Swinburne University of Technology
Sarawak Campus

The influence of $CYP2C19$ polymorphisms on clopidogrel response in a multiethnic population with coronary artery disease

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A thesis submitted in fulfilment of the requirements for the award of Master of Science by Research by Swinburne University of Technology Sarawak Campus.
Abstract

Clopidogrel plays a crucial role in preventing thrombotic events in patients undergoing percutaneous coronary intervention (PCI). CYP2C19 polymorphism involved in drug metabolism influence the antiplatelet efficacy of clopidogrel. Hence, this study aimed to determine the prevalence of CYP2C19 *2 (681G>A; rs4244285), *3 (636G>A; rs4986893) and *17 (-3402C>T; rs11188072 and -806C>T; rs12248560) alleles and their association with adenosine diphosphate – induced platelet aggregation (ADP-PA) in clopidogrel-treated multiethnic Malaysian patients (Chinese, Malay and non-Malay Bumiputras) before possible PCI. This study also aimed to assess the impact of CYP2C19 polymorphism with adverse clinical outcomes at one month and twelve months after hospital discharge in patients who had PCI. A total of 237 consecutive patients, who were electively admitted for possible PCI, were recruited from a Malaysian tertiary cardiology referral centre. These patients underwent either dual antiplatelet therapy, DAPT (clopidogrel plus aspirin) or aspirin monotherapy. CYP2C19 *2 (681G>A), *3 (636G>A) and *17 (-3402C>T) genotypes were determined by PCR-RFLP assay; while CYP2C19 *17 (-806C>T) genotype was detected by high resolution melt analysis. ADP-PA was assessed by Multiplate analyzer. Our study cohort had a mean age of 57.6 ± 11.1 years and 77.6% were male. The allelic frequencies of CYP2C19 *2, *3 and *17 variant alleles were 18.1%, 1.9%, and 0.4% for Chinese; 5.9%, 1.5% and 0.2% for Malay; 3.2%, 1.9% and 0.2% for native Iban; and 2.1%, 0.8% and 0.8% for other races respectively. The patients were categorized into three predicted metabolizers which were poor metabolizers (PMs), normal metabolizers (NMs) and ultrarapid metabolizers (UMs) according to the observed CYP2C19 genotypes. Majority of the PMs were Chinese followed by Malay, native Iban and other races. In this study, only 63.3% patients received DAPT. There was no significant difference in the post-treatment ADP-PA levels among the patients who received DAPT (p = 0.056). However, there was a significant difference in the post-treatment ADP-PA levels among the Chinese clopidogrel-treated patients (p = 0.031), but not among the other ethnic groups. From the 150 clopidogrel-treated patients, only 39.3% of the patients underwent PCI. Nevertheless, our study
demonstrated that clinical outcomes were not significantly associated with clopidogrel antiplatelet therapy and \textit{CYP2C19} genotypes. In conclusion, \textit{CYP2C19} *2 carriers, but not \textit{CYP2C19} *3 and *17 carriers, were highly prevalent in Malaysians. Most of the \textit{CYP2C19} *2 variant allele carriers were Chinese patients. \textit{CYP2C19} polymorphism was not significantly associated with ADP-PA levels and clinical outcomes in those undergoing PCI in Malaysian population.
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Declaration

I, Chua Jia Ni, hereby declare that this thesis contains no material which has been accepted for the award to the candidate of any other degree or diploma, except where due reference is made in the text of the thesis. To the best of my knowledge, this thesis contains no material previously published or written by another person except where due reference is made in the text of this thesis; and where the work is based on joint research or publications, the relative contributions of the respective workers or authors has been disclosed.

__________________________
Chua Jia Ni
Date: 25 November 2015
Conference Presentation


Acronyms

ACS  Acute Coronary Syndrome
ADP  Adenosine Diphosphate
ADP-PA  Adenosine Diphosphate – induced Platelet Aggregation
AU   Arbitrary Units
AUC  Aggregation Curve
BGI  Beijing Genomic Institute
BLAST Basic Local Alignment Search Tool
BMS  Bare Metal Stents
CAD  Coronary Artery Disease
CI   Confidence Interval
CVDs Cardiovascular Diseases
CYP2C19 Cytochrome P450 2C19
D    Disequilibrium
DAPT Dual Antiplatelet Therapy
DES  Drug Eluting Stents
DNA  Deoxyribonucleic Acid
dNTP Deoxyribonucleotide Triphosphate
dsDNA Double-stranded DNA
EDTA Ethylenediaminetetraacetic Acid
FDA  U.S. Food and Drug Administration
GOF  Gain-of-function
GP   Glycoprotein
GUSTO  Global Use of Strategies to Open Occluded Arteries
HRM  High Resolution Melting
LOF  Loss-of-function
MACE Major Adverse Cardiac Events
MEA  Multiple Electrode Platelet Aggregometry
MgCl$_2$ Magnesium Chloride
MREC Medical Research and Ethics Committee
NCBI National Center for Biotechnology Information
NCVD National Cardiovascular Disease Database
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>NM</td>
<td>Normal Metabolizer</td>
</tr>
<tr>
<td>NTC</td>
<td>No Template Control</td>
</tr>
<tr>
<td>PCI</td>
<td>Percutaneous Coronary Intervention</td>
</tr>
<tr>
<td>PCR-RFLP</td>
<td>Polymerase Chain Reaction – Restriction Fragment Linked Polymorphism</td>
</tr>
<tr>
<td>PM</td>
<td>Poor Metabolizer</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single Nucleotide Polymorphisms</td>
</tr>
<tr>
<td>ssDNA</td>
<td>Single-stranded DNA</td>
</tr>
<tr>
<td>SUHREC</td>
<td>Swinburne’s Human Research Ethics Committee</td>
</tr>
<tr>
<td>TIMI</td>
<td>Thrombolysis In Myocardial Infarction</td>
</tr>
<tr>
<td>TxA₂</td>
<td>Thromboxane A₂</td>
</tr>
<tr>
<td>UM</td>
<td>Ultrarapid Metabolizer</td>
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<tr>
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Chapter 1 Introduction
1.1 Research Background

Cardiovascular diseases (CVDs) have become the leading cause of death in Asia Pacific countries including Malaysia (Khor 2001; Lu et al. 2013). World Health Organization (WHO) has estimated that coronary artery disease (CAD) will be the largest cause of mortality in many countries world-wide by the year 2020 (WHO 2012). With CAD accounting for the most deaths globally, finding proper treatments for CAD has emerged as a major challenge in the cardiology practice.

A person will suffer from CAD when there is an inadequate supply of oxygen-rich blood and nutrients to the heart muscles due to the narrowing or even blockage of the coronary arteries caused by atherosclerosis (Zaret et al. 1992). In Malaysia, percutaneous coronary intervention (PCI) is a recognized and popular treatment for CAD (Ministry of Health Malaysia et al. 2009; Ahmad et al. 2011). It is a non-surgical procedure that uses stent implantation to widen narrowed or blocked coronary arteries without open-heart surgery (Davis 2015). Bare metal stents (BMS) and drug eluting stents (DES) are the commonly preferred stents, but DES is a more popular option to significantly reduce the risk of restenosis and improve clinical outcomes (Morice et al. 2002; Kastrati et al. 2007; Pasceri et al. 2007; Ahmad et al. 2011).

The use of dual antiplatelet therapy (DAPT) with aspirin and clopidogrel is an established practice for CAD patients undergoing PCI with stent placement (Ahmad et al. 2011). Platelet aggregation plays a pivotal role in atherothrombosis and stent-related complications. Therefore, inhibition of the platelet aggregation pathway by DAPT is important to inhibit platelet activation and aggregation (Zaret et al. 1992; Wenaweser et al. 2005). DAPT has proven to be efficacious in reducing the risk of further major adverse cardiac events (MACE) and stent-related complications (Mehta et al. 2000). However, a substantial number of subsequent thrombotic events continue to occur even with DAPT, which might be associated with the wide inter-individual and inter-
ethnic variabilities in clinical response towards the antiplatelet effect of antiplatelet drugs (Kuliczkowski et al. 2009; Simon et al. 2009; Chan 2012).

Clopidogrel, which is the most commonly prescribed thienopyridine antiplatelet drug, is an inactive prodrug which requires several biotransformation steps by various hepatic cytochrome P450 (CYP) isoenzymes to convert it into active metabolites. These active metabolites will bind selectively and irreversibly to the P2Y\textsubscript{12} receptor on the platelet surface, which will then inactivate the glycoprotein (GP) IIb/IIIa receptor, hence inhibiting adenosine diphosphate (ADP)-mediated platelet activation and aggregation (Savi et al. 2000). Among the various CYP450 isoenzymes, CYP2C19 plays an important role and contributes significantly in two sequential oxidative steps during the biotransformation of clopidogrel into its active metabolites (Kazui et al. 2010).

Hence, genetic polymorphism of CYP2C19 which is involved in drug metabolism can influence the variation of pharmacodynamic response to clopidogrel (Brandt et al. 2007). Several single nucleotide polymorphisms (SNPs) of the gene that encodes CYP2C19 isoenzyme have been reported, which include the CYP2C19 *2 (681G>A; rs4244285), *3 (636G>A; rs4986893) and *17 (-3402C>T; rs11188072 and -806C>T; rs12248560) alleles. CYP2C19 *1 allele refers to a normal allele or wild type allele, and is associated with normal functional CYP2C19-mediated drug metabolism (Li et al. 2010). Both CYP2C19 *2 and *3 alleles are loss-of-function (LOF) variant alleles and are associated with decreased functional metabolic activity. The genetic defect of CYP2C19 *2 is caused by the 681 G>A substitution in exon 5, resulting in a splice-defective site; while CYP2C19 *3 has a point mutation of 636 G>A in exon 4, leading to premature stop codon (leiri et al. 1996; Yin et al. 2004). Conversely, CYP2C19 *17 gain-of-function (GOF) allele, which carries two different SNPs of -3402C>T and -806C>T, is a polymorphism in the 5’ flanking region of the CYP2C19 gene and is associated with increased rate of transcription. Both SNPs of -3402C>T and -806C>T are in complete linkage disequilibrium with each other (Sim et al. 2006; Li et al. 2010).
Therefore, individuals who are carriers of LOF variant alleles of CYP2C19 *2 and *3 will have lower levels of active metabolites of clopidogrel compared to non-carriers (CYP2C19 *1). Carriers of these LOF variant alleles will experience higher risk of thrombotic events due to the decreased antiplatelet effect of clopidogrel (de Morais et al. 1994). On the other hand, individuals with GOF variant allele of CYP2C19 *17 will experience increased CYP2C19 gene expression and enhanced platelet response to clopidogrel. Hence, carriers of this GOF variant allele might have an increased risk of bleeding (Sim et al. 2006; Sibbing et al. 2010).

Due to inter-individual variability in clinical response towards clopidogrel efficacy, individuals can be categorized into three predicted CYP2C19 metabolic phenotypes, namely poor metabolizers (PMs), normal metabolizers (NMs) and ultrarapid metabolizers (UMs) (Gurbel et al. 2010).

Many studies have reported that there is a wide inter-ethnic variability in CYP2C19 polymorphism. Asian populations (~ 55% to 70%) have higher prevalence rate of CYP2C19 LOF variant alleles (CYP2C19 *2 and *3) compared with white populations (~ 25% to 35%) and black populations (~ 35% to 45%) (Desta et al. 2002; Lee et al. 2009; Man et al. 2010; Hwang et al. 2011). On the other hand, Asian populations (~ 4%) have low prevalence of CYP2C19 GOF variant allele (CYP2C19 *17) as compared to white populations (~ 18%) (Sim et al. 2006; Sugimoto et al. 2008).

The role of genetic polymorphisms in a wide inter-individual variability in clopidogrel response has led to the application of cardiovascular pharmacogenomics in the management of CVDs and CAD. The main aims of cardiovascular pharmacogenomics are to use genetic information as a guide to help in patients’ treatment decisions and improve antiplatelet therapies for patients with CAD or patients who had undergone PCI with stent placement (Johnson et al. 2011). Pharmacogenomics will become an important approach as a standard practice in clinical settings to predict drug responsiveness in the next five to ten years (Collin et al. 2001; Johnson 2013).
1.2 Research Problem Statements

Atherothrombosis is a complex process. It is impossible to completely prevent the mechanisms of platelet function even with proper antiplatelet therapy guidelines and antiplatelet drugs (Corti et al. 2003; Tantry et al. 2005). Hence, despite the chronic DAPT, the risk of recurrent thrombotic and ischemic events still remains. This might be associated with the wide inter-individual and inter-ethnic variabilities in clinical response towards the antiplatelet effect of clopidogrel (Kuliczkowski et al. 2009; Simon et al. 2009; Chan 2012).

