

**Curriculum Vitae**  
**Sarah Katherine Bromberek**  
**skbromberek@gmail.com**

**Degree and Date to be Conferred:** M.S. December 2021

**Collegiate Institutions Attended:**

University of Maryland, Baltimore. 2021  
M.S. Human Genetics  
Saint Joseph's College, Indiana. 2017  
B.S. Biology and Chemistry, Cum Laude

**Professional Positions Held:**

Environmental Lab Technician, Pace Analytical Services 7726 Moller Rd. Indianapolis, IN 46268. 2017-2018

**Teaching Experience:**

Genetics Teaching Assistant at Saint Joseph's College, Jan. 2017 – May 2017  
Organic Chemistry Teaching Assistant at Saint Joseph's College, Aug. 2015 – May 2017

**Current Memberships:**

The American Society of Clinical Pharmacology and Therapeutics Pending  
The American Society of Human Genetics 2017  
American Chemical Society 2014

**Special Awards:**

2020 ASCPT Annual Meeting Presidential Trainee Award.  
Scholarship Winner, Saint Joseph's College 13<sup>th</sup> Annual Colloquium 2016  
Scholarship Winner, Saint Joseph's College 11<sup>th</sup> Annual Colloquium 2014

## Abstract

**Title of Thesis:** Pharmacometabolomics of clopidogrel: Determining the genetic and metabolic contributors to clopidogrel response

**By:** Sarah Bromberek, Master's Human Genetics, 2021

**Thesis Directed by:** Dr. Amber L. Beitelshes, *Associate Professor, Department of Medicine.*

Clopidogrel is a commonly prescribed antiplatelet drug and there is considerable variability in response. The PAPI study was conducted in 687 individuals to determine the genetic predictors of clopidogrel response. Targeted metabolomic profiling of 42 amines was performed in a subset of 198 PAPI subjects with genetics data available.

We identified the metabolic signature of clopidogrel and determined metabolites associated with clopidogrel-induced changes in platelet reactivity.

We found tyrosine was most significantly changed after exposure to clopidogrel, and BCAAs baseline levels were associated with clopidogrel-induced changes in platelet aggregation. Gaining insights into factors that influence variability in antiplatelet response is important for identifying novel antiplatelet mechanisms or biomarkers for predicting response. These findings can have long-term implications toward advancing precision medicine in antiplatelet therapy.

Pharmacometabolomics of Clopidogrel: Determining the Genetic and Metabolic Contributors to Clopidogrel Response

By:  
Sarah Bromberek

Dissertation submitted to the faculty of the Graduate School of the  
University of Maryland, Baltimore in partial fulfillment  
of the requirements for the degree of  
Master of Science  
2021

©Copyright 2021 by Sarah Bromberek

All Rights Reserved

## Acknowledgements

This graduate school journey has allowed me to work with the most incredible people. A countless number of people have supported me throughout this process; many nearby, and many miles away. I'd like to take this page to acknowledge those who have influenced me, guided me, and supported me while completing this degree.

Thank you to my parents, Brian and Patricia, for your unconditional love and support of my decision to move to Baltimore to pursue a master's degree in human genetics. You were always a phone call away when I needed advice and guidance. LYB

Dr. Amber Beitelshees, thank you for mentoring me, your ability to teach and guide me through this research in a positive environment made this possible, and enjoyable. Thank you for all the support and patience you've had for me. Throughout this project you have been a role model for how to conduct my research, and how to live life outside of research.

Thank you to my committee members, Dr. Lewis, Dr. Mitchell, and Dr. Montasser for your time and effort, your valuable insights, and always pointing me in the right direction.

Dr. Toni Pollin, thank you for advising me while I completed my degree. The effort you put into your students shows, and I want to thank you for support during my transition into graduate school.

Dr. Joshua Lewis, thank you for coaching me on public speaking.

## Table of Contents

Chapter.....	Page
Acknowledgments.....	vi
Table of Contents.....	vii
List of Tables.....	viii
List of Figures.....	x
Abbreviations.....	xi
Chapter 1: Introduction.....	1
Chapter 2: Determine the Metabolic Signature of Clopidogrel.....	10
2.1 Introduction.....	10
2.2 Methods.....	11
2.3 Results.....	13
2.4 Discussion.....	16
Chapter 3: Determine the Metabolites Associated with Clopidogrel-induced Change in Platelet Aggregation....	21
3.1 Introduction.....	21
3.2 Methods.....	22
3.3 Results.....	26
3.4 Discussion.....	45
Chapter 4: Conclusions.....	50
References.....	53

## List of Tables

Table	Page
Table I: Baseline Characteristics for Metabolomic Subset of PAPI Study Participants.....	12
Table II: Wilcoxon Signed-Rank Test Results, Delta Metabolite Levels.....	14
Table III: Correlation between Baseline Metabolites and Change in Platelet Aggregation (post <i>minus</i> pre).....	26
Table IV: Linear Regression Association between Baseline Leucine and Clopidogrel-Induced Change in Platelet Aggregation, Adjusted for Age/Sex.....	30
Table V: Linear Regression Association between Baseline Valine and Clopidogrel-induced Change in Platelet Aggregation, Adjusted for Age/Sex.....	31
Table VI: Linear Regression Association between Baseline Isoleucine and Clopidogrel-induced Change in Platelet Aggregation, Adjusted for Age/Sex....	32
Table VII: Correlation between Baseline Metabolites and Baseline Platelet Aggregation.....	33
Table VIII: Linear Regression Association between <i>CYP2C19</i> and Baseline Leucine, Adjusted for Age/Sex.....	34
Table IX: Linear Regression Association between <i>CYP2C19</i> and Baseline Valine, Adjusted for Age/Sex.....	35
Table X: Linear Regression Association between <i>CYP2C19</i> and Baseline Isoleucine, Adjusted for Age/Sex.....	36
Table XI: Linear Regression Association Cardiometabolic Traits and Baseline BCAAs.....	37
Table XII: Correlation Between Delta Metabolites and Change in Platelet Aggregation (post-pre).....	38
Table XIII: Linear Regression Association between <i>CYP2C19</i> and Delta 2-Aminoadipic Acid, Adjusted for Age/Sex.....	42
Table XIV: Linear Regression Association between <i>CYP2C19</i> and Delta O-Phosphoethanolamine, Adjusted for Age/Sex.....	43

Table XV: BCAA PRS Association with Baseline BCAA Levels with a Threshold of 1.00E-07 and No LD Clumping	44
.....	
Table XVI: Leucine PRS Association with Clopidogrel Response with Threshold Of 1.00E-07.....	44

## List of Figures

Figure	Page
Figure I: Platelet Aggregation.....	3
Figure II: Blood Coagulation Cascade.....	4
Figure III: Clopidogrel Metabolic Pathways.....	6
Figure IV: Clopidogrel Mechanism of Active Metabolite.....	7
Figure V: PAPI Design Schematic.....	12
Figure VI: Histogram of Baseline Platelet Aggregation with ADP 10 $\mu$ M.....	23
Figure VII: Histogram of Post-Clopidogrel Platelet Aggregation with ADP 10 $\mu$ M.....	23
Figure VIII: Histogram of Change in Platelet Aggregation with ADP 10 $\mu$ M in Response to Clopidogrel.....	24
Figure IX: Linear Regression Association between Baseline Leucine and Clopidogrel-induced Change in Platelet Aggregation.....	30
Figure X: Linear Regression Association between Baseline Valine and Clopidogrel-induced Change in Platelet Aggregation.....	31
Figure XI: Linear Regression Association between Baseline Isoleucine and Clopidogrel-induced Change in Platelet Aggregation.....	32
Figure XII: Boxplot: Association between <i>CYP2C19</i> (rs4244285) and Baseline Leucine.....	34
Figure XIII: Boxplot: Association between <i>CYP2C19</i> (rs4244285) and Baseline Valine.....	35
Figure XIV: Boxplot: Association between <i>CYP2C19</i> (rs4244285) and Baseline Isoleucine.....	36
Figure XV: Boxplot: Association between <i>CYP2C19</i> (rs4244285) and Delta 2- Aminoadipic Acid.....	42
Figure XVI: Boxplot: Association between <i>CYP2C19</i> (rs4244285) and Delta O- Phosphoethanolamine.....	43
Figure XVII: BCAAs Mechanisms Driving Cardiometabolic Disease Pheno- types.....	46

Figure XVIII: Summary Figure..... 69

## List of Abbreviations

ADP:	Adenosine Diphosphate
ATP:	Adenosine Triphosphate
BCAA:	Branched-Chain Amino Acid
BMI:	Body Mass Index
CAD:	Coronary Artery Disease
CTM:	Active Thiol Metabolite of Clopidogrel
CVD:	Cardiovascular Disease
CYP450:	Cytochrome P450
ECM:	Extracellular Matrix
GWAS:	Genome Wide Association Study
HDL:	High-Density Lipoprotein
LDL:	Low-Density Lipoprotein
LOF:	Loss-of-Function
MMAF	Mixed Model Analysis for Pedigree and Population
OASIS:	Omics Analysis, Search and Information System
OOA:	Old Order Amish
PAPI:	Pharmacogenomics of Anti-Platelet Intervention
PRP:	Platelet Rich Plasma
PRS:	Polygenic Risk Score
SAS:	Statistical Analysis System
SNP:	Single-Nucleotide Polymorphism
TSH:	Thyroid Stimulating Hormone

## **Chapter 1: Introduction**

### **Clopidogrel Overview**

Cardiovascular disease (CVD) is a group of heart disorders and is the number one cause of death globally. The World Health Organization estimates 17.9 million people died of CVD in 2016.<sup>[1]</sup> Clopidogrel is an antiplatelet drug in the thienopyridine class. It is characterized by its selective targeting of the adenosine diphosphate (ADP) 2 receptor (P2Y<sub>12</sub>) on the platelet surface. It is commonly prescribed as part of a dual antiplatelet therapy used as the standard of care for reducing recurrent thrombotic events in patients with CVD, transient ischemic attack, and stroke. Antiplatelet therapy with P2Y<sub>12</sub> inhibitors such as clopidogrel is considered a standard practice to prevent thrombosis in patients undergoing percutaneous coronary interventions.<sup>[2]</sup> However, there is a wide range of inter-individual variability in response to clopidogrel.<sup>[2]</sup> Therefore, the goal of this project is to better understand some of the contributors to this variability.

### **Metabolomics/Pharmacometabolomics Overview**

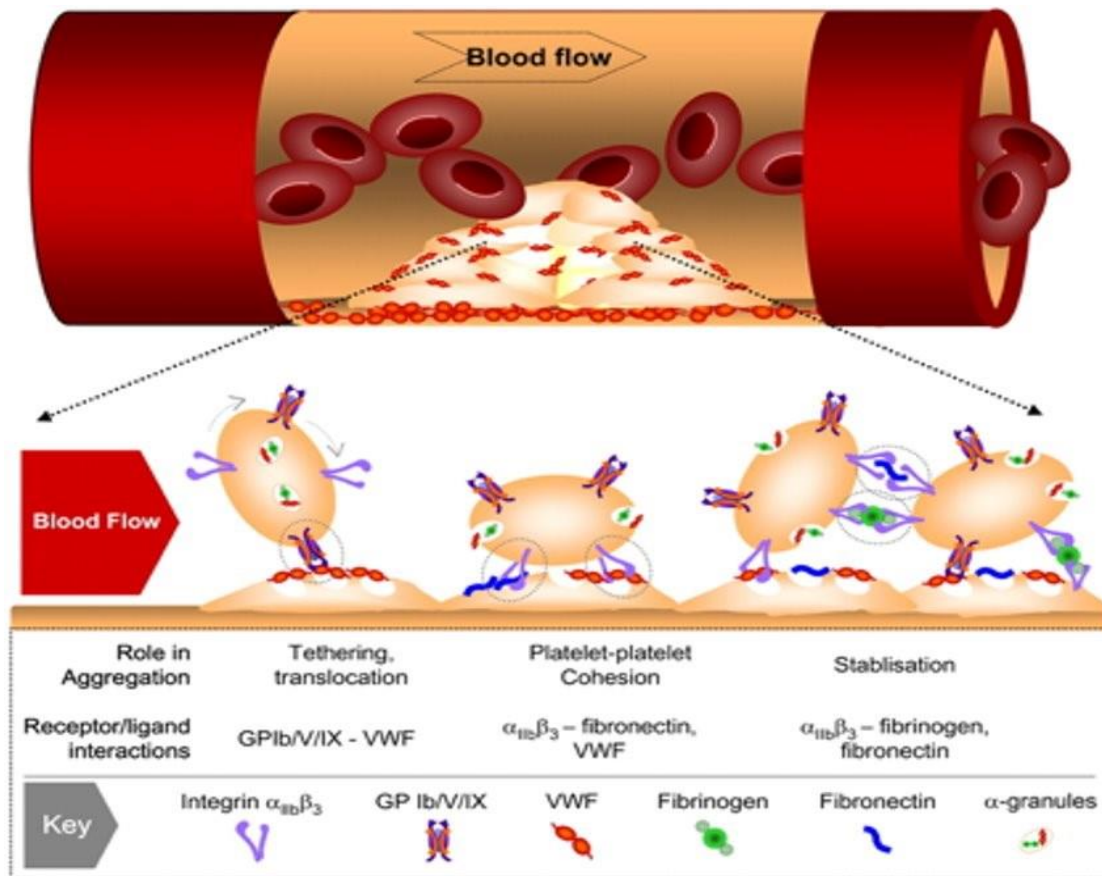
There are multiple omics technologies that can be used to gain insights into the understanding of disease processes and drug response (e.g. genomics, transcriptomics, metabolomics, and proteomics). Metabolomics is the comprehensive study of small molecules, or metabolites, present in the blood, cells, tissues or other biofluids. These metabolites are impacted both by genetics and the environment, including medications.<sup>[3]</sup> The use of pharmacometabolomic studies can help define drug actions by mapping metabolites that are changed in response to medication intake. These metabolites can serve as biomarkers

for drug response or adverse effects.<sup>[4]</sup> Samples, typically plasma or serum, are collected from clinical studies at baseline prior to treatment and after treatment with the drug of choice. Targeted and/or non-targeted metabolomics platforms are used to profile these samples to test hypotheses or create new hypotheses about pathways implicated in variation in response. The targeted approach is useful for looking at known metabolites in a particular pathway of interest. In the non-targeted analysis, all measurable metabolites, both known and unknown can be analyzed. Metabolomics and pharmacometabolomics are utilized in this project to determine the contributors to clopidogrel-induced change in platelet aggregation.

