.58 ± 13.94
Diastolic blood pressure ± SD (mm Hg)	70.85 ± 7.43	69.88 ± 7.45
No. with hypertension (%)*	17 (4.99)	23 (6.65)
Total cholesterol ± SD (mg/dl)	206.21 ± 43.56	214.72 ± 51.29
LDL cholesterol ± SD (mg/dl)	137.32 ± 40.14	137.69 ± 47.51
HDL cholesterol ± SD (mg/dl)	55.24 ± 14.66	62.05 ± 15.20
Triglycerides ± SD (mg/dl)†	68.39 ± 39.82	74.8 ± 41.86
No. with hypercholesteremia (%)‡	80 (23.46)	84 (24.28)
No. with self-reported diabetes (%)	3 (0.88)	2 (0.58)
Hematocrit ± SD (%)	41.67 ± 2.4	37.68 ± 2.27
White blood cell count ± SD (n x 1000)	6.07 ± 1.49	6.09 ± 1.42
Platelet count ± SD (n x 100,000)	234.57 ± 45.53	245.21 ± 51.81
No. of current smokers (%)§	69 (20.23)	0 (0)
No. taking aspirin (%)	6 (1.76)	3 (0.87)
No. taking lipid-lowering medications (%)	3 (0.88)	3 (0.87)
No. taking anti-hypertensive medications (%)	1 (0.29)	0 (0)

Abbreviations: BMI, body mass index; PAPI, Pharmacogenomics of Anti-Platelet Intervention; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation.
SI conversion factors: To convert HDL-cholesterol, LDL-cholesterol, and total cholesterol values to mmol/L, multiply by 0.0259; triglycerides to mmol/L, multiply by 0.0113.
*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.
†Logarithm-transformed for analysis and back-transformed for presentation.
‡Defined as LDL-cholesterol greater than 160 mg/dl or taking prescription medication for previously diagnosed hypercholesterolemia.
§Self-reported history of smoking cigarette, pipe, or cigar.
Only men report smoking in the OOA community.

On following page – **Figure 1**: Distribution of Clopidogrel Response. Measured in 688 Amish participants of the Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study.

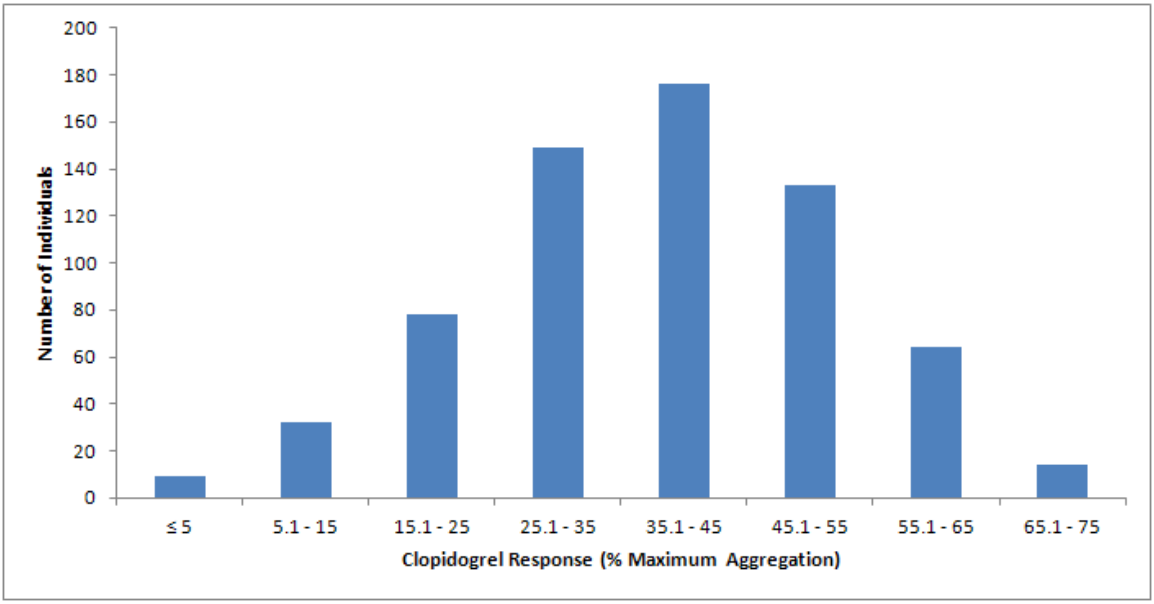

Table 2: Predictors of Variance in Clopidogrel Response in PAPI Study Participants.

Predictor (Units)	Beta	Standard Error	P-value	Variance Explained
Age (years)	-0.09	0.04	0.04	0.44%
Sex (female)	0.47	1.12	0.68	
BMI (kg/m^2)	-0.43	0.12	4.60×10^{-4}	2.35%
Current smoking (%)*	-1.83	1.91	0.34	
Self-reported diabetes (%)	-23.8	9.9	0.02	0.62%
HDL cholesterol (mg/dl)	0.06	0.04	0.14	
LDL cholesterol (mg/dl)	-0.04	0.01	6.24×10^{-3}	0.66%
Total cholesterol (mg/dl)	-0.04	0.01	3.44×10^{-3}	0.58%
Triglycerides (mg/dl)	-0.07	0.01	3.26×10^{-6}	3.06%
Diastolic blood pressure (mm Hg)	-0.23	0.08	6.94×10^{-3}	1.24%
Systolic blood pressure (mm Hg)	-0.17	0.05	5.39×10^{-4}	2.24%
Mean arterial pressure (mm Hg)	-0.25	0.07	1.00×10^{-3}	1.93%
Heart rate (beats/min)	-0.09	0.08	0.25	
Hypertension (%)	-0.98	2.84	0.73	
rs11249454 genotype (G allele)	-10.27	2.44	2.92×10^{-5}	2.70%

All correlations are age- and sex-adjusted, except for age and sex.
Abbreviations: BMI, body mass index; PAPI, Pharmacogenomics of Anti-Platelet Intervention; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
*Observed only in men to account for the OOA community's male-only smoking cohort.
Variance explained was only calculated for traits with significant correlations to clopidogrel response.

2. Association Analyses

Consistent with previous investigations, we observed strong evidence of association between clopidogrel response and the *CYP2C19**2 variant (rs4244285; β = 7.93 ± 1.08; P = 5.34 x 10^{-13}). When examining association between clopidogrel response and the previously reported clopidogrel response variant *ABCB1* C3435T (rs1045642), there was no significant association observed.[30,138] We observed a significant novel association between clopidogrel response and rs11249454, an intronic SNP in UDP glucuronosyltransferase 2A1 and 2A2 (*UGT2A1/2*) (Figure 2). The rs11249454 minor

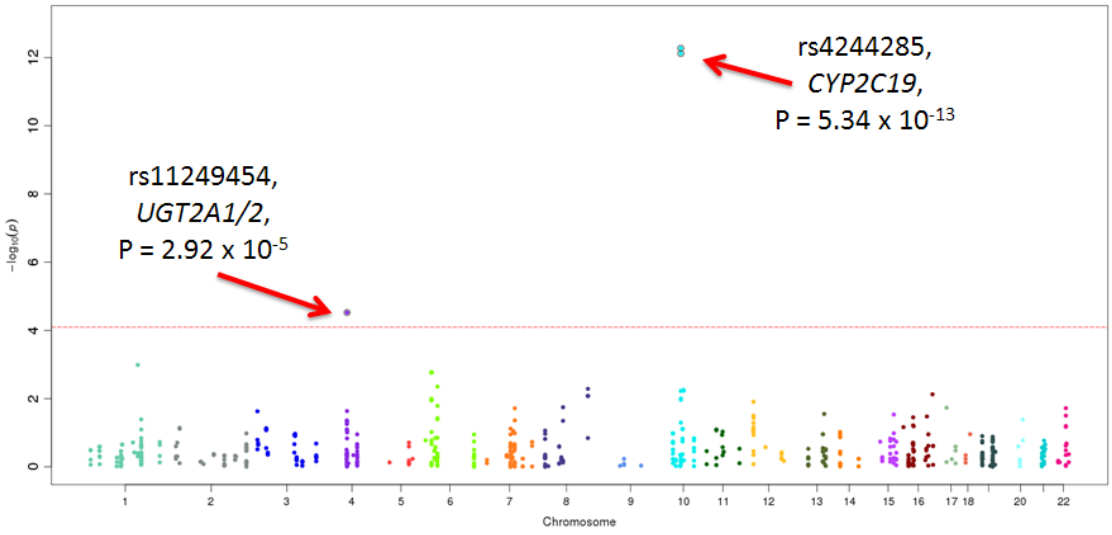

Figure 2: DMET Manhattan Plot. Plot of associations between DMET SNPs and change in platelet aggregation between baseline and post-clopidogrel measurements.

allele (G) had a frequency of 3.1% in this population (607 major allele homozygotes ([AA], 40 heterozygotes [AG], and 0 minor allele homozygotes [GG]), and adhered to Hardy-Weinberg equilibrium (P = 0.45). The results of our association analysis, adjusting for age, sex, and relatedness among the OOA, revealed a significant effect of rs11249454 genotype on clopidogrel response (β = -10.27 ± 2.44; P = 2.92 x 10^{-5}) (Figure 3)

indicating that the G-allele is associated with decreased clopidogrel response compared to AA homozygotes. Based on the results of the correlation analyses described above, traits found to be significantly correlated with clopidogrel response were included as covariates in a post-hoc association analysis in order to account for effects they may have on the association analysis of rs11249454. The additional covariates used in this analysis included BMI, self-reported diabetes, LDL, total cholesterol, triglycerides, SBP, DBP, and MAP, and the results of the analysis were similar to the minimally adjusted model ($\beta = -9.75 \pm 2.41$; $P = 5.80 \times 10^{-5}$).

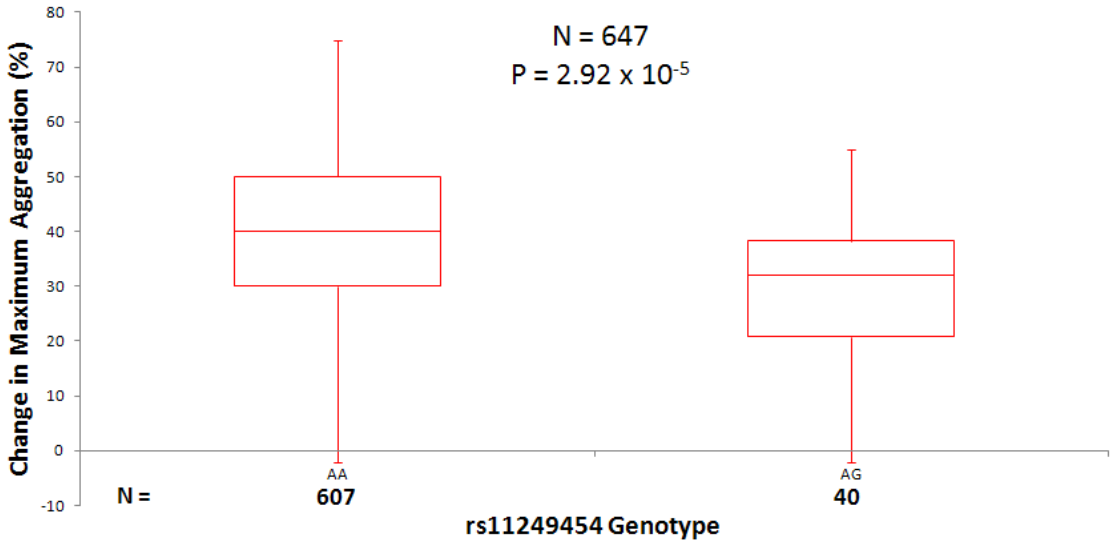

Figure 3: UGT2A1/2 Association Box Plot. Box plot of age- and sex-adjusted association in PAPI between rs11249454 and change in platelet aggregation between baseline and post-clopidogrel measurements.