CYP2C19 polymorphism, which plays an essential role in the two oxidative steps in bioactivation of clopidogrel into active metabolites, can influence clopidogrel efficacy. The SNPs that encode the CYP2C19 isoenzymes gene are CYP2C19 *2 (681G>A; rs4244285), *3 (636G>A; rs4986893) and *17 (-3402C>T; rs11188072 and -806C>T; rs12248560). CYP2C19 *2 and *3 are LOF variant alleles which have higher prevalence in Asian populations. Inversely, CYP2C19 *17, GOF variant allele has lower prevalence in Asian populations (Li et al. 2010).

To date, the studies reporting the prevalence of CYP2C19 *2, *3 and *17 variant alleles in Malaysian multiethnic populations still remains insufficient (Yang et al. 2004; Sani et al. 2013; Mejin et al. 2013). Besides, the use of the pharmacogenetic testing and platelet function testing to predict the patients' platelet aggregation levels in response to clopidogrel and subsequently to determine a proper personalized individual antiplatelet therapy remains debatable. Further investigations and confirmations are required to provide more insight into the prevalence of these variant alleles, as well as efficient antiplatelet therapy (Gurbel et al. 2012; Krishna et al. 2012).
1.3 Research Aims

Knowing that atherothrombosis is a complex process and inhibition of platelet aggregation is the essential focus and strategy in cardiovascular disease research, there are many ongoing studies and clinical trials to provide better evidence and guidelines to improve healthcare among CAD patients undergoing PCI with stent implantation. Besides, the existence of wide inter-individual and inter-ethnic variations in response to clopidogrel antiplatelet effect have made the goals of cardiovascular disease research, which is to determine optimal antiplatelet treatment strategies, even more challenging. Similarly, our study aims to further investigate the influence of *CYP2C19* polymorphism on clopidogrel response in multiethnic Malaysian patients with CAD. These findings will aid in advancing our understanding to benefit Malaysian CAD patients by providing them better and appropriate antiplatelet therapies. Therefore, the objectives of this study are summarized as below.

a) To determine the prevalence of *CYP2C19* *2* (681G>A; rs4244285), *3* (636G>A; rs4986893) and *17* (-3402C>T; rs11188072 and -806C>T; rs12248560) variant alleles in clopidogrel-treated multiethnic Malaysian patients (Chinese, Malay and non-Malay Bumiputras).

b) To understand the linkage disequilibrium between *CYP2C19* *2* (681G>A; rs4244285), *3* (636G>A; rs4986893) and *17* (-3402C>T; rs11188072 and -806C>T; rs12248560) variant alleles.

c) To evaluate the association between *CYP2C19* genetic polymorphism with ADP-induced platelet aggregation (ADP-Pa) in a multiethnic Malaysian population pre-treated with clopidogrel before possible PCI.

d) To assess the impact of *CYP2C19* polymorphism on adverse clinical outcomes at one month and twelve months after hospital discharge in multiethnic patient group who had undergone PCI.
Chapter 2 Literature Review

2.1 Cardiovascular Diseases (CVDs) and Coronary Artery Disease (CAD)

With the rapid urbanization, industrialization, improved socio-economic status and lifestyle changes of this modern world, CVDs especially CAD have become the most common cause of death in Malaysia since the 1980s (Chen 1980; Khor 2001). WHO has even estimated that CAD will be the largest cause of mortality in many countries world-wide by the year 2020 (WHO 2012).

The heart is a strong muscular pump that pumps blood through the whole human body every day. Like the other organs, the heart is an aerobic organ that requires continuous supply of oxygenated blood and essential nutrients from the coronary arteries in order to maintain its muscles and enable its electrical conduction system to function properly (Zaret et al. 1992; Libby et al. 2005).

Coronary arteries are like hollow tubes that allow blood to flow freely to the heart muscles. The inner wall of the coronary arteries is smooth and elastic, lined with a layer of endothelium cells. These endothelium cells help in regulating the function of the coronary arteries by releasing chemical signals in response to various stimuli (Zaret et al. 1992).

Hence, a person will suffer from CAD when there is an inadequate supply of oxygen-rich blood and nutrients to the heart muscle due to the narrowing or even blockage of the coronary arteries caused by atherosclerosis. Atherosclerosis, or hardening of the arteries, is the progressive buildup of plaques on the inner wall of the coronary arteries. This buildup of plaques is waxy substances that are made of cholesterol, fatty substances, cellular waste products, calcium and a clotting material in the blood called fibrin (Zaret et al. 1992).
The buildup of plaques is a gradual process. The plaques can develop at different sizes on the inner wall of the coronary arteries. Over time, the coronary arteries can become too narrow until they are unable to supply sufficient oxygenated blood and nutrients to the heart muscles for the heart to pump properly. This can lead to chest pain or discomfort called angina (Figure 2-1) (Zaret et al. 1992).

However, if the plaques rupture, it would activate the blood clotting system. Platelets, which are disc-shaped particles in the blood that aid in the blood clotting system, will form blood clots (thrombus) around the ruptured plaques. These blood clots can block the coronary artery and greatly decrease the supply of oxygenated blood and nutrients to the heart muscles. The heart muscles would then be permanently damaged, causing myocardial infarction (or more commonly called as heart attack) and even death (Zaret et al. 1992; Libby et al. 2005).

Figure 2-1: Coronary artery disease - narrowed or blocked coronary arteries caused by atherosclerosis could lead to angina and heart attack (Johns Hopkins Medicine 2014).
2.2 Percutaneous Coronary Intervention (PCI)

In Malaysia, PCI has become a recognized and popular treatment for patients with atherosclerotic CAD (Ministry of Health Malaysia et al. 2009; Ahmad et al. 2011). PCI, which is commonly known as angioplasty with stent, is a non-surgical procedure that uses a catheter, balloon and stent to help to open and widen narrowed or blocked coronary arteries without open-heart surgery (Davis 2015).

During the process of PCI, a thin flexible hollow tube called the catheter is inserted into the blood vessels, either through the femoral artery or hand radial artery. The catheter is threaded through the blood vessels and is moved up into the affected coronary artery using the help of an X-ray called fluoroscopy. The tip of the catheter contains a tiny balloon covered with a small expandable metallic tube called stent. When the catheter reaches the site of the narrowed or blocked coronary artery, the balloon is inflated to compress the plaques in the coronary artery and hence widen the coronary artery. The stent is expanded and placed permanently in the coronary artery after the initial balloon dilation. Then, the balloon will be deflated and withdrawn from the coronary artery. This stent can help to reduce the risk of recurrent narrowing or blockage of the coronary artery. Thus, PCI will help to restore and improve the blood flow from the coronary artery to the heart muscles, hence reducing angina and preventing heart attacks (Figure 2-2) (Davis 2015).

Stents are now commonly used in PCI procedures followed by balloon angioplasty. There are five types of stents available in Malaysia which are bare metal stents (BMS), drug eluting stents (DES), endothelial progenitor cell capture stents, covered stents and biodegradable (bioabsorbable) stents. The functions of the five types of stents are summarized in Table 2-1 (Ministry of Health Malaysia et al. 2009). BMS and DES were commonly preferred compared to the other stents. Many studies reported that endothelial progenitor cell capture stents and covered stents are associated with higher rates of MACE and stent-related complications which require further investigations and
evaluations (Cervinka 2009; Ministry of Health Malaysia et al. 2009). However, DES is a more popular option to significantly reduce the risk of restenosis and improve clinical outcomes when compared to BMS (Morice et al. 2002; Kastrati et al. 2007; Pasceri et al. 2007; Ahmad et al. 2011).

**Figure 2-2:** Percutaneous coronary intervention (PCI) and stent placement (Johns Hopkins Medicine 2014).
Table 2-1: Summarized functions of the five types of coronary stents used in Malaysia (Ministry of Health Malaysia et al. 2009).

<table>
<thead>
<tr>
<th>Type of Coronary Stents</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bare metal stents (BMS)</td>
<td>A metal stent with no special coating. It is a metal mesh tube that is made of either stainless steel or cobalt chromium used to help open and widen the narrowed or blocked coronary artery.</td>
</tr>
<tr>
<td>Drug eluting stents (DES)</td>
<td>A metal stent coated with a drug (antiproliferative agents). The drug will be slowly released into the inner walls of the coronary artery to inhibit neointimal proliferation. This can help to reduce the risk of restenosis.</td>
</tr>
<tr>
<td>Endothelial progenitor cell capture stents</td>
<td>A metal stent coated with an antibody that attracts endothelial progenitor cells in the blood circulation. These cells can rapidly transform into endothelial cells which will speed up natural healing by forming new functional endothelium and hence reduce the risk of thrombosis and restenosis.</td>
</tr>
<tr>
<td>Covered stents</td>
<td>A durable fabric (such as polyester) coated stent. It is a contained tube and is expandable like the bare metal stent. This stent is useful for sealing coronary perforations and excluding aneurysms, but are associated with higher rates of thrombosis and restenosis.</td>
</tr>
<tr>
<td>Biodegradable (bioabsorbable) stents</td>
<td>It is a stent designed to provide temporary support and create an opening in the affected coronary artery. Then, it will gradually be resorbed by the body over time. This stent is helpful in preventing stent thrombosis.</td>
</tr>
</tbody>
</table>
2.3 Complications from Percutaneous Coronary Intervention (PCI)

PCI is known to be an effective non-surgical treatment in opening and widening narrowed or blocked coronary arteries, which helps to relieve angina and prevent heart attacks. Nevertheless, PCI is not risk-free. There are certain stent-related complications that might occur from PCI. Two common stent-related complications are stent thrombosis and in-stent restenosis (Ministry of Health Malaysia et al. 2009).

2.3.1 Stent Thrombosis

Coronary stents are foreign bodies in the coronary artery which can induce platelet adhesion, activate coagulation cascade and cause stent thrombosis. Besides, stent thrombosis will also occur when there are certain tears or injuries in the inner wall of the coronary artery, which can cause blood clotting (thrombosis) to happen at the site of balloon angioplasty or stent placement (Figure 2-3). The formation of blood clots can cause sudden recurrent narrowing or complete blockage of the coronary artery, which may result in myocardial infarction and death (Wenaweser et al. 2005; Ministry of Health Malaysia et al. 2009). Stent thrombosis can be classified as acute stent thrombosis, subacute stent thrombosis, late stent thrombosis and very late stent thrombosis according to the timeframe after the stent implantation, as shown in Table 2-2. Moreover, stent thrombosis can be defined as definite stent thrombosis, probable stent thrombosis and possible stent thrombosis according to event certainty, as shown in Table 2-3 (Cutlip et al. 2007).
Table 2-2: Classification of stent thrombosis according to timeframe after stent implantation (Cutlip et al. 2007).

<table>
<thead>
<tr>
<th>Type of Stent Thrombosis</th>
<th>Timeframe after Stent Implantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute stent thrombosis</td>
<td>0 to 24 hours after stent implantation</td>
</tr>
<tr>
<td>Subacute stent thrombosis</td>
<td>&gt; 24 hours to 30 days after stent implantation</td>
</tr>
<tr>
<td>Late stent thrombosis</td>
<td>&gt; 30 days to 1 year after stent implantation</td>
</tr>
<tr>
<td>Very late stent thrombosis</td>
<td>&gt; 1 year after stent implantation</td>
</tr>
</tbody>
</table>

Table 2-3: Definition of stent thrombosis according to event certainty (Cutlip et al. 2007).

<table>
<thead>
<tr>
<th>Definition of Stent Thrombosis</th>
<th>Event Certainty</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definite stent thrombosis</td>
<td>Acute Coronary Syndrome (ACS) with angiographic or autopsy confirmation of stent thrombosis.</td>
</tr>
<tr>
<td>Probable stent thrombosis</td>
<td>Unexplained death within 30 days of stent implantation without autopsy; acute myocardial infarction in the area of stent implantation but without angiographic confirmation.</td>
</tr>
<tr>
<td>Possible stent thrombosis</td>
<td>Unexplained death after 30 days of stent implantation without autopsy.</td>
</tr>
</tbody>
</table>

Figure 2-3: Stent thrombosis (Meier et al. 2013).
2.3.2 In-stent Restenosis

After stent placement, scar tissue will form around the coronary stent during the healing process. Over time, the stent could be completely covered by the excess scar tissue and cause re-narrowing or re-blockage of the treated coronary artery. This is called in-stent restenosis which can obstruct the blood flow to the heart muscles, causing myocardial infarction and death (Figure 2-4). In-stent restenosis usually occurs within six months after stent placement (Serruys et al. 1988; Dangas et al. 2002). In-stent restenosis might be due to elastic recoil, vascular remodeling and neointimal hyperplasia. There are four types of in-stent restenosis identified, which are focal, diffuse, proliferative and total occlusion. They are classified according to the length and pattern of the lesions, as shown in Table 2-4 and Figure 2-5 (Mehran et al. 1999; Ministry of Health Malaysia et al. 2009).