### **Platelet Aggregation/Blood Coagulation Overview**

To understand clopidogrel's mechanism, a brief overview of blood clotting and platelet aggregation is useful. Hemostasis is the spontaneous stop of blood flow from a damaged blood vessel.<sup>[5]</sup> Normal vascular endothelial cells are not thrombogenic and circulating platelets do not normally adhere to the cells. Impaired hemostasis results in spontaneous bleeding, and stimulated hemostasis results in thrombus formation, or clot.<sup>[5]</sup> During the aggregation process, platelets adhere to the von Willebrand Factor (via glycoprotein 1b) on the extracellular matrix (ECM), as well as the exposed collagen of the damaged endothelium, activating the platelet.<sup>[5]</sup> The activated platelet releases granules, dense bodies with only ADP and calcium, alpha granules with ATP, histamine, serotonin, adrenaline, fibrinogen, factor V, factor VIII, and thromboxane A<sub>2</sub>.<sup>[5]</sup> Platelet aggregation is due, in part, to ADP released from the platelet and reacting with the receptor on P2Y<sub>12</sub>. This

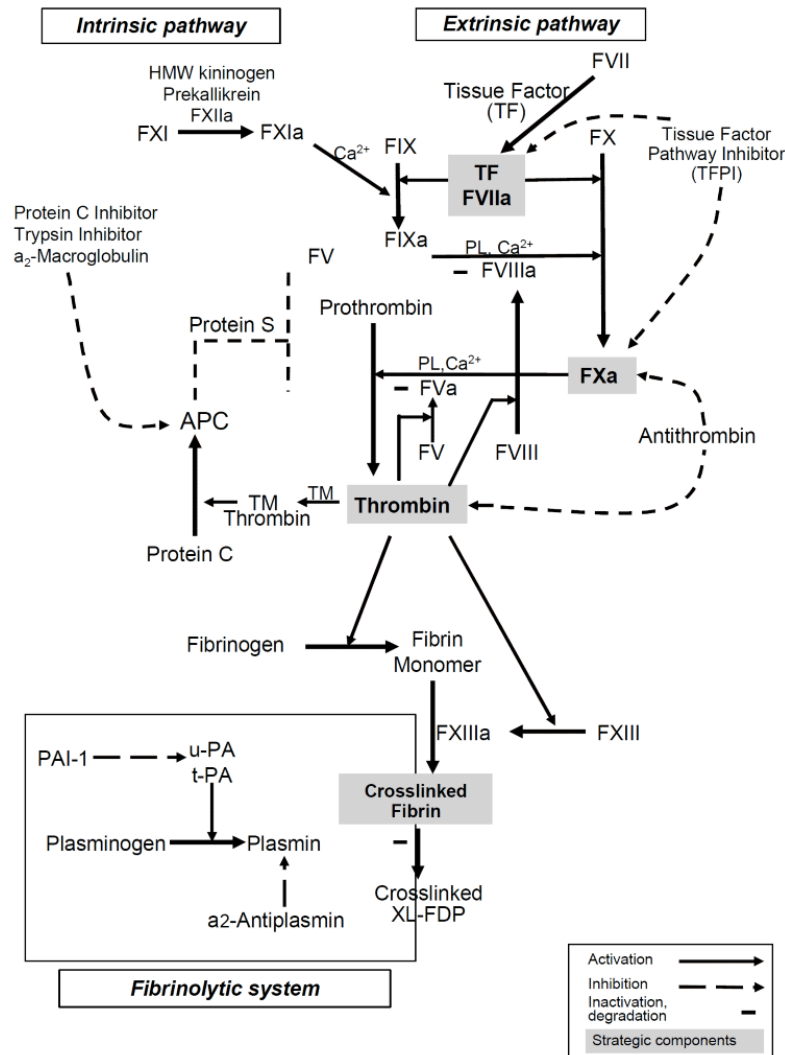
leads to glycoprotein IIb/IIIa, and thromboxane A<sub>2</sub> enlarging platelets and forming a gelatinous mass called the primary hemostatic plug<sup>[6]</sup> (Figure 1).



**Figure I:** Platelet Aggregation  
 Jackson, Shaun P. “The Growing Complexity of Platelet Aggregation.”

To create hemostasis, the blood must coagulate by transforming soluble fibrinogen into insoluble fibrin. Fibrinogen (factor I) is the substrate for the enzyme thrombin (factor IIa). The coagulation cascade goes as follows<sup>[7]</sup> (Figure 2): The main initiator of blood clotting is the tissue factor (TF)/factor VIIa pathway. The exposure of TF on damaged endothelium binds and activates circulating factor VII. This complex will activate factors X and IX and generate thrombin. Thrombin will activate upstream proteins, factors V, VIII, and XI, resulting in further thrombin generation. Thrombin, a potent activator of

platelets, converts fibrinogen to fibrin, and activates factor XIII. This results in an insoluble cross-linked fibrin molecule.<sup>[5]</sup> Platelet aggregation along with the simultaneous activation of the coagulation cascade coming to fruition, platelet contraction creates an irreversible secondary hemostatic plug. Deposition and activation of fibrin serves to cement the adhered platelets to each other to reach hemostasis.



**Figure II:** Blood Coagulation Cascade  
 “Coagulation.” *Diapharma*, 2020

### **Clopidogrel Pharmacology:**

Platelet function is regulated by three categories of substances.<sup>[5]</sup> The first group of agents are generated outside of the platelet and interact with the platelet membrane receptors, such as collagen, thrombin, and prostacyclin. The second group consists of agents generated within the platelet that interact with membrane receptors, such as ADP and serotonin. The third group contains agents generated within the platelet that act within the platelet, examples include prostaglandin endoperoxides, and thromboxane A<sub>2</sub>.<sup>[5]</sup>

Clopidogrel targets group two by the inhibition of ADP-induced platelet aggregation.

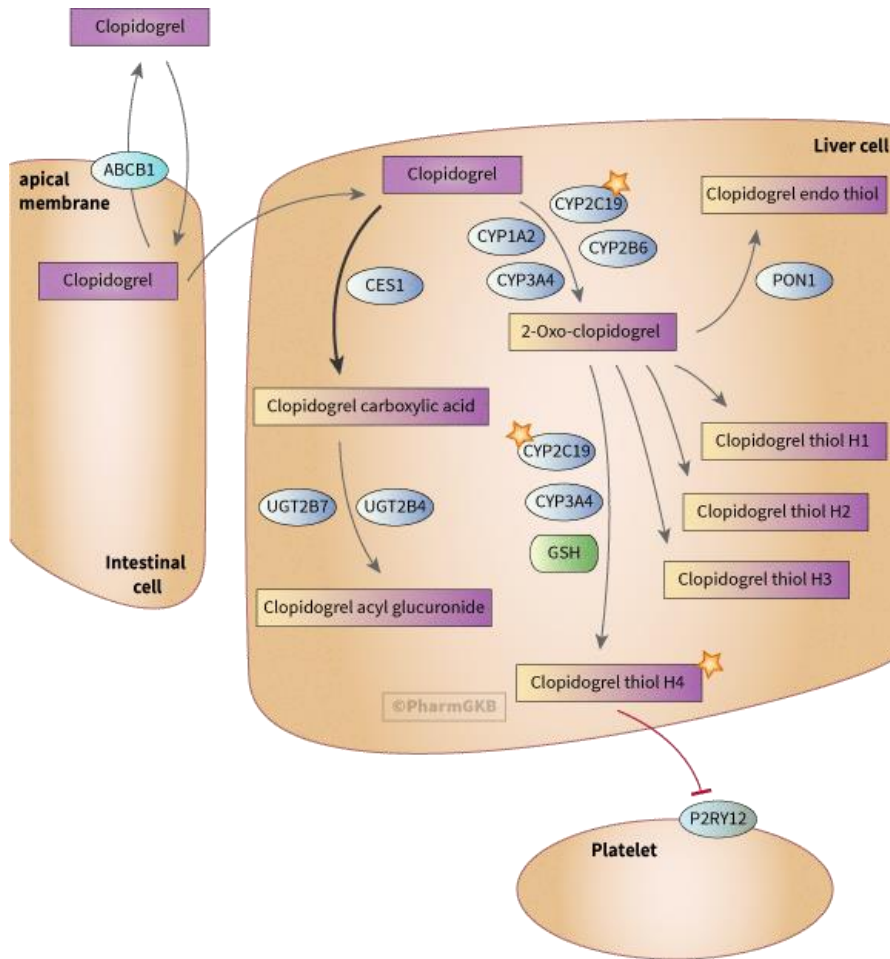
### **Pharmacokinetics:**

Clopidogrel is a prodrug; it must be metabolized in the liver to become pharmacologically active.<sup>[8]</sup> Clopidogrel is absorbed in the intestine by P-glycoprotein mediated efflux.<sup>[9]</sup> After taking a standard oral dose of 75mg, clopidogrel absorption is at least 50% 45 minutes later, based on urinary excretion of clopidogrel metabolites.<sup>[10]</sup>

Clopidogrel is extensively metabolized by two main metabolic pathways: one mediated by esterases (CES1) leading to hydrolysis into an inactive carboxylic acid derivative, SR26334 (85% of circulating metabolites), and one mediated by multiple cytochrome P450 enzymes (CYP2C19, CYP1A2, CYP2B6 and CYP3A) which lead to the active metabolite via a two-step process<sup>[11]</sup> (Figure 3).

Only 15% of the absorbed clopidogrel is transformed to the active metabolite, a thiol derivative of clopidogrel (CTM).<sup>[12]</sup> CYP450s first oxidize clopidogrel to a 2-oxo-clopidogrel intermediate metabolite. Subsequent metabolism of the 2-oxo-clopidogrel in-

intermediate metabolite through CYP2C19 and contributions from several other CYP enzymes, including CYP1A2, CYP2B6 and CYP3A4 results in formation of CTM.<sup>[11]</sup> CTM is a chiral compound existing as four isomers (H1-H4). The H4 isomer is the only active circulating isomer of CTM.<sup>[13,14]</sup>

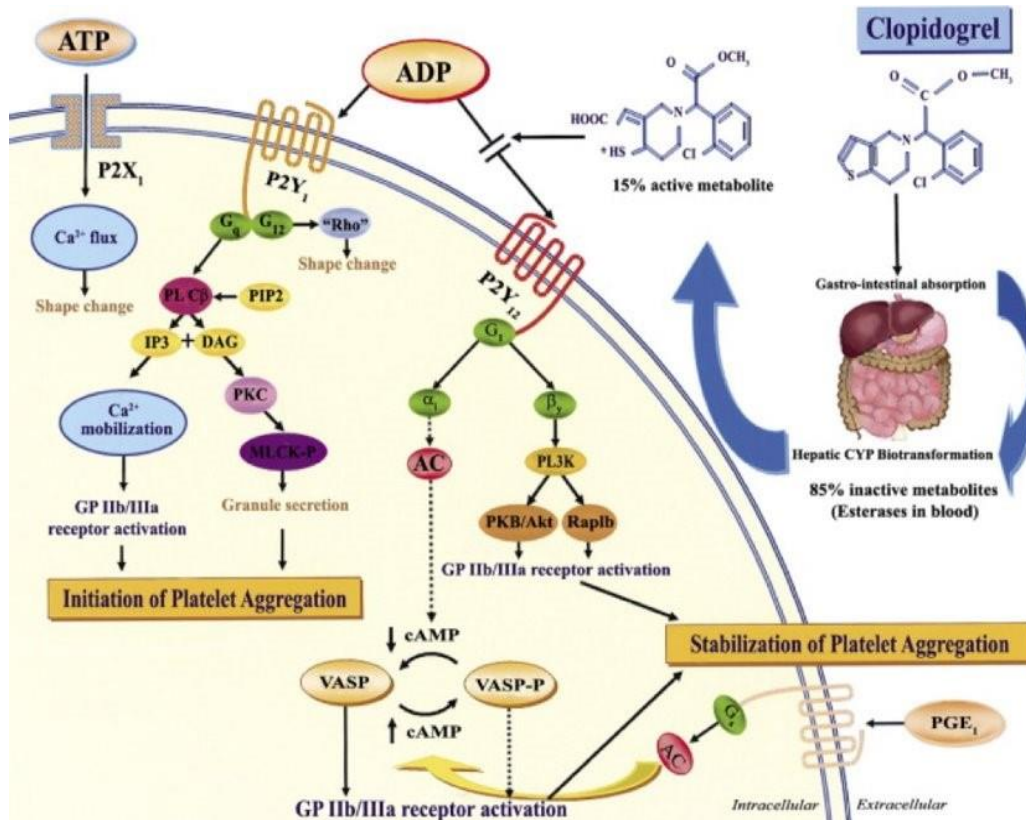


**Figure III:** Clopidogrel Metabolic Pathways  
Sanguhl, Katrin, et al. “Clopidogrel Pathway.”

**Pharmacodynamics:**

The active thiol metabolite of clopidogrel, CTM, (see Pharmacokinetics section above) selectively inhibits the binding of ADP to its platelet P2Y<sub>12</sub> receptor and the subsequent ADP-mediated activation of the glycoprotein GPIIb/IIIa complex, thereby inhibiting

platelet aggregation<sup>[15]</sup> (Figure 4). This action is irreversible. Consequently, platelets exposed to clopidogrel's active metabolite are affected for the remainder of their lifespan (about 7 to 10 days).<sup>[10]</sup> Platelet aggregation induced by agonists other than ADP is also inhibited by blocking the amplification of platelet activation by released ADP.



**Figure IV:** Clopidogrel Mechanism of active metabolite  
 Angiolillo, Dominick J., et al. "Variability in Individual Responsiveness to Clopidogrel."

### CYP2C19 and Clopidogrel:

Most of the studies investigating clopidogrel-induced change in platelet aggregation have focused on genetics, particularly *CYP2C19*. The *CYP2C19* enzyme contributes to both steps of the metabolic activation of clopidogrel. The *CYP2C19* gene is highly polymor-

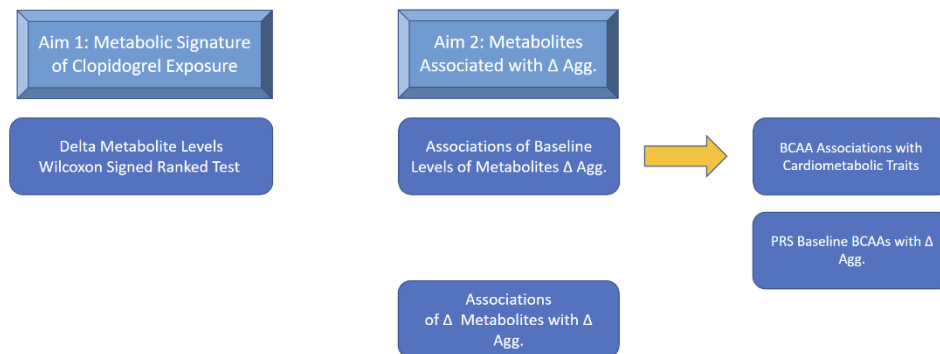
phic, having over 25 known variant alleles; the wild type being designated as the \*1 allele.<sup>[16]</sup> The two most common loss-of-function (LOF) alleles are *CYP2C19*\*2 (rs4244285) which has a frequency of approximately 15% in those of European and African descent, and 29-35% in those of Asian descent,<sup>[17]</sup> and *CYP2C19*\*3 (rs4986893) which has a frequency of less than 1% in those of European and African descent and approximately 2-9% in the Asian population.<sup>[17]</sup> There is also a gain-of-function allele leading to increased transcription of *CYP2C19* designated *CYP2C19*\*17 (rs12248560) present in 21% of European, 22% of African, and 3% of Asian descent individuals.

*CYP2C19* LOF alleles are inherited in an autosomal codominant fashion. Response to clopidogrel varies widely with inhibition of ADP-induced platelet aggregation being normally distributed across a broad range. Many studies have shown that *CYP2C19* LOF carriers have reduced active clopidogrel metabolite concentration, higher on-treatment platelet aggregation, and increased risk for ischemic events as compared with individuals homozygous for the wild type alleles.<sup>[18,19,20,21]</sup> The heterozygotes have platelet responsiveness that lie between wild type homozygotes and the LOF homozygotes.<sup>[21]</sup> *CYP2C19* genotypes are categorized as normal metabolizers (NM, \*1/\*1), intermediate metabolizers that have one normal or increased function allele and one LOF allele (IM, e.g. \*1/\*2 and \*1/\*3), poor metabolizers that have two LOF alleles (PM, e.g. \*2/\*2 and \*2/\*3), and rapid/ultra-rapid metabolizers (RM/UM, e.g. \*1/\*17 or \*17/\*17).<sup>[17]</sup>

There is considerable variability in clopidogrel response, and *CYP2C19* LOF alleles account for 12% of the variability.<sup>[21]</sup> Gaining insights into additional factors that influence variability in antiplatelet response could be important for identifying novel antiplatelet mechanisms or biomarkers for predicting response.