3. Sex-Stratified Results

Because of *UGT2A1/2*'s role in sex hormone metabolism and the reports of disparate antiplatelet therapy response and cardiovascular risk across sexes,[126,139-145] we also performed sex-stratified association analyses between rs11249454 and clopidogrel

response. Interestingly, these analyses suggest that the association signal observed in the full PAPI population is driven entirely by women ($\beta = -13.66 \pm 3.02$; $P = 8.40 \times 10^{-6}$), while no association between rs11249454 and clopidogrel response were observed in men ($\beta = -4.68 \pm 3.75$; $P = 0.21$) (Figure 4). To confirm the sex-specific manner in which rs11249454 genotype seems to mediate its effect on clopidogrel response, we performed a gene-by-sex interaction analysis. The results of this analysis showed sex as a significant

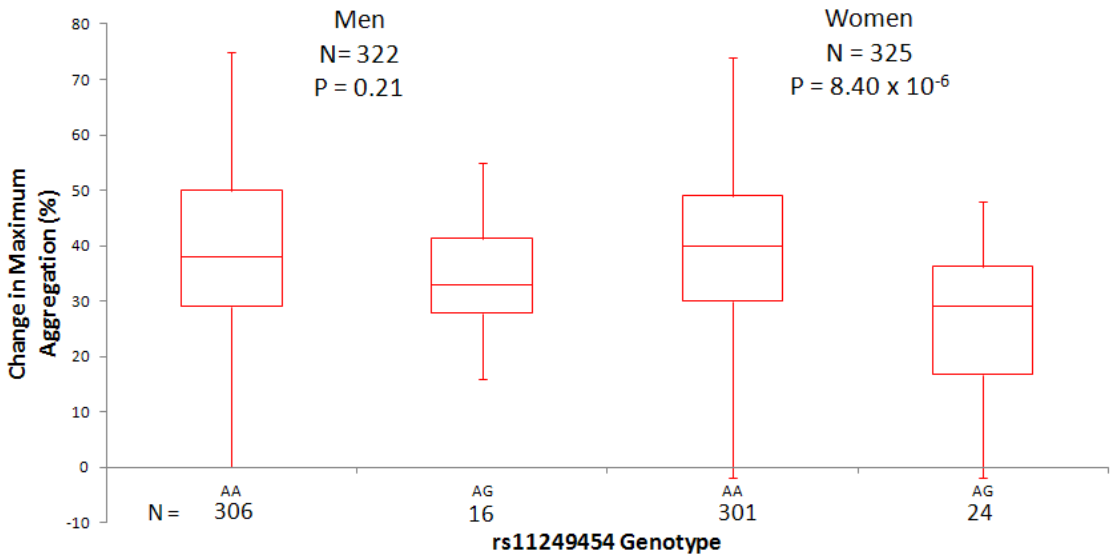

Figure 4: Sex-stratified UGT2A1/2 Box Plot. Box plot of sex-stratified, age-adjusted association in PAPI between rs11249454 and change in platelet aggregation between baseline and post-clopidogrel measurements. Gene-by-sex interaction analysis P-value = 0.049.

interaction term ($P = 0.049$) for the association between rs11249454 and clopidogrel response. After adjusting for additional clinical variables that influence clopidogrel response (BMI, self-reported diabetes, HDL, triglycerides, MAP, DBP, and SBP) in each sex independently, the effect of rs11249454 genotype on clopidogrel response was similar to models that did not contain these covariates ($\beta = -12.63 \pm 3.00$; $P = 3.40 \times 10^{-5}$)

(Table 3), and men (LDL, SBP, total cholesterol, and triglycerides-adjusted, $\beta = -5.16 \pm 3.68$; $P = 0.16$) (Table 4).

Table 3: Predictors of Variance in Clopidogrel Response in Male PAPI Study Participants.

Predictor (Units)	Beta	Standard Error	P-value	Variance Explained
Age (years)	-0.1	0.06	0.11	
BMI (kg/m^2)	-0.35	0.21	0.1	
Current smoking (%)	-1.8	2.02	0.37	
HDL cholesterol (mg/dl)	-0.02	0.05	0.7	
LDL cholesterol (mg/dl)	-0.06	0.02	6.71×10^{-3}	2.10%
Total cholesterol (mg/dl)	-0.06	0.02	2.25×10^{-3}	2.49%
Triglycerides (mg/dl)	-0.04	0.02	0.04	1.13%
Diastolic blood pressure (mm Hg)	-0.13	0.13	0.3	
Systolic blood pressure (mm Hg)	-0.18	0.07	0.02	2.30%
Mean arterial pressure (mm Hg)	-0.19	0.11	0.08	
Heart rate (beats/min)	-0.07	0.11	0.51	
Hypertension (%)	0.64	4.75	0.89	
rs11249454 genotype (G allele)	-4.68	3.75	0.21	

Correlations for all traits are age-adjusted, except for age.
Abbreviations: BMI, body mass index; PAPI, Pharmacogenomics of Anti-Platelet Intervention; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
Variance explained was only calculated for traits with significant correlations to clopidogrel response.

Table 4: Predictors of Variance in Clopidogrel Response in Female PAPI Study Participants.

Predictor (Units)	Beta	Standard Error	P-value	Variance Explained
Age (years)	-0.06	0.06	0.26	
BMI (kg/m^2)	-0.51	0.15	7.06×10^{-4}	3.57%
Self-reported diabetes (%)	-22.59	9.99	0.02	1.08%
HDL cholesterol (mg/dl)	0.16	0.05	2.51×10^{-3}	3.18%
LDL cholesterol (mg/dl)	-0.01	0.02	0.49	
Total cholesterol (mg/dl)	-0.01	0.02	0.63	
Triglycerides (mg/dl)	-0.09	0.02	9.11×10^{-6}	5.44%
Diastolic blood pressure (mm Hg)	-0.33	0.12	4.32×10^{-3}	2.23%
Systolic blood pressure (mm Hg)	-0.2	0.07	2.99×10^{-3}	2.73%
Mean arterial pressure (mm Hg)	-0.32	0.1	1.68×10^{-3}	2.89%
Heart rate (beats/min)	-0.1	0.11	0.38	
Hypertension (%)	-3.88	3.68	0.29	
rs11249454 genotype (G allele)	-13.66	3.02	8.40×10^{-6}	5.98%,

Correlations for all traits are age-adjusted, except for age.
Abbreviations: BMI, body mass index; PAPI, Pharmacogenomics of Anti-Platelet Intervention; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
*Observed using a sex-stratified analysis to account for the OOA community's male-only smoking cohort.
Variance explained was only calculated for traits with significant correlations to clopidogrel response.

D. Discussion

In this study we used DMET array genotyping to explore associations between clopidogrel response and known pharmacogenomic variants. We found the heritability of clopidogrel response to be 39%, lower than some estimates in the literature, which might be explained by our use of a slightly different trait, namely clopidogrel response relative to baseline rather than post-clopidogrel platelet aggregation.[30,146] Also, we observed significant correlations between clopidogrel response and common clinical traits with cardiovascular implications, including age, BMI, DBP, self-reported diabetes, LDL, triglycerides, MAP, SBP, and total cholesterol. Before exploring novel associations of DMET SNPs with drug response, we confirmed the association of clopidogrel response with *CYP2C19**2, which has been previously reported to affect post-clopidogrel platelet aggregation in both men and women, while failing to see an association between clopidogrel response and *ABCB1* C3435T, a variant with mixed reports of significance in affecting post-clopidogrel platelet aggregation.[64,65,138,147,148] For the first time, we observed a significant association between a novel variant, *UGT2A1/2* rs11249454, and clopidogrel response ($P = 2.92 \times 10^{-5}$), as measured by change between baseline and post-clopidogrel platelet aggregation. Interestingly, we also report that the rs11249454 variant appears to exert its effect on clopidogrel response in a sex-specific manner, with women maintaining a significant association ($P = 8.40 \times 10^{-6}$) and men having no significant contribution ($P = 0.21$) after cohort stratification by sex. This is consistent with the significant gene by sex interaction term ($P = 0.049$) when added into the model.

The rs11249454 variant is in the first intron of the *UGT2A1/2* genes on chromosome 4q13. The SNP is intronic for both genes because *UGT2A1* and *UGT2A2*

are splice variants, sharing identical exons 2-6 at the 3' end, and differing only in the 1st exon at the 5' end. These two genes also share some similarities in expression profiles, including expression in nasal epithelia, trachea, and larynx.[149-153] The difference between genes expression levels indicate that *UGT2A1* is found in the lung, tonsil, colon, and minimally in the liver, while *UGT2A2* is expressed in the breast, kidney, and small intestine.[150-152,154]

Both genes code for endoplasmic reticulum-bound detoxifying enzymes that mediate the excretion of various compounds by addition of UDP-glucuronic acid adducts, with *UGT2A1* having higher levels of glucuronidation for most compounds compared to *UGT2A2*.[155,156] Among exogenous compounds, the two enzymes have been primarily shown to metabolize indomethacin, bisphenols, phenol odorants, and tobacco carcinogens, specifically polycyclic aromatic hydrocarbons, which follow logically with the genes' expression in the nasal epithelia, lung, trachea, and larynx.[152,157,158] In addition to exogenous compounds, reports have shown these enzymes have a role in bile acid glucuronidation and, most pertinent to the sexually dimorphic trends we observed in our data, the metabolism of sex hormones, including both androgens and estrogens.[139-145,159]

The role of *UGT2A1* and *UGT2A2* in sex hormone metabolism is complex, with a homeostatic role that both influences and is influenced by sex hormone levels. *UGT2A1* and *UGT2A2* affect sex hormone levels through their roles in the metabolism of natural androgens, including testosterone, epitestosterone, adrosterone, and etiocholanolone, *ent*-androgens including *ent*-etiocholanolone, and estrogens including estradiol (17β-estradiol) and epiestradiol (17α-estradiol).[139,142-144] In contrast, there is evidence regarding the stimulation of *UGT2A1* expression by the presence of sex hormones and

similar compounds. This includes the increased expression of *Ugt2a1* in adult rare minnow livers following 17α-methyltestosterone, and the increased expression of *UGT2A1* in women with early-stage invasive breast cancer following soy supplementation. The recorded effect of soy supplementation is most likely caused by the genistein in soy that functions as an estrogen analog and can transactivate estrogen receptors.[140,160] *UGT2A1* expression seems to also be induced by a wider range of molecules, including steroids like dexamethasone, and non-steroidal compounds such as triazole anti-fungals, observed in male rat livers.[145,161]