Table 2-4: Classification of in-stent restenosis according to lesion length and pattern (Mehran et al. 1999).

<table>
<thead>
<tr>
<th>Type of In-stent Restenosis</th>
<th>Lesion Length and Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Focal</td>
<td>Lesion length is ≤ 10 mm.</td>
</tr>
<tr>
<td>Diffuse</td>
<td>Lesion length is &gt; 10 mm and the lesions are within the stent(s).</td>
</tr>
<tr>
<td>Proliferative</td>
<td>Lesion length is &gt; 10 mm and the lesions are extending outside the stent(s).</td>
</tr>
<tr>
<td>Total occlusion</td>
<td>Lesion length is &gt; 10 mm and the lesions cause complete obstruction of blood flow in the coronary artery; no perfusion; TIMI flow grade of 0.</td>
</tr>
</tbody>
</table>
Figure 2-4: In-stent restenosis (National Institutes of Health 2014).

Figure 2-5: Types of in-stent restenosis (Ong et al. 2004).
2.4 Platelet Aggregation Pathway and Dual Antiplatelet Therapy (DAPT)

Platelet activation and aggregation play a pivotal role in atherothrombosis and stent-related complications such as stent thrombosis, which can result in myocardial infarction and death (Zaret et al. 1992; Wenaweser et al. 2005).

Under normal conditions, platelets circulate freely within the bloodstream in their resting and non-active form. The healthy endothelium wall of the coronary artery prevents the adhesion and activation of the platelets by releasing antithrombotic factors such as CD39 (ectoADPase), prostaglandin I$_2$, nitric oxide, heparin, matrix metalloproteinase-9, protein S and thrombomodulin (Egbrink et al. 2005; Tantry et al. 2006). Hence, atherosclerotic plaque rupture or injuries to the endothelium wall of the coronary artery after stent placement will expose the subendothelial matrix and release prothrombotic factors, which will induce platelet adhesion and platelet activation (Aleil et al. 2005; Harvey et al. 2012).

When the platelets are adhered to the exposed collagen of the subendothelial matrix, the activated adhering platelets will release platelet granules containing chemical mediators such as thromboxane A$_2$ (TxA$_2$), ADP and thrombin. These signalling molecules will bind to the receptors on the outer membrane of other resting platelets that circulate nearby and thus activate these platelets. TxA$_2$ is produced from arachidonic acid, which is released from phospholipids of the cell membrane to bind to Tx receptors. ADP on the other hand is produced from dense granules and binds to P2Y$_{12}$ and P2Y$_1$ receptors. These two secondary platelet agonists will lead to sustained activation of glycoprotein (GP) IIb/IIIa receptors. These activated glycoprotein (GP) IIb/IIIa receptors regulate platelet-platelet interaction and platelet-rich thrombus formation. This will result in platelet aggregation (Aleil et al. 2005; Tantry et al. 2006; Harvey et al. 2012).
Platelet activation will also result in the membrane exposure of phosphatidylserine which would provide a binding site for coagulation factors and induce the production of thrombin. Thrombin catalyzes the hydrolysis of fibrinogen to fibrin and leads to the formation of a platelet-fibrin clot (Tantry et al. 2006).

Hence, inhibition of the TxA2-platelet and ADP-platelet pathway by DAPT can thereby inhibit platelet activation and aggregation, which is necessary to reduce the risk of atherothrombosis, stent thrombosis and recurrent cardiovascular events (Savi et al. 2000; Wenaweser et al. 2005; Bousser 2009; Tantry et al. 2009). DAPT with aspirin and clopidogrel is the most commonly used therapy for patients with CAD undergoing PCI in Malaysia (Ministry of Health Malaysia et al. 2009; Ahmad et al. 2011). DAPT has proven to be efficacious in managing atherothrombosis and reducing the risk of further MACE and stent thrombosis (Mehta et al. 2000).

Figure 2-6: Platelet activation and aggregation (Harvey et al. 2012).
2.5 Antiplatelet Agent

ADP initiates and induces platelet aggregation by binding to P2Y\textsubscript{12} and P2Y\textsubscript{1} receptors. The activated P2Y\textsubscript{1} receptor initiates platelet aggregation through calcium mobilization; while the activated P2Y\textsubscript{12} receptor contributes in stabilizing platelet aggregation and mediates full platelet aggregation. Therefore, the ADP and P2Y\textsubscript{12} receptors, both which play a major role in platelet aggregation, have become an important focus and strategy in cardiovascular disease research to inhibit ADP-PA (Gachet 2001).

2.5.1 Clopidogrel: Overview

Clopidogrel is the most commonly prescribed thienopyridine antiplatelet drug for patients with CAD undergoing PCI in Malaysia (Ministry of Health Malaysia et al. 2009; Ahmad et al. 2011). It is an ADP-receptor inhibitor that inhibits platelet activation and aggregation, and hence is useful in reducing the risk of heart attack, stent-related complications as well as recurrent of cardiovascular events (Savi et al. 2000; Pereillo et al. 2002; Wenaweser et al. 2005).

Ticlopidine is the predecessor of clopidogrel. However, clopidogrel provides better tolerance and antiplatelet effects compared to ticlopidine (Maffrand 2012). Clopidogrel chemically is methyl (+)-(S)-α-(2-chlorophenyl)-6,7-dihydrothieno[3,2-c]pyridine-5(4H)-acetate sulfate (1:1), has the molecular formula of C\textsubscript{16}H\textsubscript{16}ClNO\textsubscript{2}S·H\textsubscript{2}SO\textsubscript{4} (Clinical Drug Information 2015). Clopidogrel is marketed by Sanofi and Bristol-Myers Squibb using the trade name Plavix (Maffrand 2012).

Based on the clinical practice guidelines on management of PCI 2009, the prescription of loading dose of clopidogrel 300 – 600 mg for patients with CAD before PCI is recommended (Yusuf et al. 2001). Patients who had undergone PCI with BMS placement will be given clopidogrel 75 mg daily for at
least one month (Collet et al. 2008). On the other hand, patients with DES implantation will receive clopidogrel 75 mg for at least a year (Ho et al. 2008). The increased dosage of clopidogrel to 150 mg will be considered if the platelet function test indicates less than 50% of platelet inhibition (Angiolillo et al. 2007). A prolonged clopidogrel antiplatelet therapy (more than a year) will also be arranged for patients with high risk of very late stent thrombosis (Grines et al. 2007).

Nevertheless, certain groups of clopidogrel-treated patients experienced adverse effects from clopidogrel, which include poor clopidogrel responsiveness, hypersensitivity and severe active bleeding (Kuliczkowski et al. 2009; Clinical Drug Information 2015). Alternative antiplatelet drugs or antiplatelet therapies should be recommended for these groups of patients (FDA 2015).

### 2.5.2 Clopidogrel: Pathway and Mechanism of Action

Clopidogrel is an inactive prodrug that is absorbed in the intestine and activated in the liver through several biotransformation steps by various hepatic cytochrome P450 (CYP) isoenzymes (Savi et al. 1992; Caplain et al. 1999; Lins et al. 1999). After the intestinal absorption of clopidogrel, 85.0% of the clopidogrel will be inactivated by esterases. Only 15.0% of the clopidogrel will undergo metabolism by CYP450 isoenzymes \((\text{CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4})\). \text{CYP1A2, CYP2B6 and CYP2C19 are responsible for the oxidation of the thiophene ring of the clopidogrel into intermediate 2-oxo-clop}dipogrel metabolites. Then, \text{CYP2B6, CYP2C9, CYP2C19 and CYP3A4} will further oxidize these intermediate metabolites into active thiol metabolites \((2\{1-[(1S)-1-(2-chlorophenyl)-2-methoxy-2-oxoethyl]-4-sulfonyl-3-piperidinyl-diene\} acetic acid\) by opening the thiophene ring and forming both a carboxyl group and a thiol group (Pereillo et al. 2002). These active metabolites will bind selectively and irreversibly to the \(\text{P2Y}_{12}\) receptor on the platelet surface, which will then inactivate the glycoprotein (GP) IIb/IIIa receptor and thereby inhibit
ADP-mediated platelet activation and aggregation (Figure 2-7 and Figure 2-8) (Savi et al. 2000; Ding et al. 2003).

The role of CYP1A2, CYP2B6 and CYP2C19 in the formation of 2-oxo-clopidogrel was 35.8%, 19.4% and 44.9% respectively; while the role of CYP2B6, CYP2C9, CYP2C19 and CYP3A4 in the formation of the active thiol metabolites was 32.9%, 6.8%, 20.6% and 39.8% respectively. Among the various CYP450 isoenzymes, CYP2C19 plays an important role and contributes significantly in the two sequential oxidative steps in the biotransformation of clopidogrel into active metabolites, as illustrated in Figure 2-7 (Kazui et al. 2010).

![Figure 2-7: The roles and contributions of each CYP450 isoenzymes in the conversion of clopidogrel into active metabolites (Kazui et al. 2010).](image-url)
Figure 2-8: Clopidogrel pathway and mechanism of action (Simon et al. 2009).
2.6 Genetic Polymorphism of CYP2C19

Despite the fact that clopidogrel antiplatelet therapy has become an established therapy to inhibit ADP-PA, a substantial number of subsequent thrombotic events continue to occur. This might be associated with the possible clinical resistance of these individuals towards the antiplatelet effect of clopidogrel, which leads to insufficient inhibition of platelet function by clopidogrel (Kuliczkowski et al. 2009; Simon et al. 2009; Chan 2012).

The genetic variations in CYP450 isoenzymes genes (CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A4), which are involved in drug metabolism, can influence the variation of pharmacodynamic response to clopidogrel, especially the genetic variation in the CYP2C19 isoenzyme, which contributes significantly in the two sequential oxidative steps in the biotransformation of clopidogrel into active metabolites (Brandt et al. 2007; Kazui et al. 2010). Hence, genetic polymorphism of CYP2C19 could play a crucial role in wide inter-individual and inter-ethnic variabilities in clinical response towards clopidogrel (Kuliczkowski et al. 2009; Simon et al. 2009; Chan 2012).

2.6.1 Single Nucleotide Polymorphisms (SNPs): CYP2C19 *2, *3 and *17

There are more than 28 SNPs identified in the CYP2C19 isoenzyme gene (Sim 2014). However, most of these SNPs are relatively low frequency and the commonly studied SNPs include CYP2C19 *2 (681G>A; rs4244285), *3 (636G>A; rs4986893) and *17 (-3402C>T; rs11188072 and -806C>T; rs12248560) alleles (Helsby et al. 2012). CYP2C19 *1 allele refers to a normal allele or wild type allele, and is associated with normal functional CYP2C19-mediated drug metabolism (Li et al. 2010). Both CYP2C19 *2 and *3 alleles are the LOF variant alleles which are associated with decreased functional metabolic activity. The genetic defect of CYP2C19 *2 is caused by the 681 G>A substitution in exon 5, resulting in a splice-defective site; while CYP2C19 *3 has
a point mutation of 636 G>A in exon 4, leading to premature stop codon and truncated protein (Ieiri et al. 1996; Yin et al. 2004). Conversely, CYP2C19 *17 GOF allele is a polymorphism in the 5’ flanking region of the gene which is associated with increased gene transcription of CYP2C19. CYP2C19 *17 variant allele carries two different SNPs of -3402C>T and -806C>T in the 5’ flanking region. These two polymorphisms are reported to be in complete linkage disequilibrium with each other (Sim et al. 2006; Li et al. 2010).

Hence, individuals who are carriers of LOF variant alleles of CYP2C19 *2 and *3 will have decreased formation of active metabolites of clopidogrel compared to non-carriers (CYP2C19 *1). Carriers of these LOF variant alleles will experience higher risk of thrombotic events due to decreased antiplatelet effect of clopidogrel (de Morais et al. 1994). On the other hand, individuals with GOF variant allele of CYP2C19 *17 will experience increased CYP2C19 expression, which results in the production of higher levels of active thiol metabolites and enhanced antiplatelet effect of clopidogrel. Carriers of this GOF variant allele might have a potential risk of bleeding (Sim et al. 2006; Sibbing et al. 2010).