## Project Specific Aims

This project is comprised of two aims designed to gain a deeper understanding of factors underlying clopidogrel responsiveness using metabolomics. Aim 1, described in Chapter 2, will evaluate the metabolic signature of clopidogrel exposure. We will accomplish this aim by assessing changes in levels of 42 specific metabolites before and after a seven-day course of clopidogrel therapy. These metabolites were selected for their inclusion on a targeted metabolomic panel and included biogenic amines, neurotransmitters, purines, and metabolites from related pathways. Aim 2, described in Chapter 3, will expand on Aim 1 by addressing whether the assessed metabolites are associated with clopidogrel-induced change in platelet aggregation. Taken together these aims will help us to better understand the inter-individual variability in response to clopidogrel through the use of pharmacometabolomics.



## **Chapter 2: Determine the metabolic signature of clopidogrel.**

### **Introduction:**

Clopidogrel is an important antiplatelet drug, prescribed for reducing thrombotic events in patients with CVD and stroke. It is characterized by its selective inhibition of the ADP 2 receptor P2Y<sub>12</sub>. P2Y<sub>12</sub> inhibitor treatment, often with clopidogrel, is considered a standard practice in patients undergoing percutaneous coronary interventions in order to prevent thrombosis.<sup>[21]</sup> While generally effective, a subset of patients do not respond adequately to clopidogrel administration resulting in an increase in adverse clinical events in those individuals.<sup>[19]</sup>

Metabolites are small molecules present in blood, cells, tissues, or other biofluids including amino acids, lipids, and some sugars. By investigating circulating metabolite levels pre- and post-drug administration, we hypothesize that we can gain insights into the mechanisms and factors that influence variability in response to clopidogrel. Metabolic profiles integrate genetic and environmental influences.<sup>[22]</sup> Metabolites represent the downstream expression of the genome, and therefore can provide information that bridges the gap between the genome and the specific drug-response phenotype.<sup>[23]</sup>

In this investigation, we used a targeted metabolomic platform, developed at Leiden University in Dr. Thomas Hankemeier's laboratory, which includes biogenic amines, neurotransmitters, purines, and related pathways (n= 42 distinct metabolites). The purine pathway has previously been implicated in antiplatelet response to aspirin,<sup>[24]</sup> and we know that clopidogrel targets the purine pathway by inhibiting purinergic signaling through its inhibition of the ADP P2Y<sub>12</sub> receptor. Investigating metabolites from this pathway could inform our understanding of interpatient variability in clopidogrel-induced change in

platelet aggregation. The goal of this aim is to determine the metabolic signature of clopidogrel by identifying metabolites whose levels change significantly following administration of clopidogrel. After these metabolites are identified, we can test whether any are associated with drug response and could serve as potential biomarkers for identifying good or poor responders to clopidogrel.

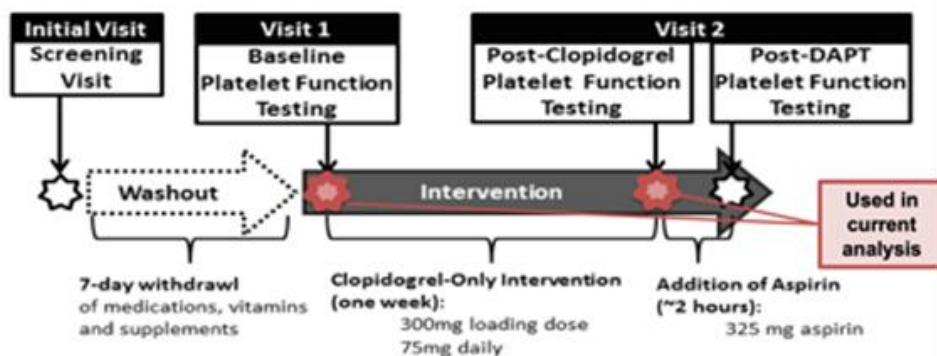
## **Methods:**

### **Study Population:**

The study population for this project is derived from a subset of the Pharmacogenetics of Antiplatelet Intervention (PAPI) study. Previous publications provide details regarding the study.<sup>[18,21,25]</sup> To summarize, the PAPI study was conducted in 687 healthy individuals of Amish descent to determine the genetic predictors of clopidogrel-induced change in platelet aggregation. The design of the PAPI study was to measure ADP-induced platelet aggregation (10  $\mu$ M) before and after 7 days of clopidogrel treatment (300 mg loading dose followed by 75 mg/d for 7d) (Figure 5).

To be eligible to participate in the PAPI study, participants had to be at least 20 years old, and free of the following conditions: severe hypertension; current pregnancy or lactation; use of prescription medications that could not be discontinued and would impact outcomes of the study; thrombocytosis or thrombocytopenia; abnormal thyroid stimulating hormone (TSH) levels; elevated liver enzymes; allergies to clopidogrel or aspirin; surgery in the 6 months prior to the study; history of gastrointestinal bleeding or other bleeding disorders; and current aspirin or clopidogrel use. Additional information regarding the inclusion and exclusion criteria have been previously published.<sup>[25]</sup>

Targeted metabolomic profiling of 42 amines was performed in serum samples in a subset of 198 PAPI subjects.<sup>[25]</sup> We used the metabolomic platform developed at Leiden University in Dr. Thomas Hankemeier’s laboratory, which includes biogenic amines, neurotransmitters, purines, and related pathways.<sup>[26]</sup> The baseline characteristics of the subset population are in Table 1.



**Figure V:** PAPI Design Schematic  
 Bozzi, Laura M., et al. “The Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study: Variation in Platelet Response to Clopidogrel and Aspirin.”

**Table I:** Baseline Characteristics for Metabolomics Subset of PAPI Study Participants

	<b>Male</b>	<b>Female</b>	<b>Combined</b>
<b>N</b>	N= 100	N= 98	N= 198
<b>Age, mean (years)</b>	44.7 ± 12.5	47.4 ± 14.4	46.0 ± 13.6
<b>BMI, mean (kg/m<sup>2</sup>)</b>	25.9 ± 3.6	27.7 ± 4.9	26.8 ± 4.5
<b>Systolic Blood Pressure, mean (mm Hg)</b>	116 ± 10	118 ± 14	117 ± 12
<b>Diastolic Blood Pressure, mean (mm Hg)</b>	71 ± 7	70 ± 6	70 ± 7
<b>HDL, mean (mg/dL)</b>	55 ± 14	61 ± 15	58 ± 15
<b>LDL, mean (mg/dL)</b>	137 ± 40	149 ± 51	141 ± 46
<b>Triglycerides, mean (mg/dL)</b>	62 ± 34	75 ± 37	69 ± 38

**Platelet Aggregometry:**

Platelet aggregation was assessed using optical aggregometry at baseline and after clopidogrel treatment to evaluate response.<sup>[25]</sup> Platelet aggregometry was completed at baseline before clopidogrel administration and post-clopidogrel alone. Platelet rich plasma (PRP) was prepared from whole blood and adjusted to a platelet count of 200,000 platelets/ $\mu$ L using a Coulter 3-part differential analyzer (Sysmex, Lancaster, PA). Platelet function was measured by optical aggregometry using a PAP8E aggregometer (Bio/Data Corporation, Horsham, PA) after stimulation of PRP samples with adenosine diphosphate (ADP; 10  $\mu$ M) using platelet-poor plasma as a referent as described previously.<sup>[25]</sup>

**Statistical Tests:**

The Wilcoxon signed-rank test is a non-parametric statistical test used to compare two related samples, matched samples, or repeated measurements on a single sample to assess whether their population mean ranks differ. This test was completed in Statistical Analysis System (SAS) (SAS version 9.4, Carey, NC) and assessed the changes in the 42 metabolites. This test identifies metabolites whose levels change in response to clopidogrel administration by testing if the change in their pre- vs post- levels differs significantly from 0. In order to adjust for multiple comparisons, false discovery rate was assessed, and q-values less than or equal to 0.05 were considered significant.

**Results:**

The Wilcoxon signed-rank test revealed that 23 metabolites were significantly changed in response to clopidogrel after false discovery rate correction (q-value <0.05). The majority

of the metabolites were decreased after clopidogrel treatment (n= 22 metabolites decreased and 1 metabolite increased). The one metabolite significantly increased in response to clopidogrel was methionine sulfoxide (q-value=0.025). Tyrosine was the metabolite most significantly changed (q-value =0.0004); with its levels decreasing approximately 8% after taking clopidogrel (Table 2). Many of these significant metabolites are classified as neutral amines (22 out of the 23) and considered hydrophobic (12 out of the 23).

**Table II:** Wilcoxon Signed-Rank Test Results, Delta Metabolite Levels

<b>Metabolite</b>	<b>Signed rank statistic</b>	<b>p-value</b>	<b>q-value</b>
<b>Tyrosine</b>	-6266.5	3.1E-17	1.3E-15
<b>Tryptophan</b>	-5540.5	3.0E-13	6.3E-12
<b>Serotonin</b>	-5084.5	3.4E-11	4.8E-10
<b>Phenylalanine</b>	-4274.5	4.32E-08	4.5E-07
<b>Methionine</b>	-3657.5	3.53E-06	2.5E-05
<b>Valine</b>	-3570.5	6.15E-06	3.5E-05
<b>Lysine</b>	-3553.5	6.85E-06	3.5E-05
<b>Isoleucine</b>	-3538.5	7.52E-06	3.5E-05
<b>Kynurenine</b>	-3381.5	1.95E-05	1.6E-05
<b>Leucine</b>	-3255.5	4.06E-05	0.0002

Table II Continued

<b>Arginine</b>	-3154.5	7.16E-05	0.0003
<b>2-Aminoadipic Acid</b>	-3118.5	8.71E-05	0.0003
<b>Alanine</b>	-2755.5	5.56E-04	0.0019
<b>Citrulline</b>	-2715.5	6.73E-04	0.0021
<b>3-Methylhistidine</b>	-2668.5	8.38E-04	0.0022
<b>Asparagine</b>	-2570.5	0.0013	0.0034
<b>Gamma-L-Glu- tamyl-L-Alanine</b>	-2494.5	0.0018	0.0044
<b>Hydroxylysine</b>	-2283.5	0.0044	0.0103
<b>Methionine Sulfox- ide</b>	2037.5	0.0113	0.0250
<b>N6 N6 N6 Trime- thyl-L-lysine</b>	-1872.5	0.0200	0.0420
<b>Threonine</b>	-1828.5	0.0231	0.0454
<b>Proline</b>	-1820.5	0.0238	0.0454
<b>4-Hydroxy-Proline</b>	-1776.5	0.0274	0.0500
<b>O-Phosphoethanola- mine</b>	-1759.5	0.0289	0.0506
<b>Serine</b>	-1493.5	0.0642	0.1079
<b>Homoserine</b>	-1295.5	0.1088	0.1758

Table II Continued

Histidine	-1208.5	0.1348	0.2097
Alpha-Aminobutyric Acid	-1192.5	0.1401	0.2102
Taurine	-1071.5	0.1851	0.2681
Glycine	-1000.5	0.2161	0.3025
Ethanolamine	-943.5	0.2435	0.3226
O-Acetyl-L-Serine	-939	0.2458	0.3226
Putrescine	-810.5	0.3166	0.4029
Glutamine	-782.5	0.3337	0.4122
Homo-L-Arginine	-633.5	0.4340	0.5208
Glutamic Acid	-547.5	0.4990	0.5822
1-Methylhistidine	-480.5	0.5531	0.6113
Aspartic Acid	-381.5	0.6377	0.6854
Glycylglycine	159.5	0.8440	0.8440
Ornithine	272.5	0.7366	0.7546
DL-3-Aminoisobutyric Acid	364.5	0.6528	0.6854
SDMA	503.5	0.5342	0.6064

**Discussion:**

CVD is the number one cause of death globally.<sup>[1]</sup> Clopidogrel is commonly prescribed as part of a dual antiplatelet therapy used as the standard of care for reducing recurrent thrombotic events in patients with CVD. Clopidogrel targets the purine pathway by inhibiting purinergic signaling through its inhibition of the ADP P2Y<sub>12</sub> receptor. There is considerable variability in clopidogrel response.<sup>[25]</sup> By studying small molecules found circulating in the blood (i.e., metabolomics), we can identify pathways perturbed by clopidogrel. We chose to utilize a targeted metabolomic approach focused on pathways related to purines including biogenic amines, neurotransmitters, and purines given the importance of the purine pathway in clopidogrel's mechanism of action. Our goal was to determine the metabolic signature of clopidogrel, so to explore the metabolites that change in response to the administration of clopidogrel.

After a week of clopidogrel treatment, we identified 23 metabolites whose levels changed significantly in response to clopidogrel. The vast majority of the metabolites were decreased in response to clopidogrel treatment, with only one metabolite, methionine sulfoxide, increasing after clopidogrel exposure. Methionine sulfoxide promotes alteration in the hydrolysis of ATP and ADP.<sup>[27]</sup> It is possible that this increase in methionine sulfoxide is a byproduct of the reaction converting clopidogrel to its active metabolite. Tyrosine was the metabolite most significantly decreased in response to clopidogrel. Other metabolites that were significantly decreased after clopidogrel include tryptophan, serotonin, phenylalanine, and methionine. Many of these metabolites that made up the clopidogrel signature have biological plausibility for impacting platelet aggregation, the most significant from our data, tyrosine and serotonin are as described below.

We observed a significant decrease in tyrosine levels after clopidogrel exposure. Thyroid hormones are tyrosine-based hormones produced by the thyroid gland. They function primarily for the regulation of metabolism. These hormones include triiodothyronine (T3) and thyroxine (T4). In a study conducted by Mousa, TSH enhanced platelet aggregation.<sup>[28]</sup> Once clopidogrel inhibits ADP-induced platelet aggregation the tyrosine levels would be predicted to decrease because T3 and T4 are not being used in platelet activity.<sup>[28]</sup> Given tyrosine's involvement in platelet activation and aggregation, it is plausible that levels could be influenced by clopidogrel. Therefore, we hypothesize that the significant decrease we saw in tyrosine levels could be due to the inhibition of platelet aggregation after clopidogrel treatment.

Serotonin was also significantly decreased after clopidogrel exposure. Serotonin has been shown to be a weak platelet activator by enhancing platelet alpha granule release and plasmatic coagulation.<sup>[29]</sup> During platelet aggregation, serotonin is released into circulation, along with other aggregating factors, and becomes a stimulus for platelet aggregation.<sup>[30]</sup> Therefore, given that clopidogrel inhibits ADP-induced platelet aggregation, it follows that circulating serotonin levels would decrease after clopidogrel administration. Other studies performed in mammal models have also seen levels of serotonin decrease in response to clopidogrel.<sup>[31,32]</sup> Interestingly, selective serotonin reuptake inhibitors (SSRIs) fluoxetine and fluvoxamine can inhibit *CYP2C19*, therefore, taking SSRIs as well as clopidogrel could lead to a decrease in clopidogrel response.<sup>[33]</sup> Some SSRIs prevent serotonin by blocking the serotonin reuptake transporter (SERT), therefore, may affect thrombosis. The inhibition of SERT is what leads to the inhibition of platelets by the SSRIs.<sup>[34]</sup> Tseng et al described a mechanism associated with SERT that may contribute

to functional effects of an SSRI (citalopram) including P2Y<sub>12</sub> signaling.<sup>[35]</sup> Another study by Roweth et al found that inhibition of SERT-dependent 5-HT uptake by citalopram does not correlate with inhibition of platelet function in vitro.<sup>[34]</sup> Thus, further research evaluating serotonin as a potential biomarker for clopidogrel response would be informative.