Because of the inherent differences in hormonal composition across sexes and the role of UGTs in metabolizing sex hormones, it is not surprising that other groups have observed sex-specific observations as well. For instance, it has been shown that *Ugt2a1* expression occurs predominantly in the nasal epithelia of female rats in comparison to males.[162] In addition, investigations have shown that variants in other UGTs (i.e. *UGT2B15* and *UGT1A1*) are significantly associated with circulating sex hormone-binding globulin concentrations and gallstone disease risk, respectively, in men only.[163,164] Furthermore, sex-specific effects by one gene have been observed in both sexes, such as the deletion of *UGT2B17*, a prominent testosterone metabolizer, being significantly associated with decreased BMI in men and increased risk for lung adenocarcinoma in women.[165,166]

Our observations from this study regarding the effect of *UGT2A1/2* rs11249454 genotype on clopidogrel response become biologically plausible when considering data in the literature regarding sex hormones and platelet aggregation. Estrogen receptors have been confirmed to be present on the platelet surface, but the observed effects of estrogen

on platelet aggregation have been mixed across studies. While some data indicate that estrogen synergizes with known platelet agonists, e.g. thrombin, to increase platelet aggregation, others' data suggest estrogen lowers the amount of platelet aggregation observed.[167-169] Further experiments, beginning with the determination of how rs11249454 affects *UGT2A1/2* function, could help clarify how altered estrogen metabolism caused by *UGT2A1/2* rs11249454 could affect platelet aggregation. In contrast to its role in estrogen metabolism, the sex-specific effect we have observed in women may be relevant to the testosterone metabolizing role of UGT2A1 and UGT2A2. In one study, a group found testosterone level was directly correlated with expression of the ADP $P2Y_{12}$ receptor in DAMI cells, a type of megakaryocytic cell line.[170] This would imply, based on the observations in our study, that women with increased testosterone, perhaps caused by a loss-of-function effect of rs11249454, may have increased $P2Y_{12}$ receptors on their platelet surfaces and thus decreased clopidogrel response in comparison to their lower-testosterone female counterparts. While connections between both sex hormones and platelet aggregation are intriguing, it is evident that understanding the direction of effect of these hormones on platelet aggregation, and the direction of effect of rs11249454 on the function of both enzymes, are of paramount importance to fully understand the nature of our observations.

The data we have presented in this investigation suggest a novel sex-specific effect of *UGT2A1/2* rs11249454 genotype on clopidogrel response. Based on the molecular function of these genes, there are many mechanisms by which these genes could be exerting their effect on clopidogrel response in women only, including through their role in sex hormone metabolism, their reciprocal induction of expression by sex

hormones, and sex-specific expression in certain tissues. Taking prior data involving sex disparities in baseline and on-clopidogrel platelet reactivity as well as sex hormones' roles in platelet aggregation, and combining it with our data regarding the effect of rs11249454 genotype on clopidogrel response in a sex-specific manner creates an interesting connection with hypothesis-generating potential.[131,167,171,172] Future studies are warranted for the confirmation of rs11249454 as the causative variant of this effect, since linkage disequilibrium between the intronic rs11249454 and exonic variants that directly alter protein function could cause the observed effects. Furthermore, examination of the association of this SNP with cardiovascular endpoints is necessary to further elucidate whether these findings are of clinical importance.

III. Genetic Variation in the Platelet Endothelial Aggregation Receptor 1 Gene Results in Endothelial Dysfunction

The work presented in this chapter has been submitted for publication and is currently under review. All data coming from GAMMA analyses was performed by our collaborator Dr. Jonathan Wren, to whom we are greatly appreciative. Dr. Laura Yerges-Armstrong was instrumental in teaching me how to acquire the data in Table 10, and Kathy Ryan as well as Mary Pavlovich both assisted me in developing Tables 9 and 11.

A. Introduction

1. PEAR1

Platelet endothelial aggregation receptor 1 (PEAR1; also known as JEDI and MEGF12) is a recently identified transmembrane receptor expressed in a number of different tissues, but most highly in endothelial cells and megakaryocytes.[100] While little is currently known regarding this receptor, prior investigations suggest that PEAR1 is important in a diverse range of biological functions, including sustained platelet aggregation through glycoprotein αIIbβ3, altered megakaryopoiesis and thrombopoiesis via PI3K/PTEN pathways, and apoptotic neuron clearance through endocytosis-dependent activities in dorsal root ganglia.[101-103] Several studies have examined the role of genetic variation in *PEAR1*, most notably the intronic single nucleotide polymorphism (SNP) rs12041331. These studies have implicated rs12041331 genotype in differential *PEAR1* expression; in platelets the minor A-allele is associated with decreased expression and lower platelet aggregation, both at baseline and in the presence of therapeutic agents such as aspirin and prasugrel.[105-108,122,173,174] However, a seemingly paradoxical effect of the rs12041331 A-allele on cardiovascular phenotypes has been observed whereby this

allele is associated with better aspirin response as measured by platelet function testing, but higher cardiovascular event rates in patients with coronary artery disease on aspirin, potentially suggesting an alternative role for PEAR1 in cardiovascular disease progression.[108]

2. Global Microarray Meta-Analysis and Follow-Up Experimentation

In an attempt to further define the role of PEAR1 in various biological and disease networks, we used a bioinformatics approach called GAMMA (Global Microarray Meta-Analysis) to identify genes consistently correlated with *PEAR1* expression across 75,000 human 1-color microarray experiments from within the publicly available datasets in National Center for Biotechnology Information's Gene Expression Omnibus.[175] Based on the results of GAMMA, we extended our findings by evaluating the effect of the *PEAR1* rs12041331 variant on endothelial cell migration using *ex vivo* assays of human umbilical vein endothelial cells (HUVECs). Finally, we clinically tested the impact of rs12041331 on endothelial function through assessment of flow-mediated dilation (FMD) of the brachial artery in 641 participants of the Heredity and Phenotype Intervention (HAPI) Heart Study.

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B. Materials and Methods

1. Global Microarray Meta-Analysis (GAMMA)

GAMMA was used to compare transcriptional co-expression across 75,000 publicly available microarrays from National Center for Biotechnology Information Gene Expression Omnibus in order to identify genes highly correlated with *PEAR1* across a broad range of experimental conditions.[175] One-color microarrays were processed to create a single gene expression used to perform meta-analysis. Microarrays were quantile-normalized and processed with an automated quality checking process that included comparison of parametric expression distributions of individual experiments to expected distributions. Unlike traditional meta-analytic approaches which evaluate genes under specific or similar experimental conditions, GAMMA utilizes heterogeneous conditions in order to generate global co-expression patterns to more accurately identify common biological responses. The set of genes highly correlated with *PEAR1* can then be queried for statistically significant biological associations they may share. This approach is useful for genes with little or no annotation. Because the use of Gene Ontology for this purpose has fallen under recent criticism and Gene Ontology covers only biological processes, molecular functions, and cellular components, we also used literature mining to identify published commonalities for the most highly correlated genes; this included other categories such as disease relevance, phenotype, and other genes predicted to be relevant to *PEAR1*'s genetic neighborhood.[176,177] GAMMA's performance has been benchmarked both by comparison of predicted annotations to known annotations and has been validated experimentally in several studies whereby predicted phenotypes were tested experimentally.[175,178-182]

2. Cell Culture

Fifty-five de-identified umbilical cords were obtained from the University of Maryland Medical Center Division of Maternal and Fetal Medicine and HUVECs were harvested as described previously.[183] HUVECS were maintained at 37^0C in a 5% CO_2 incubator using Endothelial Basal Media 2 (Lonza, Catalog #CC-3156 & CC-4176, Walkersville, Maryland) 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. DNA from each cell line was extracted using a Gentra Puregene Cell Kit (Qiagen, Valencia, California) as recommended by the manufacturer. *PEAR1* rs12041331 genotype was determined using a TaqMan SNP genotyping assay (Applied Biosystems/Life Technologies, Foster City, California), which resulted in the identification of 28 major allele homozygotes (GG), 25 heterozygotes (GA), and 2 minor allele homozygotes.

3. Endothelial Cell Migration Assay

We evaluated the impact of *PEAR1* rs12041331 genotype on *ex vivo* endothelial cell migration using methods described previously.[184] Briefly, 10 primary HUVEC lines (4 rs12041331 major allele homozygotes [GG], 4 heterozygotes [GA], and 2 minor allele homozygotes [AA]) were split at passage 3 onto a gelatin-coated 6-well plate containing Endothelial Basal Media 2. Confluent HUVEC monolayers were scraped in a uniform manner using a P1000 pipette tip to generate a scratch, which was followed by replacement of growth media. Cells were photographed at 0 and 6 hours using an Axiocam MRc 5 camera (Carl Zeiss Microscopy, Pleasanton, California) mounted on a Lumar.V12 microscope (Carl Zeiss Microscopy, Pleasanton, California). The area of the

scratch was measured using ImageJ and used to calculate endothelial cell migration as described in the Statistical Analysis section.[185] All measurements were made blinded to *PEAR1* genotype.

4. HAPI Heart Study Participants

The HAPI Heart Study recruited 868 healthy Old Order Amish (OOA) participants aged 20 years or older from 2003 to 2006 as previously described.[186] This report evaluates 641 HAPI Heart Study participants in whom brachial artery FMD measurements were recorded. Briefly, all study participants discontinued the use of medications, vitamins, and supplements 7 days prior to their initial clinic visit. Physical examinations, anthropometric measures, medical and family histories, and other phenotype information were collected at the Amish Research Clinic in Lancaster, Pennsylvania after an overnight fast. Individuals were excluded if any of the following criteria were met: pregnancy, coexisting malignancy, severe hypertension (blood pressure > 180/105 mmHg), serum creatinine > 2.0 mg/dl, AST or ALT greater than twice the upper limit of normal, hematocrit < 32%, TSH < 0.4 or > 5.5 mIU/l, or inability to safely discontinue prescription and nonprescription medications.

Complete blood count and serum lipid concentrations were assayed by Quest Diagnostics (Horsham, Pennsylvania), and levels of LDL-cholesterol were calculated using the Friedewald equation. Any participant with an LDL-cholesterol greater than 160 mg/dl or taking prescription cholesterol-lowering medications was designated hyperlipidemic. Individuals were described as hypertensive if they had one or more of the following criteria: systolic blood pressure (SBP) ≥ 140 mmHg, diastolic blood pressure (DBP) ≥ 90 mmHg, or requirement of prescription blood pressure lowering medications.

Diabetes and current smoking status (cigarette, cigar, or pipe) were obtained by self-report.

Study protocols were approved by the Institutional Review Board at the University of Maryland School of Medicine. Written informed consent was obtained from each HAPI Heart Study participant.