Inter-individual variability in clinical response towards clopidogrel efficacy can divide individuals into three predicted CYP2C19 metabolic phenotypes, which are poor metabolizers (PMs); normal metabolizers (NMs) and ultrarapid metabolizers (UMs) (Table 2-5) (Gurbel et al. 2010).

**Table 2-5:** Categorization of the predicted CYP2C19 metabolic phenotypes based on the CYP2C19 genotypes.

<table>
<thead>
<tr>
<th>Predicted Phenotype</th>
<th>Observed CYP2C19 Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor metabolizer (PM)</td>
<td>*1/*2, *1/*3, *2/*2, *3/*3, *2/*3</td>
</tr>
<tr>
<td>Normal metabolizer (NM)</td>
<td>*1/*1, *2/*17, *3/*17</td>
</tr>
<tr>
<td>Ultrarapid metabolizer (UM)</td>
<td>*1/*17, *17/*17</td>
</tr>
</tbody>
</table>
2.6.2 Prevalence of CYP2C19 *2, *3 and *17 Variant Alleles

Many studies have reported that there is a wide inter-ethnic variability in CYP2C19 polymorphism. Asian populations (~ 55.0% to 70.0%) have higher prevalence rate of CYP2C19 LOF variant alleles (CYP2C19 *2 and *3) compared with white populations (~ 25.0% to 35.0%) and black populations (~ 35.0% to 45.0%) (Desta et al. 2002; Lee et al. 2009; Man et al. 2010; Hwang et al. 2011). On the other hand, Asian populations (~ 4.0%) have low prevalence of CYP2C19 GOF variant allele (CYP2C19 *17) as compared to white populations (~ 18.0%) (Sim et al. 2006; Sugimoto et al. 2008). Table 2-6 shows the summary of the reported prevalence rate of CYP2C19 *2, *3 and *17 respectively according to different ethnic groups.

Table 2-6: Summary of the reported prevalence rate of CYP2C19 *2, *3 and *17 according to different ethnic groups.

<table>
<thead>
<tr>
<th>SNPs</th>
<th>Frequency</th>
<th>Ethnic Group</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C19 *2</td>
<td>~ 30.0%</td>
<td>Chinese</td>
<td>Xie et al. 2001</td>
</tr>
<tr>
<td></td>
<td>~ 67.0%</td>
<td>Chinese-Malaysian</td>
<td>Sani et al. 2013</td>
</tr>
<tr>
<td></td>
<td>~ 52.0%</td>
<td>Chinese-Singaporean</td>
<td>Chan et al. 2012</td>
</tr>
<tr>
<td></td>
<td>~ 17.0%</td>
<td>African-Americans</td>
<td>Xie et al. 2001</td>
</tr>
<tr>
<td></td>
<td>~ 15.0%</td>
<td>Caucasians</td>
<td>Xie et al. 2001</td>
</tr>
<tr>
<td></td>
<td>~ 24.2%</td>
<td>Japanese</td>
<td>Man et al. 2010</td>
</tr>
<tr>
<td></td>
<td>~ 14.8%</td>
<td>Koreans</td>
<td>Man et al. 2010</td>
</tr>
<tr>
<td></td>
<td>~ 5.0%</td>
<td>Chinese</td>
<td>Xie et al. 2001</td>
</tr>
<tr>
<td></td>
<td>~ 10.0%</td>
<td>Chinese-Singaporean</td>
<td>Chan 2012</td>
</tr>
<tr>
<td></td>
<td>~ 9.0%</td>
<td>Malay-Singaporean</td>
<td>Chan 2012</td>
</tr>
<tr>
<td></td>
<td>~ 1.0%</td>
<td>Indian-Singaporean</td>
<td>Chan 2012</td>
</tr>
<tr>
<td></td>
<td>~ 0.4%</td>
<td>Africans</td>
<td>Xie et al. 2001</td>
</tr>
<tr>
<td></td>
<td>~ 0.2%</td>
<td>Caucasians</td>
<td>Man et al. 2010</td>
</tr>
<tr>
<td>CYP2C19 *3</td>
<td>~ 18.0%</td>
<td>Swedish</td>
<td>Sim et al. 2006</td>
</tr>
<tr>
<td></td>
<td>~ 4.0%</td>
<td>Chinese</td>
<td>Sim et al. 2006</td>
</tr>
<tr>
<td></td>
<td>~ 1.3%</td>
<td>Japanese</td>
<td>Sugimoto et al. 2008</td>
</tr>
</tbody>
</table>
2.7 Cardiovascular Pharmacogenomics and Personalized Antiplatelet Therapy

The discovery of the role of genetic polymorphisms in the wide inter-individual variability in pharmacokinetic and pharmacodynamic response to clopidogrel has led to the application of pharmacogenomics in the management of CVDs and CAD. The main aims of cardiovascular pharmacogenomics are to determine the genetic determinants of cardiovascular drugs such as clopidogrel and to use this genetic information to help in patients’ treatment decisions. Many studies and clinical trials have been conducted to evaluate and confirm the effectiveness of using genetic tests or genetic information as a guide to improve antiplatelet therapies for patients with CAD or patients who had undergone PCI with stent placement (Johnson et al. 2011).

Cardiovascular pharmacogenomics have also caught the attention of the U.S. Food and Drug Administration (FDA). In March 2010, FDA highlighted via a boxed warning about poor clopidogrel responsiveness among carriers of CYP2C19 LOF variant alleles. FDA reminded the existence of genetic tests to determine the patients’ CYP2C19 genotypes. Alternative antiplatelet drugs and therapies should be considered for the PMs. FDA had updated the clopidogrel label three times since 2009 according to the advanced findings on the impact of CYP2C19 genotypes on clinical outcomes (Johnson et al. 2011; FDA 2014).

Many studies had investigated and reported the influence of SNPs in clopidogrel metabolism, which cause wide variation in active metabolite levels and antiplatelet effect of clopidogrel. The most commonly studied SNPs are CYP2C19 *2, *3 and *17 (Ieiri et al. 1996; Yin et al. 2004; Sim et al. 2006; Li et al. 2010). Shuldiner et al. (2009) found out that CYP2C19 *2 carriers had lower levels of clopidogrel active metabolites compared to non-carriers. Mega et al. (2010) also demonstrated that the CYP2C19 *2 variant allele had significant association with increased risk of MACE. Hwang et al. (2011) determined that CYP2C19 *3 variant allele showed similar impact with CYP2C19 *2 allele. In contrast, Frére et al. (2009) indicated that CYP2C19 *17
GOF allele carriers had enhanced antiplatelet effect of clopidogrel compared to non-carriers. Nevertheless, there are also many studies that reported inconsistent findings on CYP2C19 genotypes which showed no significant relationship between CYP2C19 polymorphism and clopidogrel platelet reactivity. Although inconsistent results have been reported, these findings are still important to aid in cardiovascular pharmacogenomics to improve treatment strategies (Johnson et al. 2011).

The understanding of cardiovascular pharmacogenomics has directed attention to personalized antiplatelet therapy especially for high risk patients. Guidelines to use CYP2C19 genotypes in deciding effective clopidogrel antiplatelet therapies for different groups of patients were published by Clinical Pharmacogenetics Implementation Consortium. These guidelines recommended standard clopidogrel doses for NMs and UMs; while alternative antiplatelet agents or therapies should be considered for PMs (Scott et al. 2011).

However, there are certain barriers to be considered when implementing cardiovascular pharmacogenomics into clinical setting and practices. The identified barriers include test-related barriers, knowledge barriers, evidence barriers and finally ethical, legal and social implications, as summarized in Table 2-7.

The completion of the Human Genome Project in 2001 led to significant advancement in medical treatment strategies. Collins et al. (2001) projected that the pharmacogenomics approach to predict drug responsiveness will become a standard practice for many disorders and drugs. Johnson (2013) also predicted that pharmacogenomics will become an important tool to guide in better and efficient treatment decisions in the next five to ten years.
Table 2-7: The types of possible barriers in cardiovascular pharmacogenomics (Johnson 2013).

<table>
<thead>
<tr>
<th>Type of Barriers</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test-related barriers</td>
<td>The concerns of these barriers emphasize that the test has to be performed in a regulated clinical laboratory with short turnaround time. The conducted test must be quick, practical, cost effective and reliable in clinical value.</td>
</tr>
<tr>
<td>Knowledge barriers</td>
<td>Many medical officers, pharmacists and other health professional students are uncertain with the pharmacogenetic test interpretation and drug therapy decision based on the test results. Hence, sufficient pharmacogenomics education is necessary. Clinical decision support tools can also help to provide clear guidance in pharmacogenetic tests and result interpretations.</td>
</tr>
<tr>
<td>Evidence barriers</td>
<td>The evidence to prove the benefit of pharmacogenetic testing is insufficient and inconsistent. More randomized controlled clinical trials and comparative effectiveness trials are required to confirm the effectiveness of pharmacogenetic-guided treatment.</td>
</tr>
<tr>
<td>Ethical, legal and social</td>
<td>These barriers show the concerns in genetic discrimination when including genetic information in medical records. However, this discrimination is unlikely to occur since pharmacogenetic tests are only to assist in predicting possible drug response.</td>
</tr>
</tbody>
</table>
Chapter 3 Research Methodology

3.1 Study Participants and Eligibility Criteria

A total of 323 consecutive patients, who were admitted electively for planned or possible ad hoc PCI, were screened and recruited in a Malaysian tertiary cardiology referral centre (Sarawak General Hospital Heart Centre, Kota Samarahan, Malaysia) from 18th October 2010 to 7th March 2011. Only 237 patients participated in this study were recruited with prior written informed consent form (refer to the Appendix 1 for the consent form). The eligible participants had a recorded clinical history of CAD and were selected for elective coronary angiography with possible ad hoc PCI. All participants were on aspirin therapy of 75 – 300 mg for at least 2 days prior to enrolment and were also given clopidogrel as adjunctive therapy prior to PCI. The participants were categorized into four different groups, according to their pretreatment or loading regimen, as presented in Table 3-1.

Table 3-1: Categorization of study participants according to their pretreatment or loading regimen.

<table>
<thead>
<tr>
<th>Group</th>
<th>Type of Pretreatment or Loading Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Patients that were given clopidogrel 75 mg daily for ≤ 3 days (n = 20).</td>
</tr>
<tr>
<td>2</td>
<td>Patients that were given clopidogrel 75 mg daily for ≥ 4 days (n = 118).</td>
</tr>
<tr>
<td>3</td>
<td>Patients that were given a single loading dose of clopidogrel 300 mg with or without a subsequent 75 mg daily dose (n = 12).</td>
</tr>
<tr>
<td>4</td>
<td>Patients that were given only aspirin (n = 87).</td>
</tr>
</tbody>
</table>

This study design and protocol was approved by the Malaysian Ministry of Health Medical Research and Ethics Committee (MREC) and Swinburne’s Human Research Ethics Committee (SUHREC) in accordance with the Declaration of Helsinki (refer to the Appendix 2 for the ethics approval letter from MREC; Appendix 3 for the ethics approval e-mail from SUHREC;
Appendix 4 for the Swinburne ethics extension e-mail from SUHREC and Appendix 5 for the approved final report from SUHREC).

3.2 Collection and Storage of Blood Samples

Prior to the planned coronary angiography with possible ad hoc PCI, the patients' whole blood samples were collected using standard venipuncture at an antecubital vein. The blood samples collected in the Ethylenediaminetetraacetic Acid (EDTA) tubes were tested for genotyping; while blood samples collected in 4.5 mL blood collection tubes containing anticoagulant hirudin (25.0 µg/mL, Refudan, Dynabyte GmbH, Minuch, Germany) were tested for platelet function analysis by determining the ADP-PA level in whole blood which was measured using Multiplate analyzer. All blood samples were divided into aliquots for batch analysis and were stored safely in a -80.0 °C freezer in the Malaysian tertiary cardiology referral centre until further use.