A major strength of this project is that metabolite levels were measured before and after a standardized administration of clopidogrel in the same individuals. The Amish population is a relatively homogenous population to study with regard to diet and lifestyle. They also take very few prescription medications. Therefore, we can minimize the impact of other potentially confounding factors while investigating changes in metabolite levels that are likely due solely as a consequence of drug administration.

Our study does have some limitations that should be acknowledged. First, a relatively small sample size of healthy volunteers was used, which may inhibit our ability to detect metabolites with small changes following clopidogrel administration. Second, our study was not designed to evaluate every metabolite that changes in response to clopidogrel given that we used a limited platform of targeted metabolites. It is certainly possible that other metabolites could be impacted by clopidogrel administration.

In summary, tyrosine was most significantly changed in response to clopidogrel, and there is biological plausibility that tyrosine plays a role in platelet aggregation which could explain the significant decrease in tyrosine levels. Serotonin was also found to significantly decrease in response to clopidogrel. Serotonin is involved as stimulus for platelet aggregation, which can explain this response. Further research should be done to replicate these findings. This study was conducted in healthy volunteers, and it would be

interesting to see if these findings would be seen in patients with CVD as well. This study was a short-term intervention study, so future research evaluating longer-term clopidogrel treatment would also be useful.

### **Chapter 3: Determine the metabolites associated with clopidogrel-induced change in platelet aggregation**

#### **Introduction:**

In Chapter 2, I determined the metabolites that were significantly changed in response to administration of clopidogrel. Levels of most purine metabolites decreased after clopidogrel exposure. The metabolite that was most significantly changed was tyrosine (Figure 6, Table 2). Having now established the metabolite changes accompanying a 7-day course of clopidogrel administration, I sought to assess whether metabolite levels (at baseline or the delta values) associate with ADP-induced *ex vivo* platelet aggregation in response to clopidogrel. Metabolites represent the downstream expression of the genome,<sup>[23]</sup> therefore analyzing the associations between metabolite levels and drug response could lead to important discoveries that could serve as novel biomarkers for antiplatelet response or lead to new insights into the mechanism of action of clopidogrel or mechanisms underlying response variability.

I have assessed both the baseline metabolite levels and delta metabolite levels for their associations with clopidogrel-induced change in platelet aggregation. Baseline metabolite levels may serve as biomarkers for predicting response or non-response while delta metabolite levels could provide mechanistic insights into variability in clopidogrel responses.

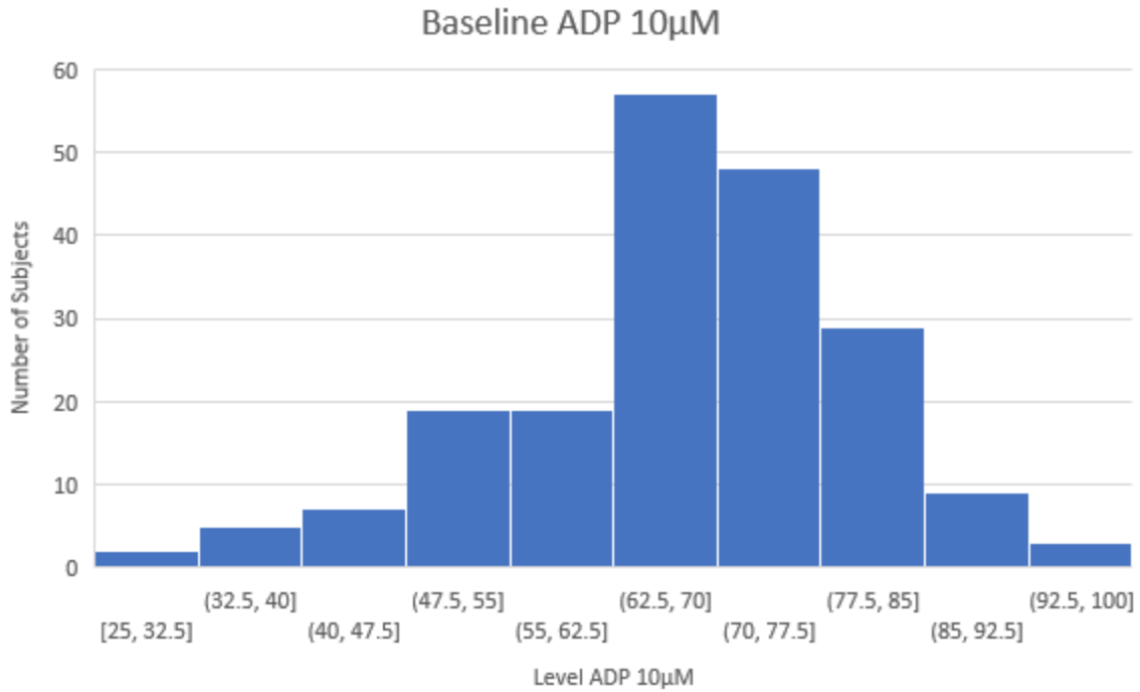
Given the importance of *CYP2C19* on clopidogrel activation (see Chapter 1), we also tested whether *CYP2C19* might impact metabolite levels. The most common LOF allele is *CYP2C19*\*2 rs4244285 (c.681G>A) which creates an abnormal splice site in exon 5,

altering the mRNA reading frame, resulting in a nonfunctional protein.<sup>[37]</sup> Thus, *CYP2C19\*2* is the variant we focused on for these analyses.

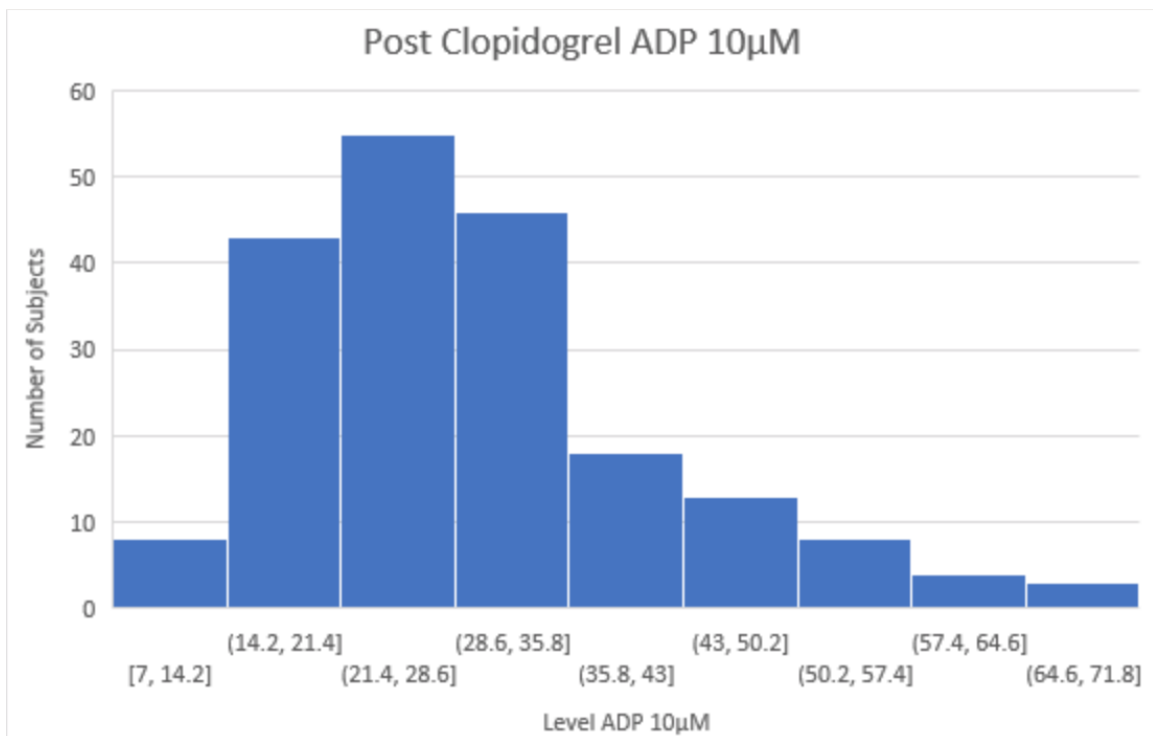
Polygenic risk scores (PRS) are a way of testing the risk of developing a complex trait, or disease, based on the burden of identified variants from many genetic loci.<sup>[38]</sup> In addition to evaluating the effect of the *CYP2C19\*2* variant, this aim also assessed the impact of a PRS for metabolite levels which are found to impact clopidogrel response. This aim will give us a more comprehensive examination of mechanisms underlying the variation in clopidogrel response, and could have long-term implications for improving our understanding of how to optimize antiplatelet therapy.

### **Methods:**

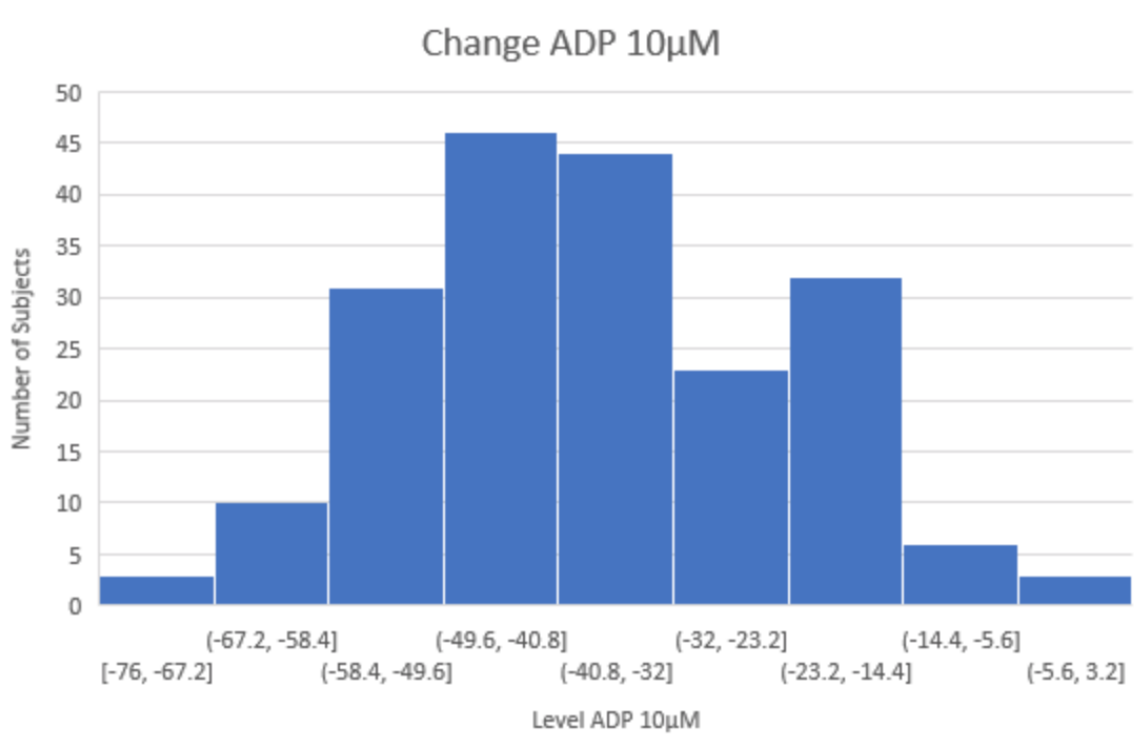
Throughout this chapter, clopidogrel-induced change in platelet aggregation was assessed through measurement of adenosine diphosphate (ADP)-induced platelet aggregation (10  $\mu$ M) post- minus pre-clopidogrel administration, thus a negative number represents a greater response to clopidogrel. As described in Chapter 2, platelet function in PAPI study participants was measured by optical aggregometry using a PAP8E aggregometer after stimulation of platelet rich plasma (PRP) samples with ADP (2, 5, 10, and 20  $\mu$ M). The ADP 10 $\mu$ M dose was chosen for these analyses because it is the middle agonist dose and provides a broad distribution of platelet aggregation to evaluate clopidogrel-induced change in platelet aggregation (Figures 6, 7, 8).



**Figure VI:** Histogram of Baseline Platelet Aggregation with ADP 10µM



**Figure VII:** Histogram of Post-Clopidogrel Platelet Aggregation with ADP 10µM



**Figure VIII:** Histogram of Change in Platelet Aggregation with ADP 10µM in Response to Clopidogrel

The baseline metabolites are defined as the metabolite levels before clopidogrel exposure. Post-clopidogrel metabolites are defined as the metabolite levels after a 300 mg loading dose as well as 7 days of 75mg clopidogrel treatment. The delta metabolites are defined as post- minus pre-clopidogrel treatment.

**Statistical Analysis:**

The metabolite levels were checked for normality prior to performing statistical analyses. To test correlations between baseline metabolites and change in platelet aggregation, as well as the delta metabolites with change in platelet aggregation, I performed Pearson

Correlation tests. In order to correct for multiple comparisons that were performed, significance was defined according to a Bonferroni correction, with a p-value less than or equal to 0.001 (0.05/42 metabolites) considered significant. P-values less than or equal to 0.05 were considered as nominally significant. To control for the influence of the population's different age and sex distributions, linear regression was performed, adjusting for age, sex, and baseline level (for delta metabolites). This analysis was performed in SAS (SAS version 9.4, Carey, NC).

We tested whether *CYP2C19\*2* might impact metabolite levels using a mixed model linear regression framework under an additive and recessive model and adjusted for age and sex. A relationship matrix was included as a random effect to account for relatedness among study subjects. The analysis was performed through MMAP and the Omics Analysis, Search and Information System (OASIS) was used to visualize the results. OASIS was created at the University of Maryland, Baltimore and is an information system for analyzing, searching and visualizing associations between phenotypes, genotypes, transcriptomics, and metabolomics.<sup>[39]</sup>

In order to generate PRSs for baseline metabolite levels associated with clopidogrel-induced change in platelet aggregation we used a published metabolomics GWAS (n=8545) to identify alleles associated with change in metabolites.<sup>[40]</sup> To generate the PRS we included SNPs with a p-value below 1.00E-07, analyzing thresholds of 1.00E-07, 1.00E-08, 1.00E-10, and 1.00E-11. We analyzed different windows of clumping for SNPs in LD, clumping window of 0.8MB, 0.2MB, and no clumping. Using PRSice Software<sup>[38]</sup> we calculated the metabolite PRSs for our Amish participants and tested the PRSs for their association with change in platelet aggregation at the ADP 10 $\mu$ M dose.

**Results:**

**Associations of baseline levels of metabolites with clopidogrel-induced change in platelet aggregation:**

After Bonferroni correction, none of the baseline metabolites reached statistical significance for association with platelet aggregation (Table 3). However, baseline levels of the branched chain amino acids (BCAAs) were the most significant metabolites and were close to the threshold for significance: leucine (p-value 0.0023), valine (p-value 0.0036), and isoleucine (p-value 0.0082). Four other metabolites reached a nominal significance level less than 0.05 for their association with Platelet Aggregation: 2-aminoadipic acid, 3-methylhistidine, glycylglycine, and 1-methylhistidine (Table 3).