5. Flow-Mediated Dilation (FMD)

Assessment of endothelial function was evaluated by FMD of the brachial artery. All brachial artery measurements were obtained after an overnight fast. Briefly, the subject's left arm was immobilized in the extended position and the left brachial artery was imaged above the antecubital fossa in the longitudinal plane by continuous 2D gray-scale imaging with an 11 MHz ultrasound (HDI 5000CV [Phillips, Andover, Massachusetts]) by a trained sonographer. A baseline rest image was acquired and blood flow was estimated by time-averaging the pulsed Doppler velocity signal obtained from a mid-artery sample volume. Arterial occlusion was created by cuff inflation to suprasystolic pressure (50 mmHg above systolic pressure) for 5 minutes, after which the cuff was deflated. The longitudinal image of the artery was recorded continuously from 30 seconds before to 2 minutes after cuff deflation. Flow images were captured on videotape, and read in a blinded fashion. From longitudinal images, the boundaries for diameter measurement were identified manually with electronic calipers at the lumen-intima interface. Five evenly spaced arterial diameter measurements were taken within a 5 cm segment of vessel at baseline and one minute after cuff deflation, and averaged for the brachial artery width measurement.

6. Genotyping

PEAR1 rs12041331 SNP genotyping in HAPI Heart Study participants was performed using a TaqMan SNP genotyping assay (Applied Biosystems/Life Technologies, Foster City, California). The mean genotype concordance rate for this polymorphism in a subset of duplicate samples was 100% and the genotype call rate was 98.3%.

7. Statistical Analyses

Statistical analyses implemented by GAMMA have been previously described.[187] Briefly, 1-color microarrays were processed to create a single gene expression matrix in which subsets can be extracted to perform meta-analyses. Microarrays were quantile normalized and noise thresholds were used to identify transcription levels that were statistically significant.

In terms of analyzing the endothelial cell migration assay, HUVEC lines were plated into 6-well plates, and 3 equidistant photographs were taken per well at 0 and 6 hours after scratch generation of the endothelial monolayer, resulting in 18 area measurements per cell line at each time point. Mean endothelial cell migration distance was calculated by dividing the area of the scratch by the height of the frame. Differences in endothelial cell migration distance between *PEAR1* rs12041331 genotype groups were assessed using two-tailed analysis of variance. P-values < 0.05 were considered statistically significant.

For the HAPI Heart Study, summary statistics and frequencies for the OOA HAPI Heart Study were calculated using SAS version 9.2 (SAS Institute Inc., Cary, North Carolina). Measures of Hardy-Weinberg equilibrium were calculated using a χ^2 test. For

HAPI Heart Study-related analyses, P-values less than 0.05 were considered statistically significant. All statistical tests were 2-sided.

Clinical correlates of FMD response were evaluated using a regression-based approach as implemented in SOLAR version 4.07 (Texas Biomedical Research Institute, San Antonio, Texas). Given the unique ancestral history of the Lancaster OOA community, all participants are related and their relationships were accounted for using the extensive genealogical records of the OOA by including a polygenic component as a random effect as previously described.[30,135] Triglyceride levels were logarithm-transformed for analysis and back-transformed for presentation. The relationship between smoking and FMD was only measured in sex-stratified analyses to account for the OOA community's cultural norms that limit smoking to males. Association analyses with *PEAR1* rs12041331 and FMD were performed under an additive model using a variance component method that assesses the effect of genotype on the quantitative trait. Analyses were adjusted for age, sex, body mass index (BMI), diabetes, SBP, DBP, and the aforementioned polygenic component, which was modeled using the relationship matrix derived from the complete OOA pedigree structure available through the Anabaptist Genealogy Database.[135,188] Secondary analyses were adjusted for the same covariates above in addition to baseline brachial artery width (D_{base}) and heart rate (HR) to account for changes in FMD caused by these variables.[189] Heritability of FMD response corresponds to the proportion of the trait variance accounted for by the polygenic component, and the heritability estimate was created with adjustments for age and sex.

C. Results

1. Global Microarray Meta-Analysis

Using the approach implemented in GAMMA, we identified genes that were most significantly associated with *PEAR1* expression (Table 5) and built a genetic neighborhood of protein-protein interactions shared by the co-expressed genes (Figure 5). The *PEAR1* genetic network identified several genes critical to platelet adhesion and aggregation, including von Willebrand factor (*vWF*), thrombospondin 1 (*THBS1*), and plasminogen activator inhibitor 1 (*SERPINE1*), as well as those a variety of vascular functions, including *ACVRL1*, *RhoJ*, and *ENG*.[190-193] The top phenotypes predicted to be affected by changes in *PEAR1* gene expression were "endothelial cell migration," "vasculogenesis," and "angiogenesis" (Table 6). Similarly, the disease most highly predicted to be influenced by alterations in *PEAR1* gene expression was "vascular disease" (Table 6). Full lists of the phenotypes and diseases that predicted to be influenced by *PEAR1* expression are shown in Tables 7 and 8, respectively.

1. Endothelial Cell Migration Assay

We functionally tested whether the well-described *PEAR1* rs12041331 variant significantly influenced endothelial cell migration. Genotypic differences in cell migration distances were assessed at 6 hours post-scratch generation. Consistent with the results of our microarray meta-analysis, we observed that the A-allele of *PEAR1* rs12041331 was significantly associated with better endothelial cell migration capability ($P = 0.04$; 143.8 ± 58.4 μm for the 4 GG cell lines, 153.1 ± 39.8 μm for the 4 GA cell lines, and 168.4 ± 34.8 μm for the 2 AA cell lines [Figure 6]). Moreover, this association remained statistically significant when we

Table 5: Genes with Expression Most Highly Correlated with *PEAR1*

Gene	Full Name	Primary Localization
CDH5	cadherin 5, type 2 (vascular endothelium)	Plasma membrane
ADCY4	adenylate cyclase 4	Plasma membrane
FAM43A	family with sequence similarity 43, member A	Unknown
MFRP	membrane frizzled-related protein	Plasma membrane
C1QTNF5	C1q and tumor necrosis factor related protein 5	Extracellular
TIE1	tyrosine kinase with immunoglobulin-like and EGF-like domains 1	Plasma membrane
COL8A1	collagen, type VIII, alpha 1	Extracellular
HHIP-AS1	HHIP antisense RNA 1	Unknown
LDLRAD2	low density lipoprotein receptor class A domain containing 2	Plasma membrane
FOXC2	forkhead box C2 (MFH-1, mesenchyme forkhead 1)	Nucleus
MGP	matrix Gla protein	Extracellular
WTAPP1	Wilms tumor 1 associated protein pseudogene 1	Unknown
RHOJ	ras homolog family member J	Endosome
ROBO4	roundabout, axon guidance receptor, homolog 4 (Drosophila)	Plasma membrane
ACVRL1	activin A receptor type II-like 1	Plasma membrane
SERPINE1	serpin peptidase inhibitor, clade E, member 1	Extracellular
SULF1	sulfatase 1	Extracellular
MMRN1	multimerin 1	Extracellular
HSPG2	heparan sulfate proteoglycan 2	Extracellular
ECSCR	endothelial cell surface expressed chemotaxis and apoptosis regulator	Plasma membrane
BTBD19	BTB (POZ) domain containing 19	Unknown
HYI	hydroxypyruvate isomerase (putative)	Nucleus
MMP1	matrix metallopeptidase 1 (interstitial collagenase)	Extracellular
VWF	von Willebrand factor	Extracellular
LAMA4	laminin, alpha 4	Extracellular
HHIP	hedgehog interacting protein	Plasma membrane
EDN1	endothelin 1	Extracellular
PTX3	pentraxin 3, long	Extracellular
LOC392536	filamin binding LIM protein 1 pseudogene	Unknown
TGFB1I1	transforming growth factor beta 1 induced transcript 1	Nucleus
BCL6B	B-cell CLL/lymphoma 6, member B	Nucleus
SPHK1	sphingosine kinase 1	Cytoplasm
ENG	endoglin	Plasma membrane
TNFRSF10D	tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	Plasma membrane
VWFP1	von Willebrand factor pseudogene 1	Unknown
MMRN2	multimerin 2	Extracellular
ESM1	endothelial cell-specific molecule 1	Extracellular
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	Extracellular
GLCE	glucuronic acid epimerase	Golgi apparatus
LOC101928281	uncharacterized LOC101928281	Unknown
CTGF	connective tissue growth factor	Extracellular
NR2F2	nuclear receptor subfamily 2, group F, member 2	Nucleus
ITGA5	integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	Plasma membrane
PLSCR4	phospholipid scramblase 4	Plasma membrane
GPR126	G protein-coupled receptor 126	Plasma membrane
THBS1	thrombospondin 1	Extracellular
ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	Nucleus
ROBO3	roundabout, axon guidance receptor, homolog 3 (Drosophila)	Plasma membrane
EGFL7	EGF-like-domain, multiple 7	Extracellular

grouped cells line containing the A-allele (i.e. GA and AA genotypes) and compared them to GG homozygotes (P = 0.048; 143.8 ± 58.4 µm for the 4 GG cell lines and 158.2 ± 38.7 µm for cell lines that carried the A-allele [N = 4]).

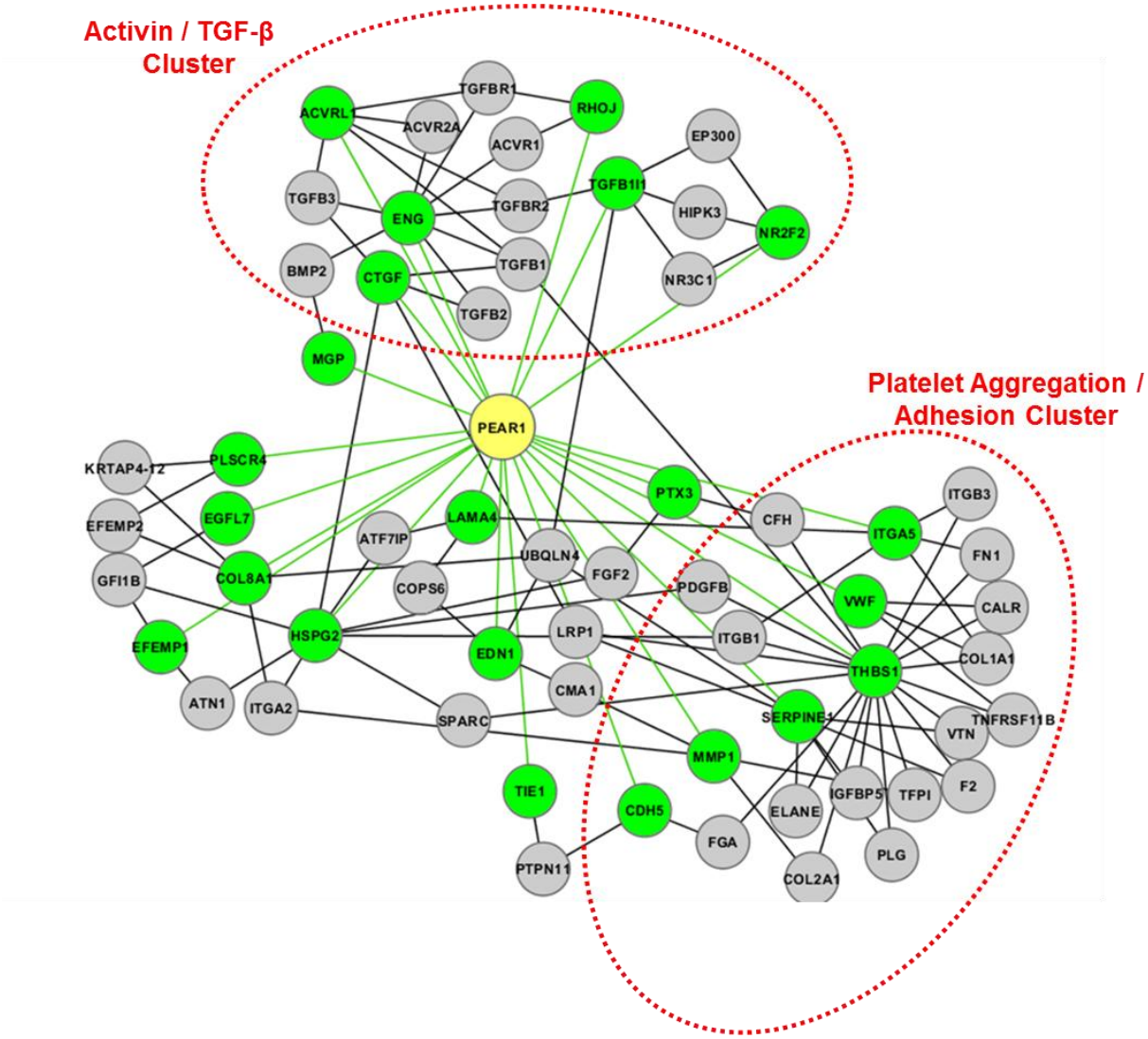

Figure 5: *PEAR1* Genetic Network. Genes highly correlated with *PEAR1* (green nodes) were evaluated for protein-protein interactions (gray nodes) that were shared by at least 2 of the 30 genes analyzed. Green lines indicate a co-expression relationship; black lines indicate a physical protein-protein interaction. Genetic neighborhoods of similar pathway or function have been highlighted and labeled.