3.3 Assessment of ADP-induced Platelet Aggregation (ADP-PA) Levels

The whole blood adenosine diphosphate (ADP, ADP test, 6.5 µM final ADP concentration) induced platelet aggregation was assessed by multiple electrode platelet aggregometry (MEA) performed with the Multiplate analyzer (Dynabyte GmbH, Munich, Germany) in the Malaysian tertiary cardiology referral centre. The measured ADP-PA levels were quantified as arbitrary units (AU) and were plotted against time (min) to obtain the area under the aggregation curve (AUC = AU*min). All of the materials used were obtained from the manufacturer, Dynabyte (Munich, Germany). The details of this method have been reported by Sibbing et al. (2008). The optimal cut-off value of 468.0 AU*min was used to determine high ADP-PA levels or poor responsiveness in patients with clopidogrel therapy (Sibbing et al. 2009).
3.4 Genomic DNA Extraction

Using the commercially available kits (Gentra Puregene Blood Kit, Qiagen, Hamburg, Germany) and facilities in the Malaysian tertiary cardiology referral centre, about 300.0 µL of genomic DNA was extracted from 3.0 ml of peripheral whole blood according to the manufacturer’s instructions and stored in a -80.0 °C freezer in the Malaysian tertiary cardiology referral centre and Swinburne University of Technology Sarawak Campus until further use.

3.5 Genotyping

3.5.1 Genotyping of CYP2C19 *2 (681G>A), *3 (636G>A) and *17 (-3402C>T)

Genotyping for CYP2C19 *2 (681G>A; rs4244285), *3 (636G>A; rs4986893) and *17 (-3402C>T; rs11188072) alleles were detected by polymerase chain reaction – restriction fragment linked polymorphism (PCR-RFLP) method according to previous protocols with some modifications, as shown in Table 3-2 (de Morais et al. 1994; Goldstein et al. 1996; Kearns et al. 2010).

CYP2C19 *2 (681G>A) amplification was performed using forward primer (5’-AAT TAC AAC CAG AGC TTG GC-3’) and reverse primer (5’-TAT CAC TTT CCA TAA AAG CAA G-3’). The PCR reactions for CYP2C19 *2 were prepared in final volume of 50.26 µL which contained 10.0 µL of 5X PCR buffer, 8.0 µL of 25 mM MgCl2 solution, 1.0 µL of 10 mM dNTP mix, 2.0 µL of 10 µM forward primer, 2.0 µL of 10 µM reverse primer, 0.26 µL of 5 u/µL GoTaq® DNA polymerase (GoTaq Flexi DNA polymerase, Promega, USA), 4.0 µL of 50 ng/µL DNA template and topped up with 23.0 µL of nuclease-free water.
PCR amplification of CYP2C19 *3 (636G>A) was conducted using forward primer (5'-AAA TTG TTT CCA ATC ATT TAG CT-3') and reverse primer (5'-ACT TCA GGG CTG GGT CAA TA-3'). The forward and reverse primers used to amplify CYP2C19 *17 (-3402C>T) were 5'-TCA AAA GAT ATA TCT GAT AAA TGA TGG-3' and 5'-ACT GTC TCC TGA AGT GTC TGT AC-3', respectively. The PCR reactions for both CYP2C19 *3 (636G>A) and *17 (-3402C>T) were carried out in final volume of 50.26 µL which consisted of 10.0 µL of 5X PCR buffer, 3.0 µL of 25 mM MgCl₂ solution, 1.0 µL of 10 mM dNTP mix, 2.0 µL of 10 µM forward primer, 2.0 µL of 10 µM reverse primer, 0.26 µL of 5 u/µL GoTaq® DNA polymerase (GoTaq Flexi DNA polymerase, Promega, USA), 4.0 µL of 50 ng/µL DNA template and topped up with 28.0 µL of nuclease-free water.

All CYP2C19 *2 (681G>A), *3 (636G>A) and *17 (-3402C>T) PCR reactions were performed in an Eppendorf thermocycler (Mastercycler Gradient, Eppendorf, Germany) with initial denaturation at 94.0 °C for 5 minutes, with 35 cycles of denaturation at 94.0 °C for 45 seconds, annealing at 53.0 °C for 40 seconds, extension at 72.0 °C for 30 seconds and a final extension at 72.0 °C for 5 minutes. No template controls (NTC) were included in every PCR amplification process. The amplified PCR products (169 bp for CYP2C19 *2; 271 bp for CYP2C19 *3 and 337 bp for CYP2C19 *17) were then analyzed on a 2.5% agarose gel with a 100 bp DNA ladder (Promega, USA) as a molecular weight marker and visualized by ethidium bromide (Vivantis, USA) staining, by using the Molecular Imager Gel Doc XR System (Biorad, USA).

The 25.0 µL of CYP2C19 *2 (681G>A), *3 (636G>A) and *17 (-3402C>T) PCR products were digested with SmaI, BamHI and MnlI restriction enzymes (Promega, USA) respectively at 37.0 °C for 4 hours. The final volume of 27.7 µL mixtures contained 2.0 µL of 10X restriction enzyme buffer, 0.2 µL of 10µg/µL acetylated bovine serum albumin, 0.5 µL of the respective restriction enzymes and 25.0 µL of the respective amplified PCR products. NTC were included in each RFLP analysis. The digested PCR products were separated by 4.5% agarose gel with both 100 bp and 50 bp DNA ladder (Promega, USA) and
visualized by ethidium bromide (Vivantis, USA) staining and Molecular Imager Gel Doc XR System (Biorad, USA).

The genetic defect of CYP2C19 *2 is caused by the 681 G>A substitution in exon 5, resulting in a splice-defective site and a defected SmaI restriction site. CYP2C19 *3 has a point mutation of 636 G>A in exon 4, leading to premature stop codon and a defected BamHI restriction site. CYP2C19 *17 polymorphism of 3402 C>T in the 5’ flanking region of the CYP2C19 gene has an absent MnlI restriction site (Ieiri et al. 1996; Yin et al. 2004; Sim et al. 2006). Therefore, homozygous mutant alleles showed single band (undigested), homozygous wild type showed two bands (digested) and heterozygous alleles showed three bands (both digested and undigested). The homozygous mutant of CYP2C19 *2, *3 and *17 yielded single band of 169 bp, 271 bp and 337 bp respectively. The homozygous wild type of CYP2C19 *2 (120 bp and 49 bp), *3 (175 bp and 96 bp) and *17 (117 bp and 220 bp) showed two bands. The heterozygous CYP2C19 *2 (169 bp, 120 bp and 49 bp), *3 (271 bp, 175 bp and 96 bp) and *17 (337 bp, 117 bp and 220 bp) displayed three bands. Table 3-3 summarized the RFLP methods for CYP2C19 *2 (681G>A), *3 (636G>A) and *17 (-3402C>T).

In addition, the genotyping of CYP2C19 *2 (681G>A), *3 (636G>A) and *17 (-3402C>T) by PCR-RFLP analysis was repeated in 10% of the randomly selected samples in order to ensure the consistency of the assays, e.g. the repeated genotyping must show 100% identical results.
Table 3-2: The PCR amplifications of CYP2C19 *2 (681G>A), *3 (636G>A) and *17 (-3402C>T).

<table>
<thead>
<tr>
<th>Allele</th>
<th>CYP2C19 *2 (681G&gt;A; rs4244285)</th>
<th>CYP2C19 *3 (636G&gt;A; rs4986893)</th>
<th>CYP2C19 *17 (-3402C&gt;T; rs11188072)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Forward primer</strong></td>
<td>5’-AAT TAC AAC CAG AGC TTG GC-3’</td>
<td>5’-AAA TTG TTT CCA ATC ATT TAG CT-3’</td>
<td>5’-TCA AAA GAT ATA TCT GAT AAA TGA TGG-3’</td>
</tr>
<tr>
<td><strong>Reverse primer</strong></td>
<td>5’-TAT CAC TTT CCA TAA AAG CAA G-3’</td>
<td>5’-ACT TCA GGG CTT GGT CAA TA-3’</td>
<td>5’-ACT GTC TCC TGA AGT GTC TGT AC-3’</td>
</tr>
</tbody>
</table>

**PCR components (GoTaq Flexi DNA Polymerase, Promega, USA)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer, 5X</td>
<td>10.0</td>
</tr>
<tr>
<td>MgCl2 solution, 25 mM</td>
<td>8.0</td>
</tr>
<tr>
<td>dNTP mix, 10 mM</td>
<td>1.0</td>
</tr>
<tr>
<td>Forward primer, 10 µM</td>
<td>2.0</td>
</tr>
<tr>
<td>Reverse primer, 10 µM</td>
<td>2.0</td>
</tr>
<tr>
<td>GoTaq® DNA polymerase, 5 u/µL</td>
<td>0.26</td>
</tr>
<tr>
<td>DNA template, 50 ng/µL</td>
<td>4.0</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>23.0</td>
</tr>
</tbody>
</table>

**Thermal cycling conditions**

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>Number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94.0</td>
<td>5 minutes</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94.0</td>
<td>45 seconds</td>
<td>35</td>
</tr>
<tr>
<td>Annealing</td>
<td>53.0</td>
<td>40 seconds</td>
<td></td>
</tr>
<tr>
<td>Extension</td>
<td>72.0</td>
<td>30 seconds</td>
<td></td>
</tr>
<tr>
<td>Final Extension</td>
<td>72.0</td>
<td>5 minutes</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 3-3: The RFLP analysis of CYP2C19 *2 (681G>A), *3 (636G>A) and *17 (-3402C>T).

<table>
<thead>
<tr>
<th>Allele</th>
<th>CYP2C19 *2 (681G&gt;A; rs4244285)</th>
<th>CYP2C19 *3 (636G&gt;A; rs4986893)</th>
<th>CYP2C19 *17 (-3402C&gt;T; rs11188072)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restriction enzyme (Promega, USA)</td>
<td>SmaI</td>
<td>BamHI</td>
<td>MnlI</td>
</tr>
<tr>
<td>Restriction enzyme digest components (Promega, USA)</td>
<td>Component</td>
<td>Final Volume (µL)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Restriction enzyme buffer, 10X</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acetylated bovine serum albumin, 10 µg/µL</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Restriction enzyme, 10 µ/µL</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PCR products</td>
<td>25.0</td>
<td></td>
</tr>
</tbody>
</table>

3.5.2 Genotyping of CYP2C19 *17 (-806C>T)

Genotyping for CYP2C19 *17 (-806C>T; rs12248560) polymorphism was detected by PCR followed by high resolution melting (HRM) analysis using the commercially available kits (Type-it HRM PCR Kit, QIAGEN, Hamburg, Germany) together with the Rotor-Gene Q 2plex HRM Platform (QIAGEN, Hamburg, Germany). The HRM analysis was performed according to the manufacturer’s instructions with minor modifications, as shown in Table 3-4 and Figure 3-1.
The HRM analysis of CYP2C19 *17 (-806C>T) was performed by using the designed forward primer (5'-CAA ATT TGT GTC TTC TGT TCT CA-3') and reverse primer (5'-ATC GTG GCG CAT TAT CTC TT-3') respectively. The forward and reverse primers were designed using the primer designing tool called Primer3. The HRM reactions were conducted in the final volume of 12.5 µL which contained 6.25 µL of 2X HRM PCR master mix, 0.88 µL of 10 µM forward primer, 0.88 µL of 10 µM reverse primer, 1.0 µL of 50 ng/µL DNA template and topped up with 3.49 µL of RNase-free water.

The HRM analysis started with 95.0 °C as initial PCR activation step for 5 minutes, with 40 cycles of denaturation at 95.0 °C for 10 seconds and annealing or extension at 55.0 °C for 30 seconds. Then HRM phase was performed at the temperature ranges from 65.0 °C to 95.0 °C with 0.1 °C of increments to cover the full range of expected melting points. Both positive control and NTC were included in each HRM analysis. All samples were amplified and analyzed in duplicate sets. The data obtained was then analyzed using the Rotor-Gene Q software (version 2.3.1) and Rotor-Gene Screen-Clust HRM software (version 1.10.1.2).

Table 3-4: The HRM analysis of CYP2C19 *17 (-806C>T).