**Table III:** Correlation between Baseline Metabolites and Change in Platelet Aggregation (post-pre)

<b>Baseline Metabolites</b>	<b>Correlation with Change in Platelet Aggregation</b> Pearson Correlation Coefficient P value
<b>Leucine</b>	<b>0.215</b> <b>2.30E-03</b>
<b>Valine</b>	<b>0.205</b> <b>3.60E-03</b>
<b>Isoleucine</b>	<b>0.187</b> <b>8.20E-03</b>
2-Aminoadipic Acid	0.167 0.01
3-Methylhistidine	0.162 0.02

Table III Continued

Glycylglycine	-0.157 0.02
1-Methylhistidine	0.145 0.04
Methionine Sulfoxide	0.137 0.05
Ethanolamine	-0.131 0.06
Ornithine	0.123 0.08
Aspartic Acid	-0.116 0.10
Serine	-0.115 0.10
Serotonin	-0.105 0.13
Kynurenine	0.102 0.14
Glutamic Acid	0.095 0.18
Arginine	-0.093 0.19
N6 N6 N6 Trimethyl-L-Lysine	0.090 0.20

Table III Continued

O-Acetyl-L-Serine	0.085 0.23
Alpha-aminobutyric acid	0.084 0.23
Phenylalanine	0.078 0.27
Hydroxylysine	0.073 0.30
Histidine	0.066 0.34
Putrescine	-0.063 0.37
Glycine	-0.055 0.43
Citrulline	0.055 0.43
4-Hydroxy-Proline	0.046 0.51
SDMA	-0.045 0.52
DL-3-Aminoisobutyric Acid	-0.037 0.60
Taurine	-0.035 0.62

Table III Continued

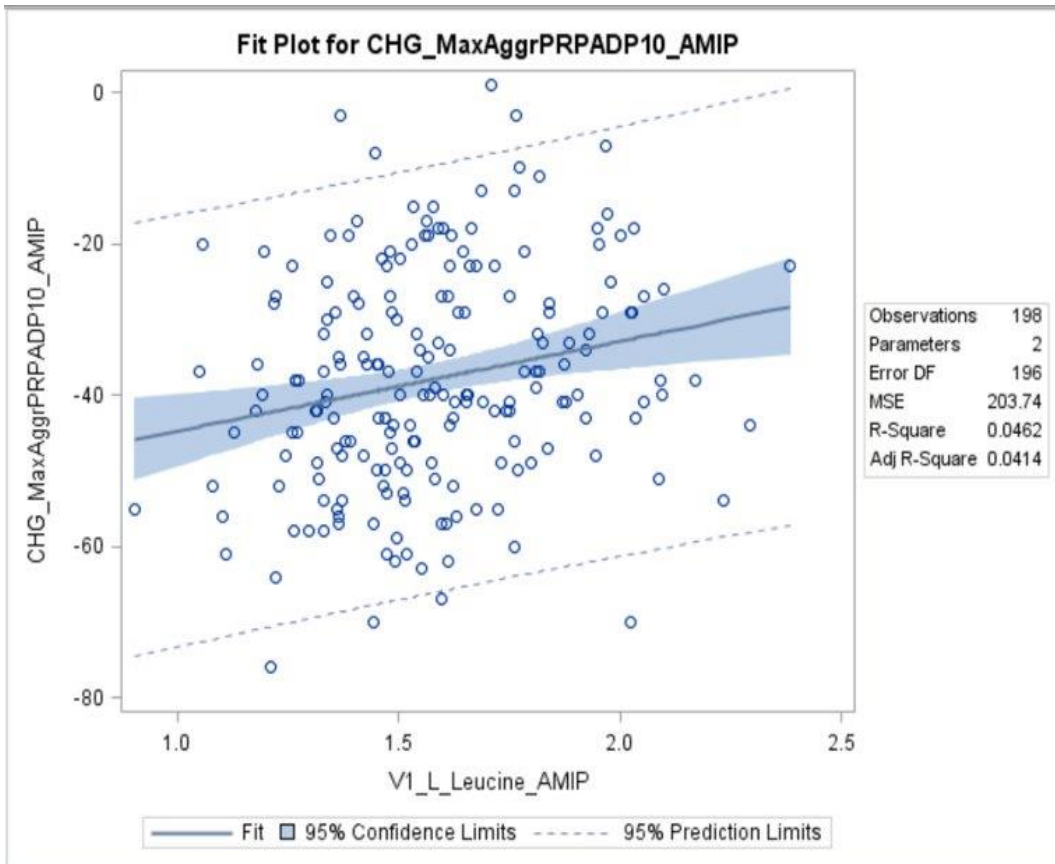
Lysine	0.034 0.63
Gamma-L-Glutamyl-L-Alanine	0.031 0.66
Tryptophan	0.031 0.66
O-phosphoethanolamine	-0.026 0.71
Tyrosine	0.026 0.71
Proline	0.026 0.71
Glutamine	-0.024 0.73
Homoserine	-0.024 0.73
Methionine	0.022 0.74
Homo-L-Arginine	-0.017 0.80
Threonine	-0.011 0.87
Asparagine	0.009 0.89

Table III Continued	
Alanine	0.008 0.90

The BCAA results were similar after adjusting for age and sex. Leucine had a suggestive p-value of 0.0032 (Table 4, Figure 9). Valine had a suggestive p-value of 0.0052 (Table 5, Figure 10). Isoleucine had a suggestive p-value of 0.0117 (Table 6, Figure 11).

**Table IV:** Linear Regression Association between Baseline Leucine and Clopidogrel-induced Change in Platelet Aggregation, Adjusted for Age and Sex

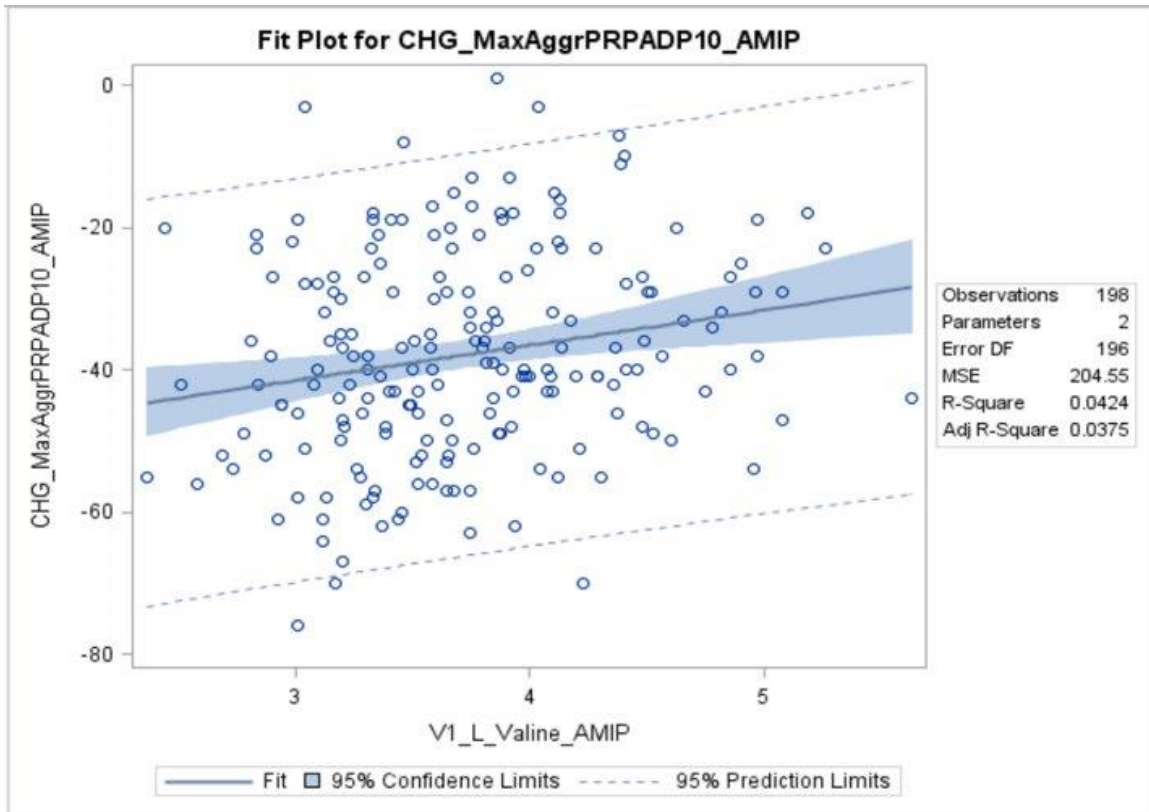
Variable	DF	Parameter Estimate	Standard of Error	t value	p value
<b>Intercept</b>	1	-58.48	8.44	-6.92	<.0001
<b>Age</b>	1	2.31E-03	7.6E-02	0.03	0.975
<b>Sex</b>	1	0.70	2.17	0.32	0.747
<b>Leucine</b>	1	12.31	4.13	2.98	3.20E-03



**Figure IX:** Linear Regression Association between Baseline Leucine and Clopidogrel-induced Change in Platelet Aggregation

**Table V:** Linear Regression Association between Baseline Valine and Clopidogrel-induced Change in Platelet Aggregation, Adjusted for Age/Sex

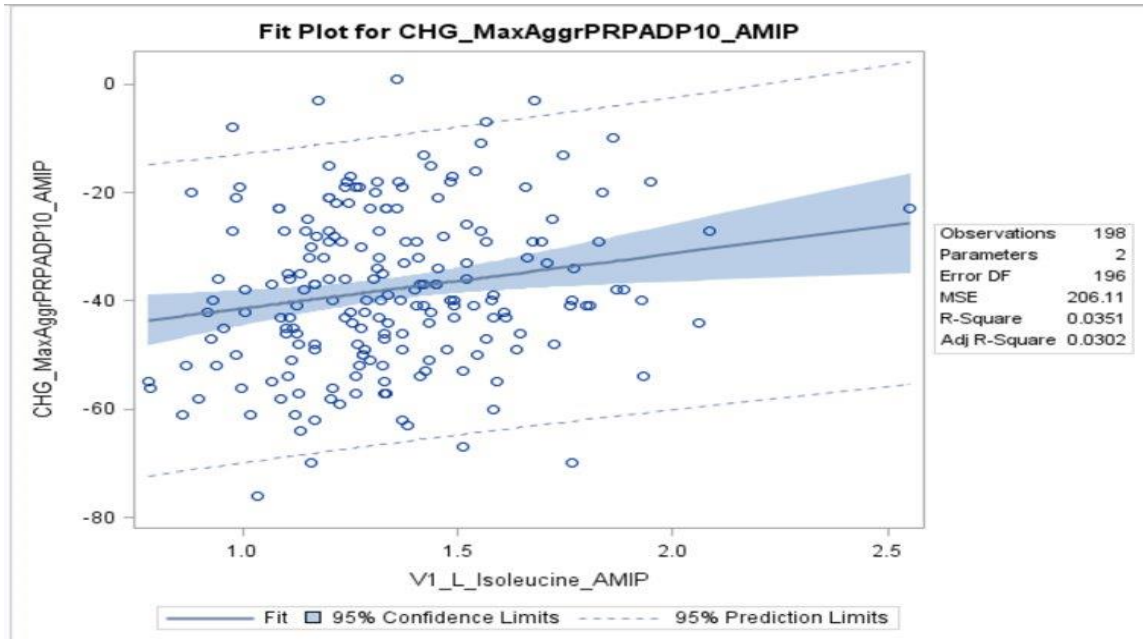
Variable	DF	Parameter Estimate	Standard of Error	t value	p value
<b>Intercept</b>	1	-55.66	7.95	-7.00	<.0001
<b>Age</b>	1	-2.53E-03	0.07	-0.03	0.9736
<b>Sex</b>	1	-0.26	2.09	-0.13	0.8982
<b>Valine</b>	1	4.91	1.73	2.82	5.20E-03



**Figure X:** Linear Regression Association between Baseline Valine and Clopidogrel-induced Change in Platelet Aggregation

**Table VI:** Linear Regression Association between Baseline Isoleucine and Clopidogrel-induced Change in Platelet Aggregation, Adjusted for Age/Sex

Variable	DF	Parameter Estimate	Standard of Error	t value	p value
<b>Intercept</b>	1	-51.49	7.21	-7.13	<.0001
<b>Age</b>	1	0.01	0.07	0.18	0.855
<b>Sex</b>	1	-0.27	2.11	-0.13	0.897
<b>Isoleucine</b>	1	9.94	3.90	2.55	0.011



**Figure XI:** Linear Regression Association between Baseline Isoleucine and Clopidogrel-induced Change in Platelet Aggregation

To determine whether the observed associations between metabolite levels and clopidogrel-induced change in platelet aggregation were driven by associations with baseline platelet aggregation, or whether they were only present after clopidogrel exposure, I tested the associations between baseline metabolites and baseline platelet aggregation for the metabolites with p-value less than 0.05. There was no correlation between baseline metabolites and baseline platelet aggregation (Table 7).

**Table VII:** Correlation between Baseline Metabolites and Baseline Platelet Aggregation

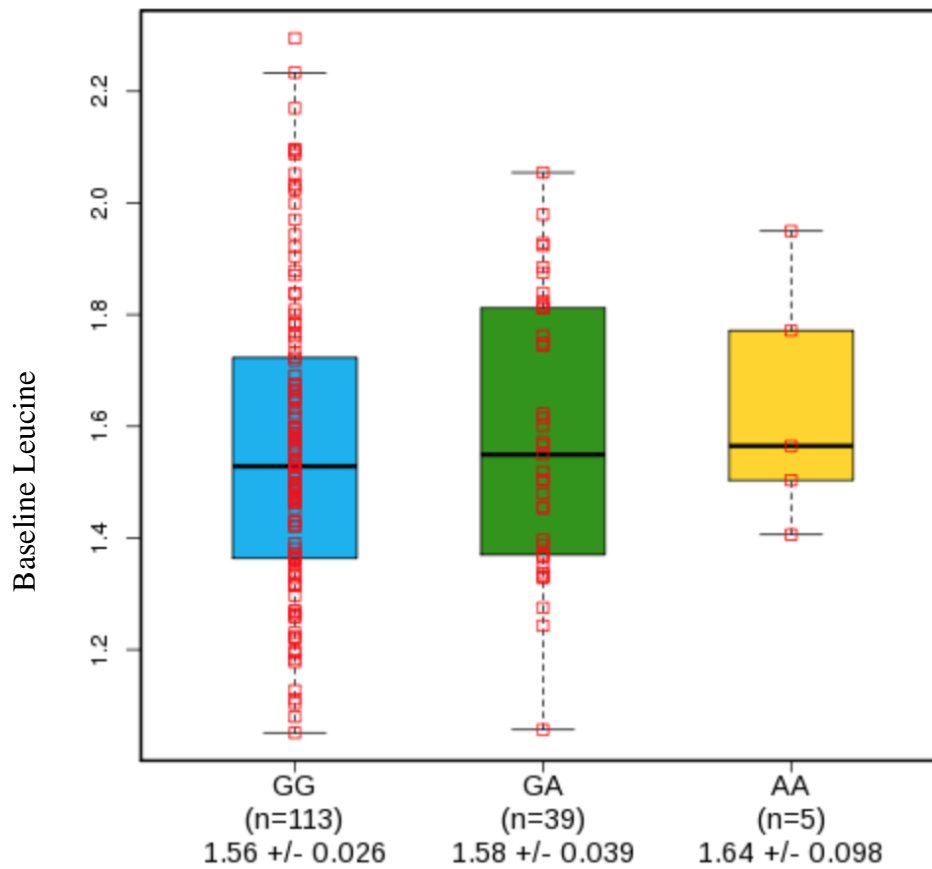
<b>Baseline Metabolites</b>	<b>Correlation with Baseline Platelet Aggregation</b> Correlation Coefficient p-value
Glycylglycine	0.104 0.14
2-Aminoadipic Acid	-0.089 0.21
Leucine	-0.088 0.21
Isoleucine	-0.077 0.28
Valine	-0.076 0.28
1-Methylhistidine	-0.052 0.46
3-Methylhistidine	-0.030 0.67

***CYP2C19* effect on Baseline Metabolites:**

The *CYP2C19*\*2 allele had no significant effect on the baseline levels of BCAAs as shown in Table 8, Figure 12, Table 9, Figure 13, and Table10, and Figure 14.