Table 6: Meta-Analysis Results of Publicly Available Microarray Datasets

Top predicted phenotypes	# Shared Relations*	Score†
Endothelial cell migration	12	136
Vasculogenesis	11	103
Angiogenesis	20	86
Lymphangiogenesis	8	70
Neovascularization	11	57
Endothelial cell proliferation	9	55
Platelet aggregation	7	55
Cell adhesion	15	52
Top predicted diseases		
Vascular disease	12	59
Non-small cell lung carcinoma	14	55
Osteoarthritis	11	46
Preeclampsia	10	42
Pancreatic cancer	12	40
Diabetic nephropathy	9	39
Colorectal cancer	14	39

*Using the 30 genes most highly correlated with *PEAR1* expression, predicted phenotypes, diseases, and genes were identified with Global Microarray Meta-Analysis (GAMMA), and the number of shared relations represents how many of the 30 genes were related to term (left column). †Score reflects the relative enrichment of observed connections within the analyzed network relative to a random network with the same number of connections per gene, enabling prediction to be prioritized.

2. Flow-Mediated Dilation

Finally, we evaluated the effect of *PEAR1* rs12041331 on *in vivo* endothelial function in 641 subjects of the HAPI Heart Study. Characteristics of the HAPI Heart Study participants are shown in Table 9. Subjects were generally healthy, middle-aged (mean age = 43.2 years), drug-naïve, and had low prevalence of disease (e.g. diabetes [0.78%], hypertension [12.8%], hypercholesterolemia [16.9%]), and obesity [mean BMI =26.3]). FMD was normally distributed in this population (Figure 7). Poorer FMD

Table 7: Most highly predicted phenotypes for *PEAR1* .

Phenotype	# of Shared Relationships	Score
Endothelial cell migration	12	136
Vasculogenesis	11	103
Angiogenesis	20	86
Lymphangiogenesis	8	70
Neovascularization	11	57
Endothelial cell proliferation	9	55
Platelet aggregation	7	55
Cell adhesion	15	52
Paracrine Signaling	13	51
Cell migration	14	51
Embryonic development	14	47
Focal adhesions	9	45
Dermal fibroblasts	9	42
Hypoxia-inducible	10	41
Endothelial cell surface	6	40
Cell motility	10	38
Endothelial cell growth	6	38
Transdifferentiation	7	37
DNA methylation	11	37
Tyrosine phosphorylation	11	36
Leukocyte extravasation	5	36
Signal transduction	15	36
Retinal endothelial cell	5	35
Carotid artery	11	34
Chemotaxis	10	34
Wound healing	11	34
Angiogenesis inhibitors	7	33
Angiogenic phenotype	5	33
Cell proliferation	16	33
Nuclear translocation	10	32
Signal transduction pathways	11	32
Cell differentiation	12	31
Regulation of angiogenesis	5	30
Heparin binding	7	30
Clopidogrel	5	29
Response to LPS	7	29
Tissue homeostasis	7	29
Adiponectin levels	6	29
Morphogenesis	10	29
Sprouting angiogenesis	4	28
Capillary morphogenesis	4	28
Glomerular filtration rate	7	19
Phagocytosis	8	18

response was associated with increasing age (0.9% of the variance; P = 0.007), male sex (17.6% of the variance; $P = 3.53 \times 10^{-30}$), increased D_{base} (29.2% of the variance; $P = 2.5 \times 10^{-54}$), increased DBP (0.6% of the variance; P = 0.026), increased SBP (1.5% of the variance; P = 0.001), decreased HR (7.5% of the variance, $P = 2.73 \times 10^{-12}$), and presence of hypertension (0.7% of the variance; P = 0.025) (Table 10). Initially, smoking was also significantly associated with FMD (1.9% of the variance; $P = 1.38 \times 10^{-3}$), but after stratifying for sex, since only men smoke in the Amish community, no association was observed (P = 0.817). Also, we found that the variance caused by sex was driven by D_{base}, as the effect of sex was diminished (P = 0.85) when adjusting for D_{base}. The estimated residual heritability of FMD after adjustment for age and sex was 0.16 ± 0.09 (P = 0.03).

Table 8: Predicted Diseases Relevant to *PEAR1* Dysregulation.

Disease	# of Shared Relationships	Score
Vascular disease	12	59
Non-small cell lung carcinoma	14	55
Osteoarthritis	11	46
Preeclampsia	10	42
Pancreatic cancer	12	40
Diabetic nephropathy	9	39
Colorectal cancer	14	39
Lymph node metastasis	9	37
Melanoma	13	36
Breast cancer	16	35
Coronary artery disease	12	33
MDA-MB-231 breast cancer	6	33
Metastatic disease	11	33
Intimal hyperplasia	6	33
Mammary tumor	11	33
Chronic kidney disease	9	32
Gastric cancer	11	32
Pulmonary arterial hypertension	7	32
Atherosclerotic plaque	7	32
Systemic sclerosis	8	31
Angiosarcoma	6	31
Insulin resistance	11	30
Renal disease	10	30
Liver fibrosis	8	29
Proliferative diabetic retinopathy	5	29
Myocardial infarction	11	28
Kidney disease	8	27

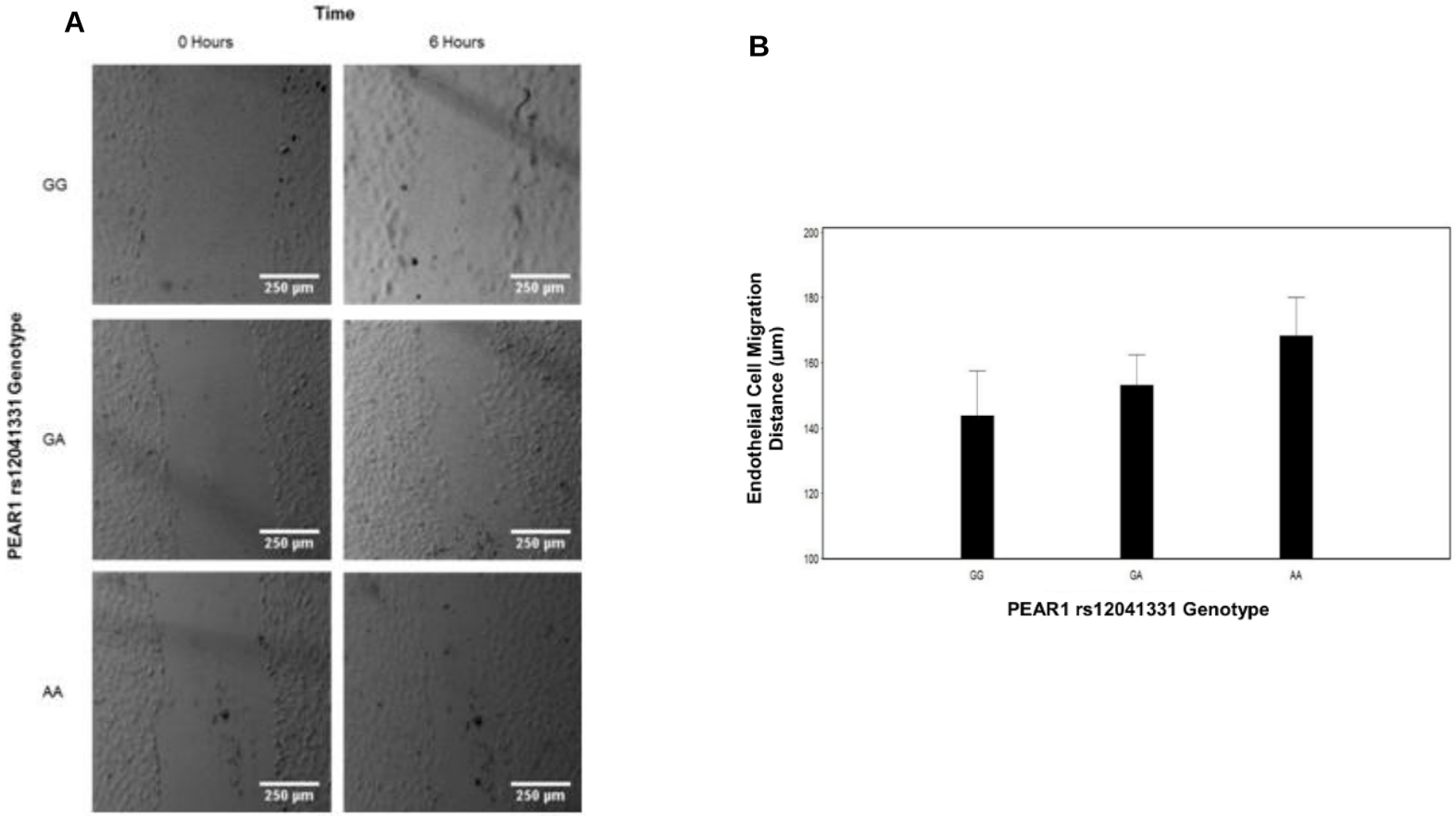

Figure 6: The Impact of *PEAR1* rs12041331 on Cell Migration in Human Umbilical Vein Endothelial Cells (HUVECs). A) Representative phase-contrast images of rs12041331-stratified HUVECs at 0 and 6 hours post-scratch generation during an *ex vivo* cell migration assay. Scale bar, 250 µm. B) Quantitative depiction of mean HUVEC cell migration. Endothelial cell migration distance was calculated by dividing the area of the scratch by the height of the frame using ImageJ. Mean endothelial cell migration distance was calculated based on 72, 72, and 36 independent measurements for GG, GA, and AA genotypes, respectively, as described in the Materials and Methods section.