<table>
<thead>
<tr>
<th>Allele</th>
<th>CYP2C19 *17 (-806C&gt;T; rs12248560)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>5'-CAA ATT TGT GTC TTC TGT TCT CA-3'</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5'-ATC GTG GCG CAT TAT CTC TT-3'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HRM PCR Components (Type-it HRM PCR Kit, QIAGEN, Hamburg, Germany)</th>
<th>Component</th>
<th>Final Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRM PCR master mix, 2x</td>
<td></td>
<td>6.25</td>
</tr>
<tr>
<td>Forward primer, 10 µM</td>
<td></td>
<td>0.88</td>
</tr>
<tr>
<td>Reverse primer, 10 µM</td>
<td></td>
<td>0.88</td>
</tr>
<tr>
<td>DNA template, 50 ng/µL</td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>RNase-free water</td>
<td></td>
<td>3.49</td>
</tr>
</tbody>
</table>

Thermal Cycling Conditions | Refer to Figure 3-1.
Figure 3-1: The optimized thermal cycling conditions of HRM analysis for CYP2C19 *17 (-806C>T).
HRM analysis is a technique that is based on DNA melting analysis. The DNA samples are characterized according to the dissociation behaviour of the transition from double-stranded DNA (dsDNA) to single-stranded DNA (ssDNA) due to increasing temperature. HRM analysis started with PCR amplifications. The target DNA sequence is amplified to a high-copy number with the presence of a dsDNA-binding fluorescent dye called EvaGreen. The dye will fluoresce when it binds only to the dsDNA. The change in fluorescence is measured to detect increased DNA concentration during PCR amplifications. During the HRM analysis, the temperature increases from a low to high temperature. The fluorescence of EvaGreen is measured continuously and is plotted against temperature. The fluorescence levels are high during the beginning of the HRM. The fluorescence levels decrease when dsDNA starts to dissociate into ssDNA, leading to DNA melting (QIAGEN 2015).

The DNA melting is influenced by GC content and overall base composition. Hence, HRM detects the difference in melting behaviour of each DNA sample to detect variations in the DNA sequence (SNP detection). In other words, different DNA samples are differentiated by different melting temperatures ($T_m$), temperature points at which the dsDNA dissociates into ssDNA (QIAGEN 2015).

Homozygous samples will show a simple curve shape; while heterozygous samples will show a complex curve shape, as shown in Figure 3-2. Positive controls were included in each HRM analysis to distinguish between homozygous wild type, heterozygous and homozygous mutant. NTC were also included to detect the presence of possible primer dimer. Figure 3-3 illustrated the overview of the HRM analysis (QIAGEN 2015).
Figure 3-2: (A): The standard normalized plot of homozygous (simple curve) and heterozygous (complex curve) samples. (B): The difference plot to differentiate between homozygous wild type, heterozygous and homozygous mutant (QIAGEN 2015).
Figure 3-3: The overview of HRM analysis (green: homozygous wild type; blue: heterozygous; red: homozygous mutant) (QIAGEN 2015).
3.5.3 Validation through Direct DNA Sequencing

All genotyping for CYP2C19 *2 (681G>A; rs4244285), *3 (636G>A; rs4986893), *17 (-3402C>T; rs11188072) alleles by PCR-RFLP method and *17 (-806C>T; rs12248560) allele by HRM analysis were completed in Swinburne University of Technology Sarawak Campus.

The genotyping of CYP2C19 *2 (681G>A), *3 (636G>A) and *17 (-3402C>T and -806C>T) were validated for all homozygous mutant and heterozygous samples by direct DNA sequencing. Prior to direct DNA sequencing, the PCR products were purified using PureLink PCR Purification Kit (Invitrogen, U.S.). The purified PCR products were sent to Beijing Genomic Institute, BGI, China and First BASE Laboratories Sdn Bhd, Malaysia for direct DNA sequencing to detect and confirm the presence of CYP2C19 *2 (681G>A), *3 (636G>A) and *17 (-3402C>T and -806C>T) variant alleles.

The sequencing results were validated against the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST). BLAST helps to compare the sequencing results against the NCBI sequence database, whereby the statistical significance of the matches (expect value, E-value) were calculated. Lower E-value or E-value that is closer to zero indicates significant match (Madden 2013). This information can help to verify the existence of CYP2C19 *2 (681G>A), *3 (636G>A) and *17 (-3402C>T and -806C>T) variant alleles. The nucleotide sequences were also analyzed using the BioEdit v7.0.9 software, which is a biological alignment editor. BioEdit helps to confirm the identified homozygous wild type, heterozygous and homozygous variant alleles as well as to determine the correct nucleotide sequence alignment (Mays et al. 2013).
3.6 Predicted Phenotypes

From the CYP2C19 genotypes, study participants can be categorized into three predicted phenotypes or metabolizers which are poor metabolizers (PMs), normal metabolizers (NMs) and ultrarapid metabolizers (UMs) (Gurbel et al. 2010). PMs have at least one copy or both of the *2 and *3 LOF alleles, NMs have either two copies of the *1 wild type alleles or both LOF and GOF alleles; while UMs have at least one copy of the *17 GOF allele, as shown in Table 3-5.

Table 3-5: Categorization of the study participants into three predicted phenotypes or metabolizers according to their CYP2C19 genotypes.

<table>
<thead>
<tr>
<th>Predicted Phenotype</th>
<th>Observed CYP2C19 Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor metabolizer (PM)</td>
<td>*1/*2, *1/*3, *2/*2, *3/*3, *2/*3</td>
</tr>
<tr>
<td>Normal metabolizer (NM)</td>
<td>*1/*1, *2/*17, *3/*17</td>
</tr>
<tr>
<td>Ultrarapid metabolizer (UM)</td>
<td>*1/*17, *17/*17</td>
</tr>
</tbody>
</table>

3.7 12-month Follow-up

The clinical outcomes at 1, 3, 6, 9 and 12 months post-hospital discharge were assessed by telephone interviews. The pre-specified clinical endpoints were cardiovascular death, an event of myocardial infarction, hospital readmission for ACS or stroke, ischemic stroke and the composite of MACE which consists of stent thrombosis, ACS and cardiac death. The bleeding endpoints were based on the Thrombolysis In Myocardial Infarction (TIMI) and Global Use of Strategies to Open Occluded Arteries (GUSTO) bleeding criteria.
3.8 Statistical Analysis

The categorical variables were showed as frequencies and percentages, and were compared using Pearson chi-square or Fisher’s exact test; while the continuous variables were presented as mean with standard deviation or median with interquartile range. The continuous variables were compared by using the independent sample t-test, Pearson chi-square or Fisher’s exact test and one-way ANOVA test. Hardy-Weinberg equilibrium was assessed using the chi-square test to compare the observed genotype frequencies and expected genotype frequencies. The linkage disequilibrium between each variant allele was evaluated, with $D \neq 0$. All of the statistical analyses were two-sided and the significance level ($\alpha$) was set at 0.05. All analyses were conducted using the Statistical Package for the Social Sciences (SPSS) software package (IBM Corp. Released 2010. IBM SPSS Statistics for Windows, Version 19.0. Armonk, NY: IBM Corp.).
3.9 Overview of the Study Design

Patients (n = 323), who were admitted electively for planned or possible ad hoc PCI, were screened in a Malaysian tertiary cardiology referral centre.

Only 237 patients participated in this study with prior written informed consent form.

12-month follow-up were pre-specified.

Patients' blood samples were collected using the standard venipuncture at an antecubital vein.

Genomic DNA was extracted from the peripheral whole blood using a commercially available kit.

Platelet aggregation level in whole blood was measured using Multiplate analyzer (468 AU*min was used as the cut-off value).

CYP2C19 *2 (681 G>A), *3 (636 G>A) and *17 (-3402 C>T) genotyping using the PCR-RFLP method.

CYP2C19 *17 (-806 C>T) genotyping using HRM analysis method.

Genotyping was validated through direct DNA sequencing.
Chapter 4 Results

4.1 Baseline Characteristics of the Study Patients

A total of 237 consecutive patients participated in this study. Our study cohort had a mean age of 57.6 ± 11.1 years and 77.6% (n = 184) were male. A majority of the patients (76.4%) were aged between 50 to 90 years old; while only 23.6% of the patients were aged less than 50 years old. Male patients had a mean age of 56.2 ± 10.7 years; while female patients had a mean age of 62.4 ± 11.4 years. Ethnic group distribution was: Chinese 50.6% (n = 120), Malay 21.1% (n = 50), native Iban 19.0% (n = 45) and other races 9.3% (n = 22) (Figure 4-1).

Figure 4-1: The ethnic group distribution in study cohort of 237 patients
In this study, only 150 (63.3%) patients received DAPT and 87 (36.7%) patients received aspirin monotherapy. These 237 patients were categorized into four different groups, according to their pretreatment or loading regimen (Table 4-1 and Figure 4-2).

**Table 4-1:** Categorization of study participants according to their pretreatment or loading regimen.

<table>
<thead>
<tr>
<th>Group</th>
<th>Type of Pretreatment or Loading Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Patients that were given clopidogrel 75 mg daily for ( \leq 3 ) days (( n = 20 )).</td>
</tr>
<tr>
<td>2</td>
<td>Patients that were given clopidogrel 75 mg daily for ( \geq 4 ) days (( n = 118 )).</td>
</tr>
<tr>
<td>3</td>
<td>Patients that were given a single loading dose of clopidogrel 300 mg with or without a subsequent 75 mg daily dose (( n = 12 )).</td>
</tr>
<tr>
<td>4</td>
<td>Patients that were given only aspirin (( n = 87 )).</td>
</tr>
</tbody>
</table>

**Figure 4-2:** Categorization of patients according to their pretreatment or loading regimen.
Among the patients who received DAPT (n = 150), 78.7% (n = 118) of the patients received a daily dose of 75 mg clopidogrel for ≥ 4 days (Group 2), 13.3% (n = 20) received a daily dose of 75 mg clopidogrel for ≤ 3 days (Group 1) and 8.0% (n = 12) received a single loading dose of 300 mg clopidogrel with or without a subsequent 75 mg daily dose prior to PCI (Group 3), as shown in Figure 4-3.

**Figure 4-3:** The distribution of patients who received DAPT (n = 150) according to group and their pretreatment or loading regimen.
The baseline characteristics of the Group 1, 2, 3 and 4 patients were well-balanced as summarized in Table 4-2.