**Table VIII:** Linear Regression Association between *CYP2C19*\*2 and Baseline Leucine, Adjusted for Age/Sex

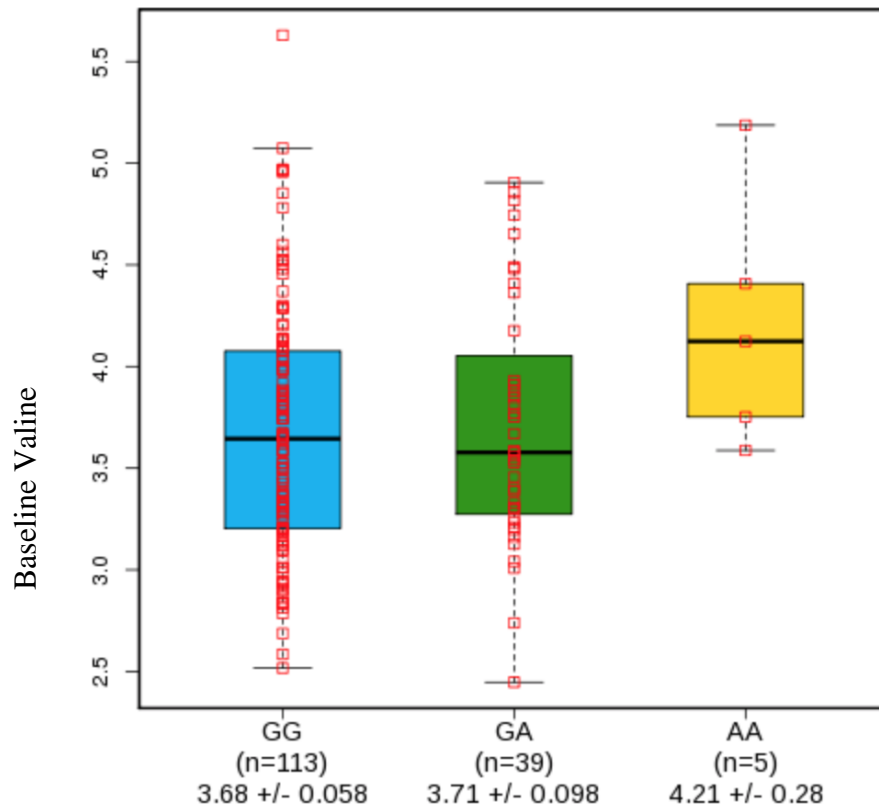
Covariates	Beta	SE	p-value
rs4244285	0.02	0.03	5.94E-01
Age	3.14E-03	1.44E-03	3.14E-02
Sex	-0.17	0.04	1.58E-05



**Figure XII:** Boxplot: Association between *CYP2C19* rs4244285 and Baseline Leucine, Additive Model (p-value 0.594), Recessive Model (p-value 0.591)

**Table IX:** Linear Regression Association between *CYP2C19*\*2 and Baseline Valine, Adjusted for Age/Sex

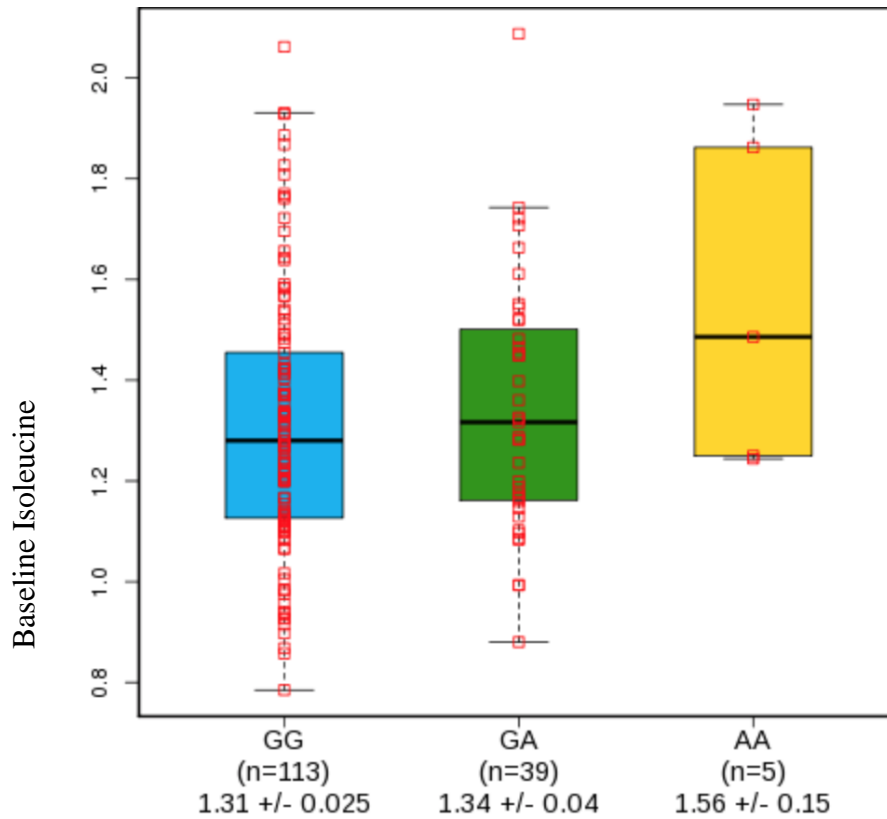
Covariates	Beta	SE	p-value
rs4244285	0.11	0.09	2.09E-01
Age	9.35E-03	3.42E-03	7.12E-03
Sex	-0.25	0.09	1.02E-02



**Figure XIII:** Boxplot: Association between *CYP2C19* rs4244285 and Baseline Valine, Additive Model (p-value 0.209), Recessive Model (p-value 0.058)

**Table X:** Linear Regression Association between *CYP2C19*\*2 and Baseline Isoleucine, Adjusted for Age/Sex

Covariates	Beta	SE	p-value
rs4244285	0.05	0.03	1.32E-01
Age	2.27E-03	1.50E-03	1.34E-01
Sex	-0.13	0.04	1.29E-03



**Figure XIV:** Boxplot: Association between *CYP2C19* rs4244285 and Baseline Isoleucine, Additive Model (p-value 0.132), Recessive Model (p-value 0.054)

**Associations of baseline levels of metabolites with cardiometabolic traits:**

Given the known association between BCAAs and cardiometabolic traits, I performed linear regression analysis to test the association between baseline BCAAs and BMI, LDL, HDL, triglycerides, and blood pressure within our PAPI study. We observed significant association between these metabolites and these cardiometabolic traits (Table 11).

**Table XI: Linear Regression Association Cardiometabolic Traits and Baseline BCAAs**

<b>Cardiometabolic Trait</b>	<b>Covariates</b>	<b>Beta</b>	<b>SE</b>	<b>p-value</b>
BMI	Leucine	5.46	1.15	4.38E-06
	Valine	3.34	0.47	2.47E-11
	Isoleucine	5.97	1.10	2.04E-07
Blood Pressure	Leucine	4.41	3.36	0.19
	Valine	2.41	1.45	9.79E-02
	Isoleucine	6.23	3.25	5.72E-02
HDL	Leucine	-23.28	3.84	6.86E-09
	Valine	-10.63	1.65	8.98E-10
	Isoleucine	-24.59	3.68	2.40E-10
LDL	Leucine	-11.05	12.44	0.37
	Valine	-2.23	5.40	0.68
	Isoleucine	-6.67	12.13	0.58
Triglycerides	Leucine	36.94	9.86	2.38E-04
	Valine	23.00	4.11	7.58E-08
	Isoleucine	47.54	9.35	8.61E-07

**Associations of delta metabolites with clopidogrel-induced change in platelet aggregation:**

After Bonferroni correction, none of the delta metabolites reached statistical significance for their association with clopidogrel-induced change in platelet aggregation (Table 12). However, I found nominal significance in the correlation between delta metabolites and change in platelet aggregation for 2-aminoadipic acid (p-value 0.02), and O-phosphoethanolamine (p-value 0.02).

**Table XII:** Correlation between Delta Metabolites and Change in Platelet Aggregation (post-pre)

<b>Delta Metabolites</b>	<b>Correlation with Change in Platelet Aggregation</b> Correlation Coefficient p-value
2-Aminoadipic Acid	-0.163 0.02
O-Phosphoethanolamine	-0.161 0.02
1-Methylhistidine	-0.127 0.07
Isoleucine	-0.127 0.07
Leucine	-0.123 0.08
O-Acetyl-L-Serine	0.094 0.18
3-Methylhistidine	-0.089 0.20

Table XII Continued

Glutamic Acid	-0.088 0.21
Homo-L-Arginine	0.083 0.24
Citrulline	-0.080 0.25
Threonine	0.077 0.27
Kynurenine	-0.067 0.34
Asparagine	0.063 0.37
Alpha-Aminobutyric Acid	-0.061 0.38
Valine	-0.061 0.39
Proline	-0.056 0.42
N6 N6 N6 Trimethyl-L-Lysine	0.056 0.42
DL-3-Aminoisobutyric Acid	-0.050 0.47
Ethanolamine	0.047 0.50

Table XII Continued

SDMA	0.046 0.51
Taurine	-0.044 0.53
Glycylglycine	0.044 0.53
Arginine	0.040 0.56
Glycine	0.036 0.61
Homoserine	0.035 0.61
Serotonin	-0.033 0.64
Methionine	0.029 0.67
Tryptophan	-0.029 0.67
Phenylalanine	-0.029 0.67
4-Hydroxy-Proline	-0.028 0.68
Serine	0.027 0.69

Table XII Continued

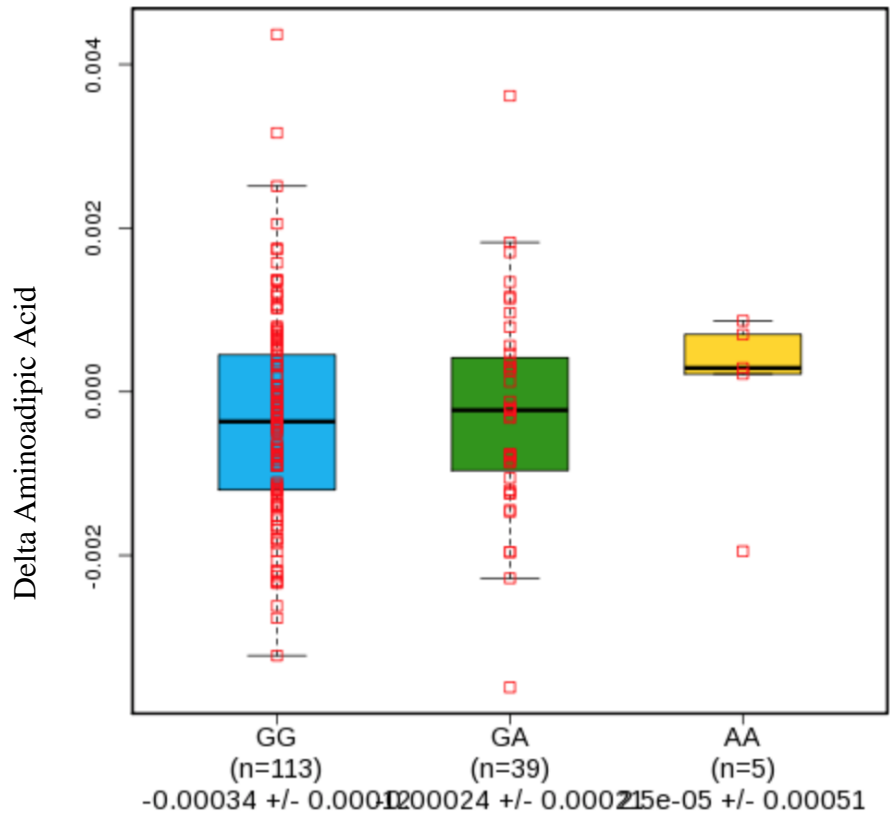
Histidine	0.024 0.72
Aspartic acid	0.018 0.79
Tyrosine	0.018 0.79
Ornithine	-0.014 0.83
Lysine	0.013 0.84
Gamma-L-Glutamyl-L-Alanine	0.009 0.89
Methionine Sulfoxide	-0.008 0.90
Glutamine	-0.008 0.91
Alanine	0.005 0.93
Putrescine	-0.003 0.96
Hydroxylysine	-0.002 0.97

### Association of *CYP2C19* with Delta Metabolites:

Given the importance *CYP2C19* has on the formation of the active metabolite of clopidogrel, we tested the influence *CYP2C19*\*2 on delta metabolite levels in response to clopidogrel. I tested this with the most suggestive findings from the correlation analysis between delta metabolites and change in platelet aggregation, 2-aminoadipic acid and O-phosphoethanolamine. The *CYP2C19*\*2 allele had no significant effect on the delta metabolites, shown in Table 13 and Table 14; and box plots are shown in Figure 15, and Figure 16.

**Table XIII:** Linear Regression Association between *CYP2C19* and Delta 2-Aminoadipic Acid, Adjusted for Age/Sex

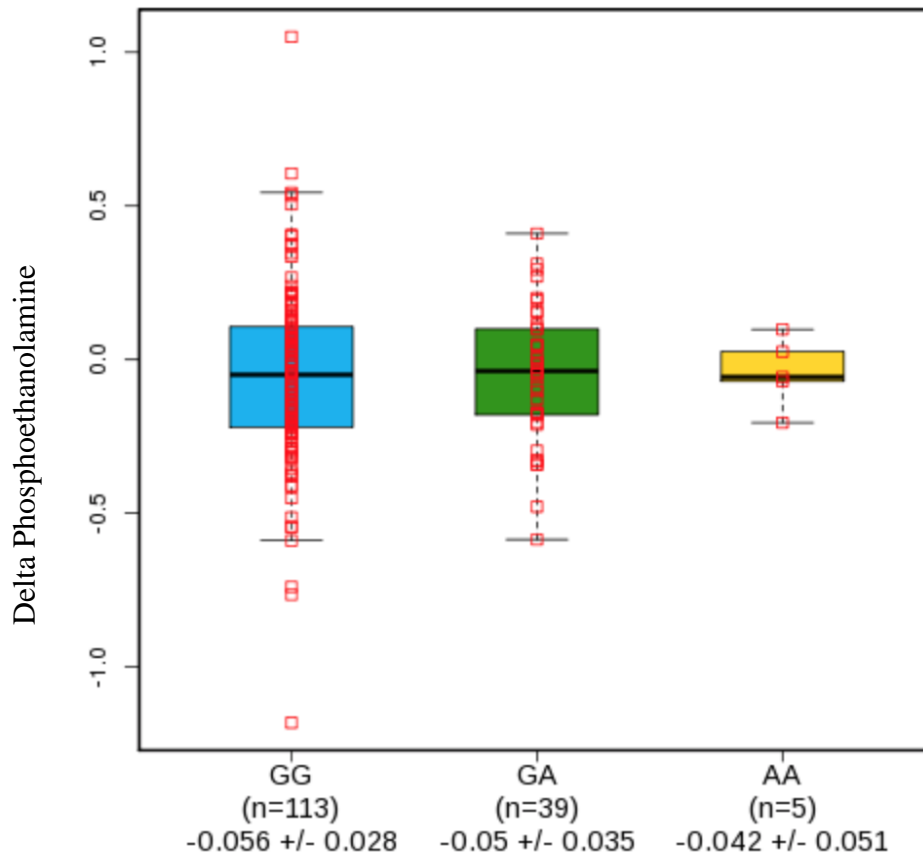
Covariates	Beta	SE	p-value
rs4244285	1.22E-04	1.93E-04	5.28E-01
Age	4.167E-06	7.532E-06	5.81E-01
Sex	-8.611E-05	2.04E-04	6.74E-01



**Figure XV:** Boxplot: Association between *CYP2C19* (rs4244285) and Delta 2-Amino adipic Acid, Additive Model (p-value 0.528), Recessive Model (p-value 0.571)

**Table XIV:** Linear Regression Association between *CYP2C19* and Delta O-Phosphoethanolamine, adjusted for Age/Sex

Covariates	Beta	SE	p-value
rs4244285	6.37E-03	0.04	8.82E-01
Age	2.02E-03	1.61E-03	2.12E-01
Sex	-0.02	0.04	5.78E-01



**Figure XVI:** Boxplot: Association between *CYP2C19* (rs4244285) and Delta O-Phosphoethanolamine, Additive Model (p-value 0.882), Recessive Model (p-value 0.841)

**PRS Results:**

To confirm our PRS instrument, we tested the PRS models for association with baseline BCAA levels and found that only the leucine PRS was significantly associated with BCAA levels in our data set with a p-value of 1.8E-03. The PRS for valine and isoleucine were not significant (p-value 0.11 and 0.17, respectively) (Table 15). Thus, only the PRS for leucine was tested for significance with clopidogrel-induced change in platelet aggregation. The leucine PRS was not associated with clopidogrel-induced change in platelet

aggregation, accounting for 1% of the variability in clopidogrel-induced change in platelet aggregation with a p-value 0.13 (Table 16). Results were similar regardless of LD clumping strategy or p-value threshold used.