Table 9: Characteristics of HAPI Heart Study Participants

Characteristic (Units)	Men	Women
Number (n)	365	276
Age ± SD (years)	42.2 ± 13.7	44.5 ± 14.1
BMI ± SD (kg/m^2)	25.6 ± 3.2	27.3 ± 4.8
Systolic blood pressure ± SD (mm Hg)	122.0 ± 12.8	120.4 ± 16.3
Diastolic blood pressure ± SD (mm Hg)	78.0 ± 8.7	75.4 ± 8.4
No. with hypertension (%)*	42 (11.5)	40 (14.5)
Total cholesterol ± SD (mg/dl)	202.3 ± 44.7	212.7 ± 48.4
LDL cholesterol ± SD (mg/dl)	136.4 ± 40.9	139.6 ± 45.8
HDL cholesterol ± SD (mg/dl)	53.3 ± 13.0	59.0 ± 14.4
Triglycerides ± SD (mg/dl)†	62.8 ± 37.9	70.9 ± 45.5
No. with hypercholesteremia (%)‡	57 (15.7)	51 (18.6)
No. with self-reported diabetes (%)	3 (0.8)	2 (0.7)
Hematocrit ± SD (%)	43.2 ± 2.5	38.5 ± 2.5
White blood cell count ± SD (n x 1000)	5.4 ±1.2	5.2 ± 1.0
Platelet count ± SD (n x 100,000)	231.4 ± 52.3	240.8 ± 50.5
No. of current smokers (%)§	73 (20.2)	0 (0)
No. taking aspirin (%)	14 (3.8)	5 (1.8)
No. taking lipid-lowering medications (%)	5 (1.4)	2 (0.7)
No. taking anti-hypertensive medications (%)	1 (0.3)	0 (0)
Brachial artery width pre-occlusion ± SD (mm)	4.1 ± 0.5	3.1 ± 0.4
Brachial artery width post-occlusion ± SD (mm)	4.4 ± 0.5	3.5 ± 0.4

Abbreviations: BMI, body mass index; HAPI, Heredity and Phenotype Intervention; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation.
SI conversion factors: To convert HDL-cholesterol, LDL-cholesterol, and total cholesterol values to mmol/L, multiply by 0.0259; triglycerides to mmol/L, multiply by 0.0113.
*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.
†Logarithm-transformed for analysis and back-transformed for presentation.

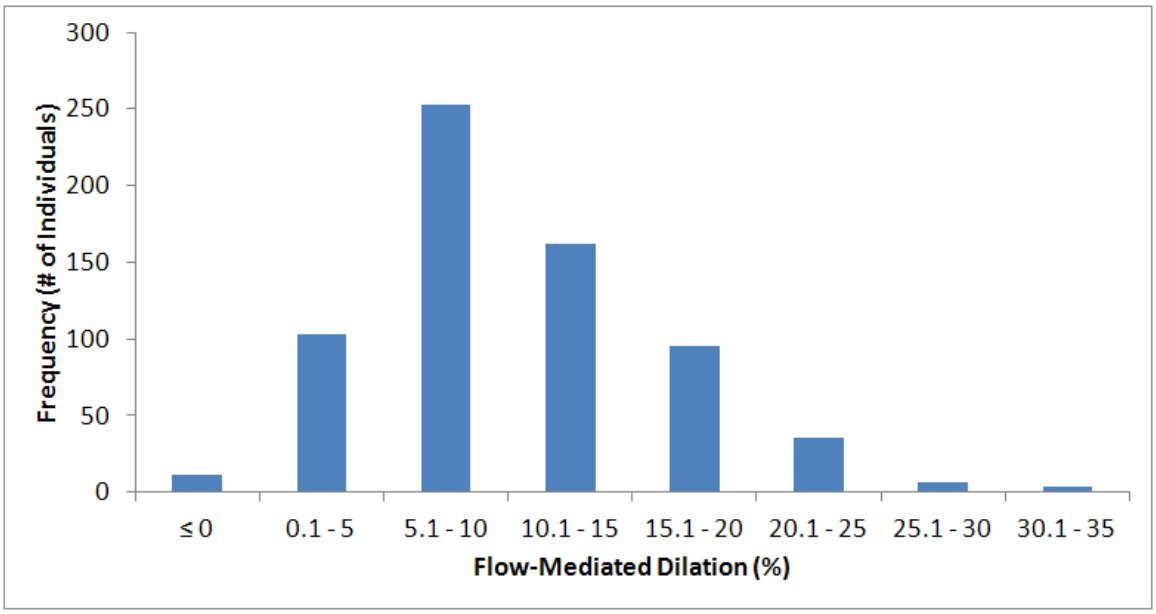

Figure 7: Distribution of Flow-Mediated Dilation. Measured in 641 Amish Participants of the Heredity and Phenotype Intervention (HAPI) Heart Study.

The minor allele (A) frequency of *PEAR1* rs12041331 in the HAPI Heart Study was 0.09, similar to that reported in other European Caucasian populations, resulting in 535 major allele homozygotes (GG), 101 heterozygotes (GA), and 5 minor allele homozygotes (AA), and conformed to expectations of Hardy-Weinberg equilibrium (P = 0.92).[106-108] Characteristics of study participants by rs12041331genotype are shown in Table 11. In our primary model accounting for clinical characteristics including age, sex, diabetes, SBP, DBP, and BMI, FMD was significantly higher in carriers of the *PEAR1* rs12041331 A-allele when compared to subjects who did not carry this allele (GG = 10.2 ± 0.3, GA = 10.8 ± 0.6, AA = 16.5 ± 5.4, P = 0.019). After accounting for variation in D_{base} (P = 0.032), HR (P = 0.019), and both D_{base} and HR (P = 0.034), *PEAR1* rs12041331 remained significantly associated with FMD.

Table 10: Predictors of Variance in Flow-Mediated Dilation in HAPI Study Participants.

Predictor (Units)	Beta	Standard Error	P-value	Variance Explained
Age (years)	-0.05	0.02	0.007	0.90%
Sex (female)	5.07	0.42	3.5×10^{-30}	17.60%
BMI (kg/m^2)	0.04	0.06	0.448	
Current smoking (%)*	0.15	0.66	0.819	
Self-reported diabetes (%)	-4.72	2.66	0.076	
Brachial artery width pre-occlusion (mm)	-0.65	0.04	2.5×10^{-54}	29.20%
HDL cholesterol (mg/dl)	0.02	0.02	0.369	
LDL cholesterol (mg/dl)	0	0.01	0.937	
Total cholesterol (mg/dl)	0	0.01	0.578	
Diastolic blood pressure (mm Hg)	-0.06	0.03	0.026	0.60%
Systolic blood pressure (mm Hg)	-0.05	0.02	0.001	1.50%
Mean arterial pressure (mm Hg)	-0.07	0.02	0.003	1.20%
Heart rate (beats/min)	0.17	0.02	2.7×10^{-12}	7.50%
Hypertension (%)	-1.54	0.68	0.025	0.70%
rs12041331 genotype (A allele)	1.22	0.62	0.047	0.50%

Abbreviations: BMI, body mass index; HAPI, Heredity and Phenotype Intervention; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
*Observed only in men to account for the OOA community's male-only smoking cohort. Variance explained was only calculated for traits with significant correlations to clopidogrel response.

Table 11: Characteristics of HAPI Study Participants by rs12041331 Genotype

Characteristic (Units)	*PEAR1* rs12041331 Genotype GG	GA	AA
Number (n)	535	101	5
Age ± SD (years)	43.2 ± 13.9	43.6 ± 13.8	33.0 ± 17.2
BMI ± SD (kg/m^2)	26.2 ± 4.0	26.9 ± 4.3	26.9 ± 2.9
No. of female gender (%)	232 (43.4)	41 (40.6)	3 (60.0)
Brachial artery width pre-occlusion ± SD (mm)	3.8 ± 3.1	3.7 ± 0.6	3.4 ± 0.6
Brachial artery width post-occlusion ± SD (mm)	4.2 ± 3.3	4.1 ± 0.6	3.9 ± 0.6
Systolic blood pressure ± SD (mm Hg)	121.5 ± 14.4	120.3 ± 14.7	123.0 ± 11.7
Diastolic blood pressure ± SD (mm Hg)*	77.2 ± 8.6	75.6 ± 8.7	68.4 ± 4.2
No. with hypertension (%)	71 (13.3)	11 (10.9)	0 (0)
Total cholesterol ± SD (mg/dl)	207.2 ± 45.7	205.4 ± 51.7	183.6 ± 18.4
LDL cholesterol ± SD (mg/dl)	138.1 ± 42.5	136.6 ± 47.2	124.4 ± 17.8
HDL cholesterol ± SD (mg/dl)	55.9 ± 13.9	55.3 ± 14.4	50.0 ± 5.6
Triglycerides ± SD (mg/dl)	66.2 ± 41.1	67.7 ± 44.5	45.8 ± 15.4
No. with hypercholesteremia (%)	94 (17.7)	14 (13.9)	0 (0)
No. with self-reported diabetes (%)	3 (0.6)	2 (2.0)	0 (0)
Hematocrit ± SD (%)	41.1 ± 3.4	41.3 ± 3.1	39.6 ± 5.8
White blood cell count ± SD (n x 1000)	5.3 ± 1.2	5.2 ± 1.2	4.8 ± 1.3
Platelet count ± SD (n x 100,000)	235.0 ± 51.8	236.5 ± 50.9	259.4 ± 57.4
No. of current smokers (%)†	56 (10.6)	17 (17.2)	0 (0)
No. taking aspirin (%)	14 (2.6)	5 (5.0)	0 (0)
No. taking lipid-lowering medications (%)	5 (0.9)	2 (2.0)	0 (0)
No. taking anti-hypertensive medications (%)	1 (0.2)	0 (0)	0 (0)

* Diastolic blood pressure was significantly different between genotypes (p=0.02) .No other traits were significantly different by genotype.
† Observed in males only using a sex-stratified analysis to account for the OOA community's male-only smoking cohort.

D. Discussion

PEAR1 was identified in 2005 by Nanda and colleagues as a type I membrane protein that is highly expressed in platelets and endothelial cells, and is involved in platelet aggregation through secondary signaling via the $\alpha IIb\beta_3$ integrin following platelet-platelet contact[1]. Several subsequent studies, including our own, have focused almost exclusively on the functional or phenotypic effects of this gene in platelets and megakaryocytes. Taken together, these investigations have yielded insights into the molecular signaling of PEAR1 as a tyrosine kinase that signals through PI3K and Akt, as well as the role of this receptor in megakaryopoiesis, platelet aggregation, antiplatelet therapy response, and cardiovascular events.[100,102,103,105-108,122,173,174]

In an attempt to gain a more comprehensive view regarding phenotypes and diseases that may be influenced by PEAR1, we used *in silico* genetic and statistical methodologies to conduct a meta-analysis of publicly available microarray datasets.[175] As expected, we observed several genes that were involved in platelet adhesion and aggregation, including von Willebrand factor (*vWF*), thrombospondin 1 (*THBS1*), and plasminogen activator inhibitor 1 (*SERPINE1*). In addition to the genes predicted by GAMMA to be associated with *PEAR1*, we also generated a list of phenotypes that are most likely to be influenced by changes in *PEAR1* expression. Among the top 8 phenotypes predicted to be affected by changes in *PEAR1* was, unsurprisingly, platelet aggregation.