Table 4-2: The demographic data, risk factors, stent types, concomitant medications and biochemical profiles of the Group 1, 2, 3, and 4 patients. The values are shown as absolute numbers (percentages) or mean ± standard deviation (SD).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1 (n = 20)</th>
<th>Group 2 (n = 118)</th>
<th>Group 3 (n = 12)</th>
<th>Group 4 (n = 87)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years): mean (SD)</td>
<td>60.8 (5.6)</td>
<td>57.3 (12.1)</td>
<td>61.3 (8.8)</td>
<td>56.7 (10.8)</td>
</tr>
<tr>
<td>Male</td>
<td>19 (95.0)</td>
<td>87 (73.7)</td>
<td>8 (66.7)</td>
<td>70 (80.5)</td>
</tr>
<tr>
<td>Female</td>
<td>1 (5.0)</td>
<td>31 (26.3)</td>
<td>4 (33.3)</td>
<td>17 (19.5)</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chinese</td>
<td>10 (50.0)</td>
<td>57 (48.3)</td>
<td>9 (75.0)</td>
<td>44 (50.6)</td>
</tr>
<tr>
<td>Malay</td>
<td>4 (20.0)</td>
<td>29 (24.6)</td>
<td>1 (8.3)</td>
<td>16 (18.4)</td>
</tr>
<tr>
<td>Native Iban</td>
<td>2 (10.0)</td>
<td>24 (20.3)</td>
<td>2 (16.7)</td>
<td>17 (19.5)</td>
</tr>
<tr>
<td>Other Races</td>
<td>4 (20.0)</td>
<td>8 (6.8)</td>
<td>0 (0.0)</td>
<td>10 (11.5)</td>
</tr>
<tr>
<td>Body mass index, kg/m²: mean (SD)</td>
<td>26.5 (3.7)</td>
<td>26.0 (4.0)</td>
<td>25.4 (3.2)</td>
<td>26.1 (3.8)</td>
</tr>
<tr>
<td>Risk factors</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>17 (85.0)</td>
<td>83 (70.3)</td>
<td>9 (75.0)</td>
<td>70 (80.5)</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>15 (75.0)</td>
<td>82 (69.5)</td>
<td>10 (83.3)</td>
<td>62 (71.3)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>6 (30.0)</td>
<td>37 (31.3)</td>
<td>3 (25.0)</td>
<td>27 (31.0)</td>
</tr>
<tr>
<td>Smoking history</td>
<td>8 (40.0)</td>
<td>69 (58.5)</td>
<td>6 (50.0)</td>
<td>48 (55.2)</td>
</tr>
<tr>
<td>Family history of CVDs</td>
<td>5 (25.0)</td>
<td>37 (31.4)</td>
<td>2 (16.7)</td>
<td>37 (42.5)</td>
</tr>
</tbody>
</table>
Table 4-2 (continued): The demographic data, risk factors, stent types, concomitant medications and biochemical profiles of the Group 1, 2, 3, and 4 patients. The values are shown as absolute numbers (percentages) or mean ± standard deviation (SD).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group 1 (n = 20)</th>
<th>Group 2 (n = 118)</th>
<th>Group 3 (n = 12)</th>
<th>Group 4 (n = 87)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Percutaneous coronary intervention</strong></td>
<td>9 (45.0)</td>
<td>45 (38.2)</td>
<td>5 (41.7)</td>
<td>33 (37.9)</td>
</tr>
<tr>
<td><strong>Stent types</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bare metal stent</td>
<td>5 (25.0)</td>
<td>18 (15.3)</td>
<td>3 (25.0)</td>
<td>15 (17.2)</td>
</tr>
<tr>
<td>Drug eluting stent</td>
<td>4 (20.0)</td>
<td>27 (22.9)</td>
<td>2 (16.7)</td>
<td>18 (20.7)</td>
</tr>
<tr>
<td><strong>Concomitant medications</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Statins</td>
<td>16 (80.0)</td>
<td>106 (89.8)</td>
<td>10 (83.3)</td>
<td>83 (95.4)</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>13 (65.0)</td>
<td>45 (38.1)</td>
<td>7 (58.3)</td>
<td>39 (44.8)</td>
</tr>
<tr>
<td>Beta blockers</td>
<td>13 (65.0)</td>
<td>65 (55.1)</td>
<td>7 (58.3)</td>
<td>45 (51.7)</td>
</tr>
<tr>
<td>Proton-pump inhibitors</td>
<td>5 (25.0)</td>
<td>42 (35.6)</td>
<td>3 (25.0)</td>
<td>17 (19.5)</td>
</tr>
<tr>
<td><strong>Haemoglobin (g/100 mL): mean (SD)</strong></td>
<td>13.8 (1.5)</td>
<td>13.6 (1.5)</td>
<td>13.6 (2.2)</td>
<td>13.9 (1.8)</td>
</tr>
<tr>
<td><strong>Hematocrit: mean (SD)</strong></td>
<td>39.8 (8.2)</td>
<td>41.7 (4.7)</td>
<td>41.3 (6.8)</td>
<td>42.1 (5.1)</td>
</tr>
<tr>
<td><strong>Platelet counts (x 10^9/L): mean (SD)</strong></td>
<td>131.8 (155.9)</td>
<td>96.5 (45.7)</td>
<td>95.8 (24.1)</td>
<td>104.4 (51.2)</td>
</tr>
<tr>
<td><strong>Creatinine clearance level (mL/min): mean (SD)</strong></td>
<td>73.7 (26.9)</td>
<td>75.0 (27.3)</td>
<td>63.5 (15.1)</td>
<td>72.6 (25.6)</td>
</tr>
</tbody>
</table>
4.2 Allele Frequencies, Genotypes and Phenotypes of CYP2C19

The allelic frequencies of the CYP2C19 *1, *2, *3 and *17 were 62.9% [95% confidence interval (CI) 67.2% to 58.6%]; 29.3% (95% CI 33.4% to 25.2%); 6.1% (95% CI 8.3% to 3.9%) and 1.7% (95% CI 2.9% to 0.5%) respectively, as presented in Table 4-3. All genotype distributions and allele frequencies were in Hardy-Weinberg equilibrium.

Table 4-3: The allele frequencies of CYP2C19 *1, *2, *3 and *17.

<table>
<thead>
<tr>
<th>Allele</th>
<th>Allele Frequency</th>
<th>95% Confidence Interval (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C19 *1</td>
<td>62.9%</td>
<td>67.2% to 58.6%</td>
</tr>
<tr>
<td>CYP2C19 *2</td>
<td>29.3%</td>
<td>33.4% to 25.2%</td>
</tr>
<tr>
<td>CYP2C19 *3</td>
<td>6.1%</td>
<td>8.3% to 3.9%</td>
</tr>
<tr>
<td>CYP2C19 *17</td>
<td>1.7%</td>
<td>2.9% to 0.5%</td>
</tr>
</tbody>
</table>

In this study cohort, 38.8% (n = 92) of the patients were non-carriers of CYP2C19 *2, *3 and *17 variant alleles (wild type homozygous of CYP2C19 *1/*1). However, 58.2% (n = 138) of the patients were carriers of CYP2C19 *2 and *3 LOF variant alleles and 3.0% (n = 7) of the patients were carriers of CYP2C19 *17 GOF variant allele. These patients were grouped into three predicted phenotypes or metabolizers which were poor metabolizers (PMs), normal metabolizers (NMs) and ultrarapid metabolizers (UMs) according to the observed CYP2C19 genotypes (Table 4-4 and Figure 4-4).

Table 4-4: Categorization of patients into three predicted phenotypes according to their observed CYP2C19 genotypes.

<table>
<thead>
<tr>
<th>Predicted Phenotypes</th>
<th>Observed CYP2C19 Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor metabolizers (PMs), n = 138</td>
<td>*1/*2, *1/*3, *2/*2, *3/*3, *2/*3</td>
</tr>
<tr>
<td>Normal metabolizers (NMs), n = 92</td>
<td>*1/*1</td>
</tr>
<tr>
<td>Ultrarapid metabolizers (UMs), n = 7</td>
<td>*1/*17, *17/*17</td>
</tr>
</tbody>
</table>
Figure 4-4: The predicted CYP2C19 phenotypes of the patients (n = 237).

From the 138 carriers of CYP2C19 *2 and *3 (PMs), 63.8% (n = 88) were heterozygous CYP2C19 *1/*2, 14.5% (n = 20) were heterozygous CYP2C19 *1/*3, 15.9% (n = 22) were homozygous CYP2C19 *2/*2, 0.7% (n = 1) were homozygous CYP2C19 *3/*3 and 5.1% (n = 7) were heterozygous CYP2C19 *2/*3. Of the seven carriers of CYP2C19 *17 (UMs), only one (14.3%) was homozygous CYP2C19 *17/*17 and six (85.7%) were heterozygous CYP2C19 *1/*17 (Figure 4-5). None of the patients were heterozygous CYP2C19 *2/*17 or *3/*17.
Figure 4-5: The distribution of genotypes among the PMs and UMs.
4.3 Prevalence of CYP2C19 Genotypes in Different Ethnic Groups

The allelic frequencies of CYP2C19 *2, *3 and *17 variant alleles were 18.1%, 1.9%, and 0.4% for Chinese; 5.9%, 1.5% and 0.2% for Malay; 3.2%, 1.9% and 0.2% for native Iban; and 2.1%, 0.8% and 0.8% for other races respectively, as listed in Table 4-5. Table 4-6 summarizes the distribution of the determined CYP2C19 genotypes according to different ethnic groups.

Table 4-5: Distribution of allele frequencies according to Chinese, Malay, native Iban and other races.

<table>
<thead>
<tr>
<th>Ethnic Group</th>
<th>Allele Frequency and (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYP2C19 *2</td>
</tr>
<tr>
<td>Chinese (n = 120)</td>
<td>18.1% (21.6% to 14.7%)</td>
</tr>
<tr>
<td>Malay (n = 50)</td>
<td>5.9% (8.0% to 3.8%)</td>
</tr>
<tr>
<td>Native Iban (n = 45)</td>
<td>3.2% (4.7% to 1.6%)</td>
</tr>
<tr>
<td>Other races (n = 22)</td>
<td>2.1% (3.4% to 0.8%)</td>
</tr>
</tbody>
</table>
**Table 4-6:** Distribution of the observed CYP2C19 genotypes according to Chinese, Malay, native Iban and other races. The values are shown as total number (n).

<table>
<thead>
<tr>
<th>Ethnic Group</th>
<th>CYP2C19 Genotypes</th>
<th>*1/*1</th>
<th>*1/*2</th>
<th>*1/*3</th>
<th>*2/*2</th>
<th>*3/*3</th>
<th>*2/*3</th>
<th>*1/*17</th>
<th>*17/*17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinese (n = 120)</td>
<td></td>
<td>41</td>
<td>53</td>
<td>6</td>
<td>16</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Malay (n = 50)</td>
<td></td>
<td>18</td>
<td>21</td>
<td>6</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Iban (n = 45)</td>
<td></td>
<td>24</td>
<td>10</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Others (n = 22)</td>
<td></td>
<td>9</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>92</td>
<td>88</td>
<td>20</td>
<td>22</td>
<td>1</td>
<td>7</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

Chinese patients showed the highest frequency of CYP2C19 *2 variant allele (18.1%) when compared to other ethnic groups. In addition, majority of the PMs were Chinese (55.8%; n = 77), followed by Malay (22.5%; n = 31), native Iban (14.5%; n = 20) and other races (7.2%; n = 10) (Figure 4-6). The allele frequencies of CYP2C19 *3 and *17 were relatively low and were quite equally distributed among the ethnic groups.
4.4 Linkage Disequilibrium

In this study, linkage disequilibrium analysis showed that CYP2C19 *2 (681G>A; rs4244285), *3 (636G>A; rs4986893) and *17 (-3402C>T; rs11188072 and -806C>T; rs12248560) variant alleles were in different linkage disequilibrium. However, both CYP2C19 *17 (-3402C>T and -806C>T) GOF variant alleles were in a complete linkage disequilibrium, where the disequilibrium, D ≠ 0. Both CYP2C19 *17 (-3402C>T and -806C>T) variant alleles displayed similar allele and genotype frequencies.
4.5 Association between *CYP2C19* Polymorphism and ADP-induced Platelet Aggregation (ADP-PA) Levels

The 150 patients who received DAPT showed an overall mean ADP-PA levels of 305.2 ± 163.8 AU*min. The ADP-PA levels ranged from 14.0 to 829.0 AU*min. The ADP-PA value of ≥ 468.0 AU*min was set as the threshold level for high ADP-PA levels or poor responsiveness in patients with clopidogrel therapy (Sibbing et al. 2009).

4.5.1 Distribution of Mean ADP-PA Levels among the Clopidogrel-treated Patients with Different Pretreatment and Loading Regimen

In 150 of the clopidogrel-treated patients, PMs showed higher mean ADP-PA levels compared to NMs (327.4 ± 166.5 vs 268.6 ± 157.4 AU*min, \( p = 0.043 \)). From these 63.3% clopidogrel-treated patients, only 14.7% (\( n = 22 \)) patients had clinical resistance towards clopidogrel (\( p = 0.695 \)). Among these 22 patients, 72.7% (\( n = 16 \)) were PMs who also showed higher mean ADP-PA levels compared to NMs (613.0 ± 107.5 vs 563.0 ± 78.6 AU*min, \( p = 0.314 \)).

There was no significant difference in the post-treatment ADP-PA levels among the Group 1, 2 and 3 patients who received DAPT (\( p = 0.056 \)), as detailed in Table 4-7. Figure 4-7 shows the mean levels of ADP-PA among the NMs and PMs in Group 1, 2 and 3 clopidogrel-treated patients.
Table 4-7: Platelet inhibition profiles of Group 1, 2 and 3 clopidogrel-treated patients.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Phenotype</th>
<th>Clopidogrel Resistance, n (%)</th>
<th>Mean ADP-PA (AU*min)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>NM (n = 5)</td>
<td>1 (5.0%)</td>
<td>378.6 ± 137.3</td>
<td>0.970</td>
</tr>
<tr>
<td></td>
<td>PM (n = 15)</td>
<td>4 (20.0%)</td>
<td>375.5 ± 163.3</td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>NM (n = 43)</td>
<td>5 (4.2%)</td>
<td>258.7 ± 156.9</td>
<td>0.228</td>
</tr>
<tr>
<td></td>
<td>PM (n = 68)</td>
<td>10 (8.5%)</td>
<td>310.6 ± 162.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>UM (n = 7)</td>
<td>0 (0.0%)</td>
<td>264.1 ± 132.2</td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>NM (n = 1)</td>
<td>0 (0.0%)</td>
<td>145.0 ± 0.0</td>
<td>0.302</td>
</tr>
<tr>
<td></td>
<td>PM (n = 11)</td>
<td>2 (16.7%)</td>
<td>365.7 ± 194.1</td>
<td></td>
</tr>
</tbody>
</table>

Figure 4-7: The mean ADP-PA levels among the NMs and PMs in Group 1, 2, and 3 clopidogrel-treated patients.
4.5.2 Distribution of Mean ADP-PA Levels among the Different Ethnic Groups

Of the 150 clopidogrel-treated patients, the ethnic group distribution was: Chinese 50.7% (n = 76), Malay 22.7% (n = 34), native Iban 18.7% (n = 28) and other races 8.0% (n = 12) (Figure 4-8). Chinese patients had the highest prevalence rate of PMs compared to other ethnic groups, as shown in Figure 4-9.