**Table XV:** BCAA PRS Association with Baseline BCAA Levels with a Threshold of 1.00E-07 and No LD Clumping

Metabolite PRS	R2	Coefficient	SE	P-value Baseline BCAA Levels	Num of SNP
Leucine	0.05	-0.02	4.9E-03	1.8E-03	72
Valine	0.01	-0.02	0.01	0.11	52
Isoleucine	0.01	-9.8E-03	7.0E-03	0.17	37

**Table XVI:** Leucine PRS Association with Clopidogrel-induced Change in Platelet Aggregation with Threshold of 1.00E-07 and No LD Clumping

Metabolite PRS	R2	Coefficient	SE	P-value Clopidogrel-induced change in platelet aggregation	Num of SNP
Leucine	0.01	-0.45	0.30	0.13	72

**Discussion:**

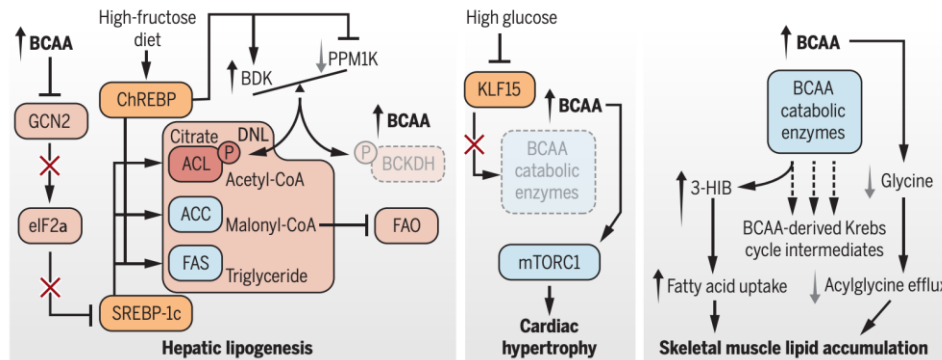
Our focus in this investigation was on finding associations between metabolite levels and response to clopidogrel as assessed by *ex vivo* ADP-induced platelet aggregation. To our knowledge, this analysis, for the first time revealed that baseline levels of the BCAAs (leucine, valine, and isoleucine) are associated with response to clopidogrel. For each BCAA, the higher the baseline level of BCAA, the poorer the clopidogrel-induced

change in platelet aggregation. In order to determine whether the observed associations were driven by baseline platelet aggregation, or whether they were only present after clopidogrel exposure; associations between baseline metabolites and baseline platelet aggregation were tested. The baseline levels of the BCAAs were not driven by baseline platelet aggregation, and as we expected, *CYP2C19\*2* did not influence the baseline levels of the BCAAs.

The mechanism by which BCAAs might influence clopidogrel-induced change in platelet aggregation is not clear. The fact that the BCAA PRS was not associated with clopidogrel-induced change in platelet aggregation could suggest that the baseline BCAA levels are not be causally related to clopidogrel-induced change in platelet aggregation and are acting indirectly through their association with cardiometabolic traits. We and others have found cardiometabolic risk factors to be associated with clopidogrel response.<sup>[21,25]</sup> However, it is also possible that the BCAA PRS was not a strong enough instrument for BCAA levels to assess shared genetic risk between BCAAs and clopidogrel-induced change in platelet aggregation. Our PRS development was based on data from a Finnish metabolomics GWAS and the Amish immigrated to the United States from Switzerland and Germany, therefore, the choice of reference population is not optimal.<sup>[41]</sup> Thus, at this point in time, we do not know the mechanism underlying the association between BCAAs and clopidogrel-induced change in platelet aggregation and whether there is a causal relationship or not.

Indeed BCAAs are becoming more established in the literature for their associations with important cardiometabolic risk factors including insulin resistance, obesity, BMI, HDL,

LDL, and triglycerides; as well as potentially having causal roles underlying cardiovascular diseases.<sup>[42]</sup> The BCAAs have direct mechanisms in which they influence cardiometabolic disease phenotypes (Figure 17).



**Figure XVII:** BCAAs Mechanisms Driving Cardiometabolic Disease Phenotypes

White et al. “Branched-Chain Amino Acids in Disease Are BCAAs a Biomarker, Causal Agent, or Both in Cardiometabolic Disease?”

For example, glucose suppresses BCAA catabolism by inhibiting Kruppel-like factor 15 transcription factor, an activator of genes encoding BCAA catabolic enzymes.<sup>[43]</sup> Also, a valine-derived metabolite, 3-hydroxy-isobutyrate (3-HIB), is generated within in the skeletal muscle in response to forced expression of peroxisome proliferator activated receptor  $\gamma$  coactivator 1a (PGC-1a), resulting in stimulation of trans-endothelial fatty acid transport.<sup>[44]</sup> Circulating 3-HIB concentrations are positively correlated with blood glucose in diabetic individuals, suggesting that this metabolite could promote excessive lipid storage and impaired insulin action.<sup>[44]</sup>

More recently the BCAAs have also been associated with CVD, particularly in those with type 2 diabetes (T2D)<sup>[42]</sup> The accumulation of BCAAs, as a result of the inhibition of the Kruppel-like factor 15 transcription factor, activates mTORC1 to drive protein synthesis

and cardiac hypertrophy.<sup>[45]</sup> mTORC1 (mammalian target of rapamycin complex 1) is a protein complex that regulates cell growth and controls protein synthesis. mTORC1 integrates four major signals: growth factors, energy status, oxygen, and amino acids.<sup>[46]</sup> Leucine is known to induce the signaling cascade that results in the activation of mTORC1.<sup>[47]</sup> Studies have shown that the Rag proteins, a family of four related small GTPases, interact with mTORC1 in an amino acid-sensitive manner and are necessary for the activation of the mTORC1 pathway by amino acids.<sup>[48,49]</sup>

Many studies have shown that metabolic and CVD traits, such as BMI, HDL, LDL, triglycerides, and blood pressure have been associated with clopidogrel response.<sup>[21,25,50,51]</sup> We were able to analyze baseline BCAA levels and their influence on these cardiometabolic traits and found that baseline BCAA levels were significantly associated with BMI, HDL, and triglycerides in the overall PAPI, findings that are consistent with what has been previously reported.<sup>[25]</sup> In the overall PAPI study, it was previously reported by Bozzi et al that a positive association between triglycerides and ADP stimulated platelet aggregation was observed after clopidogrel treatment.<sup>[25]</sup> Poor clopidogrel-induced change in platelet aggregation has been shown to be associated with higher BMI levels, higher triglyceride levels, and lower HDL levels.<sup>[21]</sup> Wagner et al compared pharmacodynamic and pharmacokinetic effects of clopidogrel in high body weight and low body weight patients. They found that patients with higher body weight had lower levels of the clopidogrel active metabolite, and higher platelet aggregation.<sup>[51]</sup> In Carreras et al, it was found that both diabetes mellitus and *CYP2C19\*2* are independently associated with elevated platelet reactivity with clopidogrel.<sup>[52]</sup>

Given the results of our analyses, one future study that could be performed is a Mendelian randomization analysis to determine causality. In this analysis, a genetic variant (instrument) that predicts BCAA level would be tested against the outcome of clopidogrel-induced change in platelet aggregation. Another study that could be conducted would be to test whether BCAAs are also associated with response to other commonly used P2Y<sub>12</sub> inhibitors, for example ticagrelor and prasugrel.

Our study does have some limitations that should be acknowledged including the relatively small sample size of 198 healthy volunteers from a single ethnic group, the use of a limited set of metabolites, and the lack of replication from independent cohorts. To better understand metabolites impacting clopidogrel response, further research should be done on other metabolomic pathways, not only the targeted purines we used. It would be helpful to conduct that research with a larger sample size, a more diverse population, and on patients not just healthy volunteers to see replication of our results. Possible explanations for the lack of association between the BCAA PRS and clopidogrel-induced change in platelet aggregation are our small sample size, and the fact that the BCAA GWAS from which the PRS was calculated from a Finnish study population which did not perform well in the Amish.

In summary, we found an association between baseline levels of BCAAs and clopidogrel-induced change in platelet aggregation. More specifically, the higher the levels of BCAAs at baseline, the poorer the clopidogrel-induced change in platelet aggregation. Consistent with the literature, BCAA levels were significantly associated with cardiometabolic traits such as triglycerides, BMI, and HDL in the PAPI study. Future studies will

need to be conducted to confirm our findings and determine whether the BCAAs are impacting clopidogrel-induced change in platelet aggregation directly or indirectly through cardiometabolic traits. Our analysis of the delta metabolites yielded fewer associations between the delta metabolites and clopidogrel-induced change in platelet aggregation, with only two nominally significant findings. In addition, *CYP2C19* did not influence the delta metabolite levels suggesting that the active metabolite of clopidogrel is not responsible for the changes in the metabolites.

## Chapter 4: Conclusions

The future of medicine is heading in a personalized direction with medicines tailored to individuals based on their predicted response to a drug, their risk of disease, or the physiologic basis of their disease. Precision medicine uses an individual's biological information, such as genetic makeup or other protein or transcriptomic biomarkers to determine the most appropriate dose or drug in order to maximize the likelihood of effectiveness and minimize the likelihood of adverse effects. Precision medicine will allow healthcare providers to focus on preventing disease by predicting susceptibility and overcoming limitations in the traditional, one-size-fits-all approach to medicine. In this pharmacometabolomic project, I wanted to gain insights into factors that influence variability in antiplatelet response to clopidogrel, because these data could be important for identifying novel antiplatelet mechanisms or future biomarkers for predicting response in patients. Better understanding of antiplatelet drug mechanisms could also lead to new drug discoveries. Gaining insights on future biomarkers that predict response will better refine the benefit: risk ratio for an individual patient, allowing the application of personalized medicine.

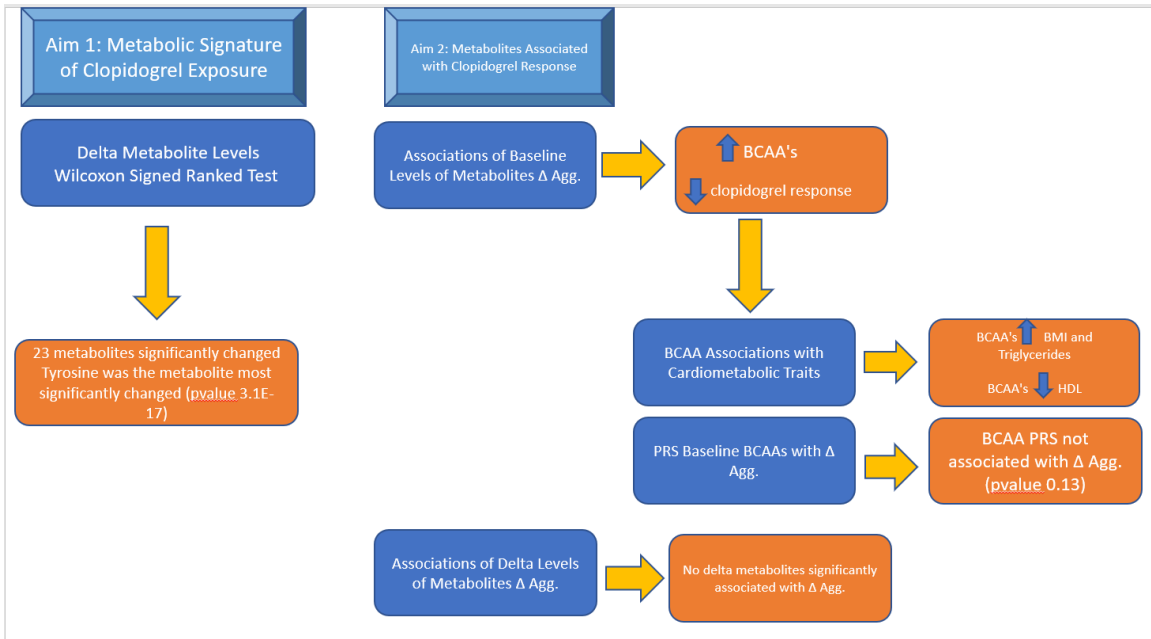
Many studies have documented a great deal of variability in clopidogrel response, largely due to genetic variation in *CYP2C19*. The goal of this project was to identify additional factors that influence this variability using metabolomics. Using metabolomics and pharmacometabolomics, I was able to determine the metabolic signature of clopidogrel and determine which metabolites are associated with clopidogrel-induced change in platelet aggregation.

In the first aim, we found that many neutral amines were changed after 7 days of clopidogrel, with tyrosine being the metabolite that changed the most. Other metabolites that were significantly decreased after clopidogrel include tryptophan, serotonin, phenylalanine, and methionine. Many of these metabolites that made up the clopidogrel signature have biological plausibility for impacting platelet aggregation including tyrosine which is involved in tyrosine phosphorylation and serotonin which is a weak platelet activator. Additional investigations assessing the role of these metabolites in clopidogrel therapy seem warranted.

In the second aim, we found that baseline BCAAs levels were associated with clopidogrel-induced change in platelet aggregation. We found that the higher the levels of BCAAs at baseline, the poorer the clopidogrel-induced change in platelet aggregation. We know of a wealth of studies that show associations between BCAAs and cardiometabolic traits, such as BMI, HDL, LDL, triglycerides, and blood pressure whereby levels of BCAAs are positively correlated with BMI and triglycerides, and inversely correlated to HDL. In addition, previous studies have found that higher levels of BMI and triglycerides were associated with lower response to clopidogrel and lower HDL levels were associated with a greater clopidogrel-induced change in platelet aggregation. We, as well as others, have found adverse cardiometabolic profiles were associated with decreased response to clopidogrel. Taken together, the data from our current metabolomic study identifying BCAA levels and previously published data identifying lipids and BMI provide compelling evidence that cardiometabolic status influences response to clopidogrel as measured by platelet aggregation. Although BCAA levels were identified as being associated with clopidogrel-induced change in platelet aggregation, our leucine PRS was

not associated with clopidogrel-induced change in platelet aggregation. It is not currently clear whether BCAAs themselves are causally related to clopidogrel-induced change in platelet aggregation or whether they are markers for cardiometabolic status which influences antiplatelet response to clopidogrel. Future studies, such as Mendelian randomization analyses, could be conducted to help define whether a causal relationship exists. Our analysis of the delta metabolites yielded fewer associations between the delta metabolites and clopidogrel-induced change in platelet aggregation, with only two suggestive findings. In addition, *CYP2C19\*2* did not influence the delta metabolite levels suggesting that the active metabolite of clopidogrel is not responsible for the changes in metabolite levels.