Interestingly, we also identified several *PEAR1* co-expressed genes that, while part of the TGF-β signaling system, are important in blood vessel formation (*ACVRL1*), endothelial cell migration and angiogenesis (*RhoJ*), endothelial cell proliferation, smooth

muscle cell recruitment, and vascular remodeling (*ENG*), as well as endothelial cell adhesion and survival (*CTGF*), showing a definite endothelial emphasis.[190-194] Of the 8 most significantly associated phenotypes predicted by GAMMA, 6 are critical in vascular endothelial function (endothelial cell migration, vasculogenesis, angiogenesis, neovascularization, endothelial cell proliferation, and cell adhesion). Similarly, a list of diseases predicted to be influenced by changes in *PEAR1* expression was generated using the same approach. Consistent with our previous report, "vascular disease" was most strongly related to changes in *PEAR1* expression.[108] Preeclampsia, a condition characterized by high blood pressure and endothelial dysfunction, was also predicted to be influenced by PEAR1. Intriguingly, other diseases predicted to be related to PEAR1 included several types of cancer (e.g. colorectal, pancreatic, and non-small cell lung carcinoma), osteoarthritis, and diabetic nephropathy. While there is currently no available data suggesting a relationship between PEAR1 and osteoarthritis, diabetic nephropathy, aortic aneurisms, or cancer risk, it is interesting to speculate given the role of the TGF-β signaling system in the progression of these disorders.

In order to experimentally validate, in part, the results of our microarray meta-analysis, we tested whether a genetic variant known to influence *PEAR1* expression (rs12041331) significantly influenced endothelial cell migration, the most highly predicted phenotype to be affected by PEAR1 based on our GAMMA results, as measured by a well-described *ex vivo* cell migration assay. Indeed, we observed that *PEAR1* rs12041331 was significantly associated with endothelial migration distance. Our results indicate that HUVECs homozygous for the A-allele of *PEAR1* rs12041331 have approximately 117% better cell migration capabilities compared to cells homozygous for

major allele (G). Additionally, when examining the effects of the rs12041331 variant in a dominant model (GA/AA vs GG) to account for the small sample minor allele homozygote (AA) sample size, the association remained significant (P = 0.048). This novel observation is highly consistent with results obtained by GAMMA, and also provides at least one mechanism by which PEAR1 influences endothelial function.

In this investigation, we also measured FMD of the brachial artery, the most widely used noninvasive test of endothelial function, in order to estimate its heritability, establish clinical predictors, and assess the potential effect of genetic variation in *PEAR1* on endothelial function *in vivo*. In relatively healthy participants of the HAPI Heart Study, we found FMD to be normally distributed (Figure 7). Gender and D_{base} accounted for over 45% of the variation in FMD, with age, smoking, SBP, DBP, hypertension, and HR jointly accounting for another 13-14%. Similar to previous reports, our heritability estimates suggest that approximately 16% of the variation in FMD is attributable to relatedness, for which genetic factors likely contribute.[195,196] To our knowledge, this investigation describes the first reported relationship between PEAR1 and clinical measures of endothelial function. Specifically, our data show that the A-allele of a common intronic variant (rs12041331) in this gene is significantly associated with greater brachial artery FMD.

To our knowledge, the only other investigation to date that has evaluated PEAR1 in the context of endothelial cell biology was a recently reported abstract presented by Vandenbriele and colleagues.[197] Consistent with the results of our meta-analysis, they observed that lentiviral-mediated knockdown of *PEAR1* resulted in 30 ± 5% faster endothelial cell migration as assessed by a cell migration assay. Furthermore, *PEAR1*

knockdown altered PI3K, AKT, and eNOS activation, significantly reduced the expression of the proliferation suppressor p21/CIP1, and doubled the rate of endothelial cell proliferation. Taken together, our results indicate *PEAR1* may be a critical determinant of endothelial homeostasis with potential implications in the development of vascular disease.

We acknowledge some limitations of this study. A potential limitation of the approach implemented in GAMMA is that it tends to be biased towards inclusion of genes that change significantly in their expression level and against genes whose transcriptional levels are too low to reliably detect. Although the analysis we performed of multiple genes for their published commonalities tends to reduce the bias towards a small number of genes skewing the results, the scientific literature itself may have its own biases in terms of preferences for what is both studied and reported. Therefore, while we functionally validated one phenotype predicted by GAMMA (i.e. endothelial migration), we believe future functional experiments to evaluate the relationship between *PEAR1* and the genes/phenotypes identified by GAMMA are warranted. Furthermore, while the use of FMD is the most commonly used method to non-invasively assess endothelial function, recent work by Atkinson and Batterham have shown that inadequate adjustment of FMD analyses, particularly of a ratio-scaling inconsistency between percent FMD and baseline vessel diameter, leads to biased results.[198] In order to minimize the effect of this potential confounder and others, we tested for association between *PEAR1* rs12041331 and FMD using several statistical models that adjusted for different clinical variables that impact FMD measurements (e.g. age, sex, $D_{base,}$ and HR). In all models, *PEAR1* rs12041331 remained significantly associated with FMD. In

addition, given the number of minor allele homozygotes (N = 5) and observed genotypic means, we also tested for association between rs12041331 and FMD using both dominant (GG vs AG/AA; P = 0.03) and recessive (GG/AG vs AA; P = 0.05) genetic models, showing a consistent direction of association.

The dynamic relationship between platelets and endothelial cells is critical in cardiovascular physiology, and dysfunction of either cell type can lead to a cardiovascular event. There is a growing body of literature indicating that PEAR1 has important effects on platelet-related processes and cardiovascular outcomes. In this investigation, we have established for the first time that genetic variation in *PEAR1* significantly impacts endothelial function as well. Looking forward, it is critical to further characterize the function(s) of PEAR1 in both platelet and endothelial cells to elucidate the mechanism by which this gene may contribute to cardiovascular risk. In addition, it will be necessary to extend the findings from this study regarding *PEAR1* in the endothelium to the context of aspirin therapy to directly address the paradoxical nature of *PEAR1* rs12041331 and its associations with on-aspirin platelet aggregation and on-aspirin cardiovascular event rates. Once understood, PEAR1 may be a novel target for treatment and prevention of cardiovascular disease.

IV. Conclusions

A. Our Findings

In recent years, substantial effort has been made to identify the genetic and non-genetic causes of variable response to antiplatelet therapy. Given that prescribing of antiplatelet agents is critical in reducing the rates of recurrent cardiovascular events in patients with CAD, a pathology that is currently the number one cause of mortality in the United States, identification of the factors that lead to differential drug response can have a significant effect on clinical outcomes, patient quality of life, and the economic burden attributed to cardiovascular disease.[4] Given the heritable nature of antiplatelet response, the overall objective of this project was to identify novel genetic variants that significantly impact response to dual antiplatelet therapy with clopidogrel and aspirin as well as to computationally and functionally evaluate the potential mechanism(s) by which a newly identified DAPT response gene (PEAR1) contributes to cardiovascular disease risk.

1. Chapter 2 Findings

In order to identify novel pharmacogenetic variants that influence clopidogrel therapy, we used DMET array genotyping to explore associations between drug response and 1936 variants previously shown to influence pharmacokinetics and pharmacodynamics of multiple other drug classes (Chapter 2). We estimated the heritability of clopidogrel response (H^2 = 39.4%), confirming that genetic variation substantially influences this phenotype, and observed significant correlations between clopidogrel response and several common clinical traits with cardiovascular implications. In addition to confirming the known association between clopidogrel response and

*CYP2C19**2, we also observed a significant association between a novel variant (rs11249454) in *UGT2A1/2* and clopidogrel response, with the variant exerting its effect in a sex-specific manner. This SNP is intronic for both genes because *UGT2A1* and *UGT2A2* are splice variants, differing only in their 1st exon.[151] Both genes code for metabolizing enzymes that catalyze compound clearance by the addition of UDP-glucuronic acid, with *UGT2A1* having the higher enzyme activity compared to *UGT2A2*.[150] Among their many exogenous and endogenous substrates, these two enzymes have important roles in the metabolism of androgens and estrogens, potentially providing a mechanism for our sex-specific findings from genetic analysis.[139-145,152,157-159]

Consistent with many other genes critical in hormone metabolism, while UGT2A1/2 metabolize androgens, estrogens, and isomers of the two sex hormones, the expression of *UGT2A1/2* is also induced by the substrates.[139,140,142-144,160] Given the dynamic relationship between *UGT2A1/2* and sex hormones along with the observation that *Ugt2a1* is expressed significantly higher in female rats than male rats, the sex-specific association between women and clopidogrel response we observed in our investigation is biologically plausible.[162] The sex-specific differences in platelet aggregation as well as response to antiplatelet therapy are well-established. In fact, in terms of the estrogen metabolizing action of *UGT2A1/2*, estrogen has been shown to affect platelet aggregation, though the direction of effect has been conflicting.[167-169] In addition, there are data that suggest *UGT2A1/2*'s actions in testosterone metabolism may be affecting platelet aggregation. Specifically, a group found testosterone level directly correlated with ADP $P2Y_{12}$ receptor expression in DAMI cells, a type of megakaryocytic cell line.[170] Future studies are warranted to further explore the exact mechanism by which

UGT2A1/2 affects clopidogrel response and evaluate its potential impact in future clinical decision making.

2. Chapter 3 Findings

In Chapter 3, we continued to probe the genetic determinants of DAPT response by evaluating a newly identified candidate gene (*PEAR1*) that has been previously shown to significantly influence on-DAPT platelet aggregation as well adverse cardiovascular event rates. Specifically, we attempted to gain novel insight regarding the genes, phenotypes, and diseases predicted to be influenced by PEAR1 using an *in silico* methodology called GAMMA that uses publicly available microarray datasets.[175] Consistent with the extensive literature regarding the role of *PEAR1* in platelet aggregation and thrombopoiesis, the results of our microarray meta-analysis identified a genetic network containing genes critical in platelet adhesion and aggregation that is predicted to be influenced by changes in PEAR1 expression.[102,103] Interestingly, however, the results of this meta-analysis also identified several genes in *PEAR1*'s genetic neighborhood whose primary functions are involved in endothelial-based pathways, including angiogenesis, endothelial cell migration and proliferation, and endothelial cell adhesion and survival. This is consistent with phenotypes predicted to be influenced by differential*PEAR1* expression, which include endothelial cell migration, vasculogenesis, angiogenesis, neovascularization, endothelial cell proliferation, and cell adhesion, as well as the top disease predicted by GAMMA to be influenced by *PEAR1*, "vascular disease." Given these results and the previous observation that PEAR1 is most highly expressed in endothelial cells (approximately 7-fold higher than megakaryocytes), we hypothesized

that the impact of *PEAR1* on cardiovascular risk may be mediated through its functions in the endothelium.[100]

To evaluate *PEAR1*'s action in the endothelium, we focused on *PEAR1*'s most impactful genetic variant in the literature, rs12041331, an intronic SNP that has been shown to affect expression in platelets.[107] We first assessed PEAR1's role in endothelial biology by functionally testing the most highly predicted phenotype by GAMMA (endothelial migration), using an *ex vivo* cell migration assay in HUVEC cells from subjects of defined rs12041331 genotype. Our observations confirmed that *PEAR1* rs12041331 does significantly influence endothelial migration distance, with the minor allele carriers having approximately 117% greater endothelial function than non-carriers. Furthermore, the cell migration assay data strengthen our confidence in the results of microarray meta-analysis, highlighting the need for further studies regarding the relationship between the other predicted genes, phenotypes, and diseases with *PEAR1*.

To clinically assess the influence of PEAR1 rs12041331 on endothelial function, we also performed an *in vivo* investigation measuring FMD of the brachial artery in 641 healthy participants of the HAPI Heart Study. Following collection of these data, we were able to estimate the heritability of FMD and further gain insights regarding its clinical significance through correlation analyses with several typical cardiovascular phenotypes. In these analyses, we observed that a significant amount of the trait variance was accounted for by sex and baseline brachial diameter. In addition, our FMD findings were consistent with the results of our cell migration data as we successfully observed significantly higher endothelial function associated with the minor allele of rs12041331. To our knowledge, our investigation describes the first report of association between

clinical measures of endothelial function and *PEAR1* genotype, complementing our *ex vivo* data as well as prior findings by others regarding the significant effect of altered PEAR1 function on endothelial cell migration.[197]

Taken together, our data regarding the role of PEAR1 in the endothelium expand upon PEAR1's known role in platelet aggregation and further emphasize the importance of PEAR1 in the development of cardiovascular disease. Follow-up studies are necessary to further clarify the dynamic functions of PEAR1 in different cardiovascular tissues and how these functions influence each other, as well as extend our endothelial observations to the context of aspirin therapy. A better understanding regarding the overall action of PEAR1 in the cardiovascular system, particularly in pathological contexts such as CAD, will be pivotal in interpreting the observed associations between PEAR1 genetic variation and cardiovascular phenotypes.

B. Antiplatelet Pharmacogenomics

The data presented in Chapters 2 and 3 of this study have significant implications for clopidogrel and aspirin pharmacogenomics, respectively. Through discovery of novel genetic variants associated with drug response (e.g. *UGT2A1/2* rs11249454 and clopidogrel), or probing biological mechanisms behind genetic associations, (e.g. *PEAR1* and endothelial function), this project furthers the long-term goal of pharmacogenomics and personalized medicine through identification of potential biomarkers used to maximize efficacy and minimize adverse drug reactions in patients. For instance, we anticipate that the findings involving *UGT2A1/2* will facilitate further interest and investigation of this variant in other cohorts. In addition, further study of the biological mechanisms underlying our observations may ultimately result in investigation that

evaluate the benefits of *UGT2A1/2* genotyping over standard of care as a proof of concept for implementation in the clinical setting. Similarly, our experiments regarding *PEAR1* in Chapter 3 is a step ahead of *UGT2A1/2* as Chapter 3 describes our follow-up studies, namely a cell migration assay and genetic analysis with FMD, which were conducted to better understand the biological mechanisms of prior observations regarding the effects of rs12041331 on cardiovascular event rate. In contrast to our variants of focus in Chapters 2 and 3, other variants whose significant effects on drug response, e.g. *CYP2C19**2's effect on clopidogrel response, have been replicated genetically and elucidated biologically and are currently at a more advanced stage of the vetting process.

C. Obstacles in Pharmacogenomics

The emergence of pharmacogenetics and personalized medicine offers great potential to improve clinical outcomes, ultimately leading to better patient quality of care. While many investigations, including the work performed in this project, have identified novel determinants of antiplatelet therapy response, adoption of pharmacogenetic testing into the clinic has been slow. The genetic variant *CYP2C19**2 is an often used example of the promise of pharmacogenomics. Its effect on *ex vivo* on-treatment platelet aggregation traits was extrapolated to *in vivo* cardiovascular event risk, and both were replicated by multiple other studies.[107,199] Unfortunately, it has proved difficult to implement *CYP2C19**2 genetic testing in the clinical setting. The gap between bench and bedside is mainly caused by the need for prospective, double-blinded, randomized, controlled, genotype-directed clinical trials focusing on hard cardiovascular endpoints so that clinicians can interpret the data and translate them into their clinical practice. Without these large-scale prospective studies, clinicians do not feel confident in the

evidence base and often choose to ignore it for the current standard of care. In one study, 499 patients with recent ACS or PCI were genotyped for *CYP2C19**2 and had CYP2C19 enzyme function measurements, with both sets of data distributed to the respective patients and their physicians. In this investigation, only 20% of clopidogrel poor metabolizers received alternative treatment (i.e. prasugrel or ticagrelor) by their physician, highlighting the disconnect between currently available pharmacogenetic information and physician decision making.[200] Furthermore, in a survey distributed to Duke University healthcare providers and faculty about the use of pharmacogenomic data in the clinic, most respondents were enthusiastic about using it but acknowledged a need for an education agenda to improve implementation and connections between clinicians and researchers.[201] It is understandable that many of the practicing clinicians today did not receive intensive pharmacogenetics training during medical school given that the technology has only existed recently and is advancing at an accelerating pace. To prevent lack of pharmacogenetic education from perpetuating, US and Canadian medical school genetics course directors were surveyed about their genetics curricula. The results showed that, on average, genetics course directors felt the amount of education devoted to genetics was inadequate for clinical practice, which is concerning in regards to the ability of future practitioners to implement these tools in the clinic. In contrast, the course directors did point out that many schools are beginning to incorporate modern genomics topics such as personalized medicine and direct-to-consumer testing into their curricula.[202]

D. Implementation of Pharmacogenomics in the Clinic

While some have suggested interesting solutions to implementing pharmacogenetic testing into the clinical setting, such as increasing collaboration between pharmacists and prescribing physicians to achieve the optimal genotype-directed therapy, others groups have begun conducting clinical studies using genotype-directed therapies to produce the data that will lay the groundwork for implementation in the clinic.[203] One example is Stimpfle et al, who implemented *CYP2C19**2 genotyping in a point-of-care (POC) setting as part of a pilot study involving 137 patients arriving to the emergency department experiencing ACS. They found that on-treatment platelet reactivity was significantly higher for *CYP2C19**2 carriers receiving clopidogrel compared to *CYP2C19**2 carriers receiving prasugrel or ticagrelor. This observation indicated that POC genotyping could provide a valuable tool in identifying patients who should receive clopidogrel reloading or an alternative antiplatelet altogether, and who would otherwise be at unnecessary risk.[204] Other groups have made the case that genotype-directed therapy is not simply good science and healthcare, but also good economics. According to a study in 2012, implementing *CYP2C19**2 genotyping does not only allow for greater protection of non-responders, but it saves money in national healthcare spending as well. The investigators in this study compared genotype-directed therapy where all patients received clopidogrel except *CYP2C19**2 carriers who got prasugrel. They found that genotype-directed therapy was more cost-effective because of the increased cost in caring for on-clopidogrel *CYP2C19**2 carriers with cardiovascular sequelae, or providing prasugrel for an entire population.[205]

Other groups, such as the Pharmacogenomics Research Network (PGRN) and the Clinical Pharmacogenetics Implementation Consortium (CPIC) have provided more support for pharmacogenomics use in the clinic by analyzing the data currently available and creating guidelines for physicians on how to use the data in their clinical practice. In the instance of clopidogrel, they divided patients into tiers of metabolizing ability based on *CYP2C19* genotype, and have drug recommendations for each type of metabolizer.[49] Ultimately, the plan for the future of pharmacogenomics is that the information gained from these studies will lead to clinical actions and contribute to personalized medicine, where unique facets of patient profiles, particularly their genomes, are taken into account when healthcare providers select a treatment. Such an initiative, called the Translational Pharmacogenetics Project, is currently being funded by the Pharmacogenomics Research Network to be carried out at the University of Maryland School of Medicine and 5 other sites. In this initiative, patients indicated for clopidogrel treatment following PCI will be offered *CYP2C19**2 genotyping, and, should they accept the genotype testing, the physicians who care for them consider altering treatments according to guidelines provided by the CPIC, all in an effort to improve personalized medicine.

E. Personalized Medicine on a National Scale

According to the United States Food and Drug Administration (FDA), the term personalized medicine refers to prescribing "the right patient with the right drug at the right time."[206] While the term is often used in the context of treatment, it is important to note that tailoring medical care of patients also includes preventative measures, diagnosis, and post-treatment follow-up. While personalized medicine has been implemented for decades without the title, e.g. blood typing prior to transfusions or the

use of alternative therapies in response to drug allergies, the personalized medicine buzzword was first introduced in the science and healthcare communities around the completion of the Human Genome Project.[207] This milestone showed scientists and physicians that substantial amounts of patients' genetic information can be attained via DNA sequencing, and with the ability to acquire massive data that are completely unique to each respective patient, the potential for tailor-made healthcare was realized. As DNA sequencing technology advanced in speed, quality, and price, an increasing number of investigators implemented genetic assays, including genotyping arrays and DNA sequencing, amassing huge datasets from their respective research cohorts. The increasing number of these sorts of experiments led to investigators combining data in the settings of consortia and publicly available datasets on the internet, empowering them statistically to observe more significant associations with the potential for implementation in the clinic.[208]

In an effort to prepare for the increasing use of DNA genotyping and sequencing technologies by research investigators in the advancement of personalized medicine, the FDA released a document in October 2013 that described their role as a regulatory body in the context of personalized medicine.[209] Specifically the document outlined their plans for developing the infrastructure and support system for managing findings from pharmacogenomics research, while also ensuring patient safety in what is essentially a whole new branch of healthcare innovation. While major steps by the FDA in recent years signify the government's appreciation for the importance of personalized medicine and the potential it brings for innovation, personalized medicine received its greatest

boost in national attention when the White House announced its plans to make it a priority earlier this year.

F. Precision Medicine Initiative

On January 30, 2015, President Obama held a press conference where he announced the White House's new Precision Medicine Initiative. As part of the initiative, the President launched a $215 million investment in the 2016 budget to spur technological advances that will move us away from "a one-size-fits-all approach," and towards healthcare that implements people's genes, lifestyles, and environments. Specifically, the initiative includes a $130 million investment at the NIH to recruit a voluntary cohort of one million or more participants who will take part in a variety of studies focusing on genetics, metabolism, microorganism composition, and medical records, similar to the UK Biobank from the Wellcome Trust.[210] Also, $70 million will be granted to the National Cancer Institute to stimulate greater understanding of genomic causes of cancer, and $10 million will go to the FDA for management of these numerous datasets, which will be pivotal for the analysis and interpretation of findings. The large-scale funding of specifically the NCI shows the emphasis on improving knowledge in cancer in the short-term, while making new discoveries for the precision medicine of broader health and diseases in the long-term.[211] The initiative makes it clear that in light of the vast data collection that will occur, patient privacy is a major priority and that many steps will be taken to insure protection of patient information.[212] The goals outlined by President Obama's Precision Medicine Initiative provide important tools that will advance knowledge forming the foundation of precision medicine, while also priming healthcare for the translation of research findings into clinical practice. As one of the

major tenets of precision medicine, the priorities set out by the President will have a major impact on the field of pharmacogenomics, including antiplatelet pharmacogenomics, making it is an exciting time to be an investigator in pharmacogenomics research.

V. References

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