To gain more insights into additional mechanisms and factors associated with clopidogrel-induced change in platelet aggregation, other omics studies could be completed in the future, such as transcriptomics and genomics. Transcriptomics studies could help us understand how changes in the level of gene activity might contribute to disease. In this case, studying gene expression in platelets or circulating plasma to identify signatures associated with variability in clopidogrel-induced change in platelet aggregation. It would also be of great interest to integrate the genomic and metabolomic data generated. For example, we could use metabolomics data to prioritize GWAS data associated with clopidogrel-induced change in platelet aggregation. These transcripts, genes/variants, and metabolomics data could be incorporated into risk scores to assess the accumulation of risk loci and/or metabolite levels and further refine what could contribute to a model for predicting clopidogrel-induced change in platelet aggregation.



**Figure XVIII:** Summary Figure

## References

1. “Cardiovascular Diseases (CVDs).” *World Health Organization*, World Health Organization, May 2017, [www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-\(cvds\)](http://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)).
2. Levine, Glenn N., et al. “2015 ACC/AHA/SCAI Focused Update on Primary Percutaneous Coronary Intervention for Patients With ST-Elevation Myocardial Infarction: An Update of the 2011 ACCF/AHA/SCAI Guideline for Percutaneous Coronary Intervention and the 2013 ACCF/AHA Guideline for the Management of ST-Elevation Myocardial Infarction.” *Circulation*, vol. 133, no. 11, 2016, pp. 1135–1147., doi:10.1161/cir.0000000000000336.
3. Rotroff, Dm, et al. “Pharmacometabolomic Assessments of Atenolol and Hydrochlorothiazide Treatment Reveal Novel Drug Response Phenotypes.” *CPT: Pharmacometrics & Systems Pharmacology*, vol. 4, no. 11, 2015, pp. 669–679., doi:10.1002/psp4.12017.
4. Kaddurah-Daouk, R, and R Weinshilboum. “Metabolomic Signatures for Drug Response Phenotypes: Pharmacometabolomics Enables Precision Medicine.” *Clinical Pharmacology & Therapeutics*, vol. 98, no. 1, 2015, pp. 71–75., doi:10.1002/cpt.134.
5. Hambleton, Julie. “Drugs Used in Disorders of Coagulation.” *Basic & Clinical Pharmacology*, 9th ed., Lange Medical Bks, 2004, pp. 543–560.

6. Jackson, Shaun P. "The Growing Complexity of Platelet Aggregation." *Blood*, vol. 109, no. 12, 2007, pp. 5087–5095., doi:10.1182/blood-2006-12-027698.
7. "Coagulation." Diapharma, 2020
8. Savi, P., et al. "Importance of Hepatic Metabolism in the Antiaggregating Activity of the Thienopyridine Clopidogrel." *Biochemical Pharmacology*, vol. 44, no. 3, 1992, pp. 527–532., doi:10.1016/0006-2952(92)90445-o.
9. Taubert, D, et al. "Impact of P-Glycoprotein on Clopidogrel Absorption." *Clinical Pharmacology & Therapeutics*, vol. 80, no. 5, 2006, pp. 486–501., doi:10.1016/j.clpt.2006.07.007.
10. "Plavix Label." *Plavix Label*, FDA, Oct. 2009, [www.accessdata.fda.gov/drugsatfda\\_docs/label/2009/020839s044lbl.pdf](http://www.accessdata.fda.gov/drugsatfda_docs/label/2009/020839s044lbl.pdf).
11. Sangkuhl, Katrin, et al. "Clopidogrel Pathway." *Pharmacogenetics and Genomics*, 2010, p. 1., doi:10.1097/fpc.0b013e3283385420.
12. Kazui, Miho, et al. "Identification of the Human Cytochrome P450 Enzymes Involved in the Two Oxidative Steps in the Bioactivation of Clopidogrel to Its Pharmacologically Active Metabolite." *Drug Metabolism and Disposition*, vol. 38, no. 1, 2009, pp. 92–99., doi:10.1124/dmd.109.029132.
13. Mega, Jessica L., et al. "Cytochrome P-450 Polymorphisms and Response to Clopidogrel." *New England Journal of Medicine*, vol. 360, no. 4, 2009, pp. 354–362., doi:10.1056/nejmoa0809171.

14. Pereillo, Jean-Marie, et al. "Structure and Stereochemistry of the Active Metabolite of Clopidogrel." *Drug Metabolism and Disposition*, vol. 30, no. 11, 2002, pp. 1288–1295., doi:10.1124/dmd.30.11.1288.
15. Angiolillo, Dominick J., et al. "Variability in Individual Responsiveness to Clopidogrel." *Journal of the American College of Cardiology*, vol. 49, no. 14, 2007, pp. 1505–1516., doi:10.1016/j.jacc.2006.11.044.
16. "CYP2C19." *PharmVar*, 14 Feb. 2020, [www.pharmvar.org/gene/CYP2C19](http://www.pharmvar.org/gene/CYP2C19).
17. Scott, S A, et al. "Clinical Pharmacogenetics Implementation Consortium Guidelines for CYP2C19 Genotype and Clopidogrel Therapy: 2013 Update." *Clinical Pharmacology & Therapeutics*, vol. 94, no. 3, 2013, pp. 317–323., doi:10.1038/clpt.2013.105.
18. Horenstein, Richard B., et al. "Effectiveness of Clopidogrel Dose Escalation to Normalize Active Metabolite Exposure and Antiplatelet Effects in CYP2C19 Poor Metabolizers." *The Journal of Clinical Pharmacology*, vol. 54, no. 8, 2014, pp. 865–873., doi:10.1002/jcph.293.
19. Mega, Jessica L., et al. "Reduced-Function CYP2C19 Genotype and Risk of Adverse Clinical Outcomes Among Patients Treated With Clopidogrel Predominantly for PCI." *Jama*, vol. 304, no. 16, 2010, p. 1821., doi:10.1001/jama.2010.1543.
20. Mega, Jessica L., et al. "Dosing Clopidogrel Based on CYP2C19 Genotype and the Effect on Platelet Reactivity in Patients With Stable Cardiovascular Disease." *Jama*, vol. 306, no. 20, 2011, doi:10.1001/jama.2011.1703.

21. Shuldiner, Alan R. “Association of Cytochrome P450 2C19 Genotype With the Antiplatelet Effect and Clinical Efficacy of Clopidogrel Therapy.” *Jama*, vol. 302, no. 8, 2009, p. 849., doi:10.1001/jama.2009.1232.
22. Holmes, Elaine, et al. “Metabolic Phenotyping in Health and Disease.” *Cell*, vol. 134, no. 5, 2008, pp. 714–717., doi:10.1016/j.cell.2008.08.026.
23. Roberts, Lee D, et al. “Targeted Metabolomics.” *Current Protocols in Molecular Biology* , 15 Apr. 2012, doi:10.1002/0471142727.mb3002s98.
24. Yerges-Armstrong, L M, et al. “Purine Pathway Implicated in Mechanism of Resistance to Aspirin Therapy: Pharmacometabolomics-Informed Pharmacogenomics.” *Clinical Pharmacology & Therapeutics*, vol. 94, no. 4, 2013, pp. 525–532., doi:10.1038/clpt.2013.119.
25. Bozzi, Laura M., et al. “The Pharmacogenomics of Anti-Platelet Intervention (PAPI) Study: Variation in Platelet Response to Clopidogrel and Aspirin.” *Current Vascular Pharmacology*, vol. 14, no. 1, 2015, pp. 116–124., doi:10.2174/1570161113666150916094829.
26. Van den Brink, W.J., et al. “Multivariate Pharmacokinetic/Pharmacodynamic (PKPD) Analysis with Metabolomics Shows Multiple Effects of Remoxipride in Rats.” *European Journal of Pharmaceutical Sciences*, vol. 109, 2017, pp. 431–440., doi:10.1016/j.ejps.2017.08.031.

27. Franceschi, T.S., Soares, M.S.P., Pedra, N.S. *et al.* Characterization of macrophage phenotype, redox, and purinergic response upon chronic treatment with methionine and methionine sulfoxide in mice. *Amino Acids* **52**, 629–638 (2020). <https://doi.org/10.1007/s00726-020-02841-4>
28. Mousa SS, Davis FB, Davis PJ, Mousa SA. Human Platelet Aggregation and Degranulation Is Induced In Vitro by L-Thyroxine, but Not by 3,5,3'-Triiodo-L-Thyronine or Diiodothyropropionic Acid (DITPA). *Clinical and Applied Thrombosis/Hemostasis*. June 2010:288-293. doi:10.1177/1076029609348315
29. Lopez-Vilchez, Irene, et al. “Serotonergic Mechanisms Enhance Platelet-Mediated Thrombogenicity.” *Thrombosis and Haemostasis*, vol. 102, no. 09, 2009, pp. 511–519., doi:10.1160/th08-12-0810.
30. Schlienger, Raymond G, and Christoph R Meier. “Effect of Selective Serotonin Reuptake Inhibitors on Platelet Activation.” *American Journal of Cardiovascular Drugs*, vol. 3, no. 3, 2003, pp. 149–162., doi:10.2165/00129784-200303030-00001.
31. Brainard, B.m., et al. “Effects of Clopidogrel and Aspirin on Platelet Aggregation, Thromboxane Production, and Serotonin Secretion in Horses.” *Journal of Veterinary Internal Medicine*, vol. 25, no. 1, 2010, pp. 116–122., doi:10.1111/j.1939-1676.2010.0647.x.
32. Hogan, Daniel F., et al. “Antiplatelet Effects and Pharmacodynamics of Clopidogrel in Cats.” *Journal of the American Veterinary Medical Association*, vol. 225, no. 9, 2004, pp. 1406–1411., doi:10.2460/javma.2004.225.1406.

33. Bykov, Katsiaryna, et al. "Impact of an Interaction Between Clopidogrel and Selective Serotonin Reuptake Inhibitors." *The American Journal of Cardiology*, vol. 119, no. 4, 2017, pp. 651–657., doi:10.1016/j.amjcard.2016.10.052.
34. Roweth, H.G., Yan, R., Bedwani, N.H. *et al.* Citalopram inhibits platelet function independently of SERT-mediated 5-HT transport. *Sci Rep* 8, 3494 (2018).  
<https://doi.org/10.1038/s41598-018-21348-3>
35. Tseng, Yu-Lun, et al. "Selective Serotonin Reuptake Inhibitors Reduce P2Y12 Receptor-Mediated Amplification of Platelet Aggregation." *Thrombosis Research*, vol. 131, no. 4, 2013, pp. 325–332., doi:10.1016/j.thromres.2013.02.007.
36. Cheng, Jie, et al. "Potential Role of CYP2D6 in the Central Nervous System." *Xenobiotica*, vol. 43, no. 11, 2013, pp. 973–984.,  
doi:10.3109/00498254.2013.791410.
37. Scott, Stuart A et al. "PharmGKB summary: very important pharmacocentic information for cytochrome P450, family 2, subfamily C, polypeptide 19." *Pharmacogenetics and genomics* vol. 22,2 (2012): 159-65.  
Doi:10.1097/FPC.0b013e32834d4962.
38. Euesden, Jack, et al. "PRSice: Polygenic Risk Score Software." *Bioinformatics*, vol. 31, no. 9, 2014, pp. 1466–1468., doi:10.1093/bioinformatics/btu848.
39. "OASIS: Omics Analysis, Search & Information System." OASIS Resources, [edn.som.umaryland.edu/OASIS/resources/index.htm](http://edn.som.umaryland.edu/OASIS/resources/index.htm).

40. Teslovich, Tanya M, et al. “Identification of Seven Novel Loci Associated with Amino Acid Levels Using Single-Variant and Gene-Based Tests in 8545 Finnish Men from the METSIM Study.” *Human Molecular Genetics*, vol. 27, no. 9, 2018, pp. 1664–1674., doi:10.1093/hmg/ddy067.
41. Palo, Jukka U, et al. “Genetic Markers and Population History: Finland Revisited.” *European Journal of Human Genetics*, vol. 17, no. 10, 2009, pp. 1336–1346., doi:10.1038/ejhg.2009.53.
42. White, Phillip J., and Christopher B. Newgard. “Branched-Chain Amino Acids in Disease Are BCAAs a Biomarker, Causal Agent, or Both in Cardiometabolic Disease?” *Science*, vol. 363, no. 6427, 8 Feb. 2019, pp. 582–583.
43. Shao, D., Villet, O., Zhang, Z. *et al.* Glucose promotes cell growth by suppressing branched-chain amino acid degradation. *Nat Commun* **9**, 2935 (2018).  
<https://doi.org/10.1038/s41467-018-05362-7>
44. Jang, Cholsoon, et al. “A Branched-Chain Amino Acid Metabolite Drives Vascular Fatty Acid Transport and Causes Insulin Resistance.” *Nature Medicine*, vol. 22, no. 4, 2016, pp. 421–426., doi:10.1038/nm.4057.
45. Shao, D., Villet, O., Zhang, Z. *et al.* Glucose promotes cell growth by suppressing branched-chain amino acid degradation. *Nat Commun* **9**, 2935 (2018).  
<https://doi.org/10.1038/s41467-018-05362-7>
46. Laplante, M., and D. M. Sabatini. “mTOR Signaling at a Glance.” *Journal of Cell Science*, vol. 122, no. 20, 2009, pp. 3589–3594., doi:10.1242/jcs.051011.

47. Nicklin, Paul, et al. “Bidirectional Transport of Amino Acids Regulates MTOR and Autophagy.” *Cell*, vol. 136, no. 3, 2009, pp. 521–534.,  
doi:10.1016/j.cell.2008.11.044.
48. Kim, Eunjung, et al. “Regulation of TORC1 by Rag GTPases in Nutrient Response.” *Nature Cell Biology*, vol. 10, no. 8, 2008, pp. 935–945.,  
doi:10.1038/ncb1753.
49. Sancak, Y., et al. “The Rag GTPases Bind Raptor and Mediate Amino Acid Signaling to mTORC1.” *Science*, vol. 320, no. 5882, 2008, pp. 1496–1501.,  
doi:10.1126/science.1157535.
50. Hochholzer, Willibald, et al. “Impact of Cytochrome P450 2C19 Loss-of-Function Polymorphism and of Major Demographic Characteristics on Residual Platelet Function After Loading and Maintenance Treatment With Clopidogrel in Patients Undergoing Elective Coronary Stent Placement.” *Journal of the American College of Cardiology*, vol. 55, no. 22, 2010, pp. 2427–2434.,  
doi:10.1016/j.jacc.2010.02.031.
51. Wagner, Henrik, et al. “Higher Body Weight Patients on Clopidogrel Maintenance Therapy Have Lower Active Metabolite Concentrations, Lower Levels of Platelet Inhibition, and Higher Rates of Poor Responders than Low Body Weight Patients.” *Journal of Thrombosis and Thrombolysis*, 2013, doi:10.1007/s11239-013-0987-8.

52. Carreras, Edward, et al. "Diabetes Mellitus, CYP2C19 Genotype, and Response to Escalating Doses of Clopidogrel." *Thrombosis and Haemostasis*, vol. 116, no. 07, 2016, pp. 69–77., doi:10.1160/th15-12-0981.