![Figure 4-8: The ethnic group distribution in 150 of the clopidogrel-treated patients.](image)
Figure 4-9: The prevalence of PMs in 150 of the clopidogrel-treated patients according to different ethnic groups.

There was a significant difference in the post-treatment ADP-PA levels among the Chinese clopidogrel-treated patients (p = 0.031), but not among the other ethnic groups, as detailed in Table 4-8. Figure 4-10 shows the mean levels of ADP-PA among the NMs and PMs according to different ethnic groups.

Table 4-8: Platelet inhibition profiles of the clopidogrel-treated patients (NMs and PMs) according to different ethnic groups.

<table>
<thead>
<tr>
<th>Ethnic Group</th>
<th>Phenotype</th>
<th>Mean ADP-PA (AU*min)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinese (n = 76)</td>
<td>NM (n = 20)</td>
<td>249.4 ± 144.4</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>PM (n = 54)</td>
<td>342.5 ± 166.8</td>
<td></td>
</tr>
<tr>
<td>Malay (n = 34)</td>
<td>NM (n = 11)</td>
<td>255.7 ± 123.5</td>
<td>0.372</td>
</tr>
<tr>
<td></td>
<td>PM (n = 22)</td>
<td>302.7 ± 148.1</td>
<td></td>
</tr>
<tr>
<td>Native Iban (n = 28)</td>
<td>NM (n = 13)</td>
<td>316.9 ± 181.2</td>
<td>0.913</td>
</tr>
<tr>
<td></td>
<td>PM (n = 14)</td>
<td>325.3 ± 214.4</td>
<td></td>
</tr>
<tr>
<td>Other races (n = 12)</td>
<td>NM (n = 5)</td>
<td>248.2 ± 226.5</td>
<td>0.869</td>
</tr>
<tr>
<td></td>
<td>PM (n = 4)</td>
<td>266.3 ± 38.8</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4-10: The mean ADP-PA levels among the NMs and PMs according to different ethnic groups.
4.6 Association between CYP2C19 Polymorphism and Clinical Outcomes

In this study cohort of 237 patients, 38.8% or 92 patients underwent PCI with either BMS (17.3%) or DES (21.5%) placement. Overall, there were seven cardiac-related deaths, three hospital readmissions due to ACS or stroke and four non-cardiac related deaths by the end of the 12-month follow-up after the antiplatelet treatment.

From the 150 clopidogrel-treated patients, only 39.3% (n = 59) of the patients underwent PCI with 17.3% (n = 26) and 22.0% (n = 33) had BMS and DES placement respectively. Among the 59 patients with stent implanted, the majority were PMs (64.6%), followed by NMs (32.2%) and UMs (3.4%). Figure 4-11 shows the distribution of NMs, PMs and UMs in 59 patients who underwent PCI with either BMS or DES placement.

![Figure 4-11: The distribution of NMs, PMs and UMs in 59 patients who had either BMS or DES placement.](image)
Among the 26 patients who had BMS placement, no patient experienced bleeding or MACE within one month of follow up after DAPT. The angiography was cancelled for only two patients after the recruitment to the study (Table 4-9).

**Table 4-9:** Clinical outcomes at 1-month follow up in patients with BMS placement after DAPT.

<table>
<thead>
<tr>
<th>Clinical Outcome at 1-month Follow-up</th>
<th>Number of Patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No MACE</td>
<td>25 (96.2%)</td>
</tr>
<tr>
<td>Procedure abandoned</td>
<td>1 (3.8%)</td>
</tr>
</tbody>
</table>

Of the 33 patients who had DES implanted, there were two cardiac-related deaths reported by the end of the 12-month follow-up. These patients were NMs. There were two patients who were lost to follow-up at 12 months; while angiography was cancelled for only one patient after the recruitment to the study (Table 4-10).

**Table 4-10:** Clinical outcomes at 12-month follow up in patients with DES placement after DAPT.

<table>
<thead>
<tr>
<th>Clinical Outcome at 12-month Follow-up</th>
<th>Number of Patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac-related death</td>
<td>2 (6.1%)</td>
</tr>
<tr>
<td>No MACE</td>
<td>28 (84.8%)</td>
</tr>
<tr>
<td>Procedure abandoned</td>
<td>1 (3.0%)</td>
</tr>
<tr>
<td>Lost to follow-up</td>
<td>2 (6.1%)</td>
</tr>
</tbody>
</table>
Chapter 5 Discussions

5.1 Baseline Patient Characteristics

According to Annual Report of the NCVD-PCI Registry Year 2007 to 2009, CAD patients in Malaysia had a mean age of 57.0 ± 10.0 years and the majority was male patients (Ahmad et al. 2011). This is consistent with our study cohort of 237 patients, where the mean age was 57.6 ± 11.1 years and 77.6% were male patients. In comparison with North American National Cardiovascular Disease Registry (mean age range of 63.9 to 64.1 years; 66.0% male) (Peterson et al. 2010) and Melbourne Interventional Group Registry (mean age of 64.9 years; 73.0% male) (Yan et al. 2009), Malaysia CAD patients were younger and the majority was male patients. The average age of CAD is 64.7 years for men; while 72.2 years for women (Go et al. 2013). Men generally possess more and higher cardiovascular risk factors which lead to greater risk in CAD as compared to women (Jousilahti et al. 1999).

Ahmad et al. (2011) reported that a majority of the CAD Malaysian patients were Malay (47.5%), followed by Chinese (23.9%), Indian (22.8%) and native Iban (2.0%). However, our study had more Chinese CAD patients (50.6%), followed by Malay (21.1%), native Iban (19.0%) and other races (9.3%). The study by Lu et al. (2013) also showed higher prevalence of Malay (49.0%) and Chinese (22.5%) patients. Lu et al. (2013) had determined that Malay patients had higher body mass index; while Chinese patients had the highest rate of hypertension and hyperlipidemia.

There are several cardiovascular risk factors that will cause and increase the risk of CVDs. The major and independent risk factors include hypertension, hyperlipidemia, diabetes and smoking history. Patients with hypertension, hyperlipidemia, diabetes and smoking history are prone to have higher risk of CVDs. Other types of risk factors that are associated with high risk of CVDs are obesity and family history of CVDs (Grundy et al. 1999). Overweight patients are the most likely to experience hypertension, hyperlipidemia and diabetes (Eckel 1997). The risk of CVDs doubles in patients with a family history of CVDs.
(Sesso et al. 2001). Most of our CAD patients had higher prevalence of multiple cardiovascular risk factors as compared to both North American National Cardiovascular Disease Registry and Melbourne Interventional Group Registry (Yan et al. 2009; Peterson et al. 2010). High prevalence of cardiovascular risk factors indicates higher complications and risk of CAD among our patients (Grundy et al. 1999).

Hence, there is need to increase cardiovascular healthcare awareness, provide more cardiovascular disease prevention programmes and improve cardiac facilities as well as services in Malaysia to aid in decreasing CAD incidence and mortality.

5.2 Antiplatelet Therapy

In this study, both aspirin and clopidogrel were the most commonly prescribed antiplatelet agents. The most common pretreatment or loading regimen was clopidogrel 75 mg daily for ≥ 4 days prior to PCI. DAPT was more popular compared to aspirin monotherapy. This was also reported in the Annual Report of the NCVD-PCI Registry Year 2007 to 2009 (Ahmad et al. 2011).

Many studies had reported the significance of DAPT in reducing the risk of atherothrombosis, stent thrombosis and recurrent cardiovascular events (Savi et al. 2000; Wenaweser et al. 2005; Bousser 2009; Tantry et al. 2009) especially when DAPT has proven to be efficacious in managing atherothrombosis and reducing the risk of further MACE and stent thrombosis as compared to aspirin monotherapy (Mehta et al. 2000; Yusuf et al. 2001; Mehta et al.2001; Gurbel et al. 2010).

Platelet activation plays a pivotal role in atherothrombosis and stent-related complications (Zaret et al. 1992; Wenaweser et al. 2005). Aspirin inhibits thromboxane production. Clopidogrel on the other hand inhibits ADP receptors. Both aspirin and clopidogrel mechanisms are complementary. Hence, inhibition
of both thromboxane production and ADP-receptors can greatly decrease the risk of atherothrombosis and stent-related complications (Savi et al. 2000; Wenaweser et al. 2005; Bousser 2009; Tantry et al. 2009).

However, there is no clearly defined optimal dosage, timing and duration of antiplatelet treatments for patients with CAD (McCann 2007; Terpening 2009; FDA 2014). The types of appropriate pretreatments, loading regimens and antiplatelet therapies are varied among different patients, since each patient has different levels of CAD, physical conditions, cardiovascular risk factors and genetic background (Ministry of Health Malaysia et al. 2009; Hamm et al. 2011; Sayols-Baixeras et al. 2014).

5.3 Linkage Disequilibrium

CYP2C19 *17 GOF variant allele is characterized by two SNPs (-3402C>T and -806C>T) in the 5’ flanking region of the gene. Our linkage disequilibrium analysis had identified that both SNPs were in complete linkage disequilibrium with each other, where the disequilibrium, D ≠ 0. Both CYP2C19 *17 (-3402C>T and -806C>T) variant alleles displayed similar allele and genotype frequencies. This complete linkage disequilibrium between CYP2C19 *17 (-3402C>T and -806C>T) was also reported by Sim et al. (2006).
5.4 Prevalence of *CYP2C19* *2, *3 and *17 in Multiethnic Malaysian Patients

The prevalence of *CYP2C19* *2, *3 and *17 variant alleles is varied by ethnicity. Studies had reported that Asian populations (~55.0% to 70.0%) have higher prevalence rate of *CYP2C19* LOF variant alleles (*CYP2C19* *2 and *3) compared with white populations (~25.0% to 35.0%) and black populations (~35.0% to 45.0%) (Desta et al. 2002; Lee et al. 2009; Man et al. 2010; Hwang et al. 2011). On the other hand, Asian populations (~4.0%) have low prevalence of *CYP2C19* GOF variant allele (*CYP2C19* *17) as compared to white populations (~18.0%) (Sim et al. 2006; Sugimoto et al. 2008).

Man et al. (2010) and Chan et al. (2012) had demonstrated high prevalence of *CYP2C19* *2 carriers among the Han Chinese (52.5%), Koreans (50.5%), Japanese (50.8%) and Chinese-Singaporean (52.0%). Our study showed similar higher prevalence of *CYP2C19* *2 LOF variant allele, especially among the Chinese-Malaysian.

Carriers of *CYP2C19* *3 were found frequently among the Koreans (14.8%), Japanese (24.2%) and Chinese-Singaporean (10.0%), but were rare among the (0.2%) Caucasians and (0.4%) Africans (Xie et al. 2001; Man et al. 2010; Chan et al. 2012). However, our study showed lower prevalence of *CYP2C19* *3 carriers in multiethnic Malaysian patients.

Both Yang et al. (2004) and Sani et al. (2013) had reported that there were more Chinese PMs compared to other ethnic groups (Malay and Indian). Our study also showed higher prevalence of Chinese PMs compared to Malay, native Iban and other races.

In contrast, *CYP2C19* *17 carriers were more prevalent in (26.0%) Caucasians and (18.0%) Swedish when compared to Asians (4.0% in Chinese; 1.3% in Japanese) (Sim et al. 2006; Sugimoto et al. 2008; Ragia et al. 2009). In our study cohort, there were only seven carriers of *CYP2C19* *17 GOF variant
allele, which confirmed the low prevalence of CYP2C19 *17 in Asian populations.

5.5 Association between CYP2C19 Polymorphism and ADP-induced Platelet Aggregation (ADP-PA) Levels

This study showed that there was no significant influence of the clopidogrel pretreatment types or loading regimen on ADP-PA levels or platelet inhibition. Patients who received clopidogrel 75 mg daily for ≥ 4 days prior to PCI had lower mean ADP-PA levels compared to patients who were given clopidogrel 75 mg daily for ≤ 3 days and patients who received a single loading dose of 300 mg clopidogrel with or without a subsequent 75 mg daily dose prior to PCI (p = 0.056). Most of our patients were given at least 4 days of 75 mg clopidogrel to ensure that the patients had the minimum three daily maintenance dose of clopidogrel to inhibit platelet aggregation and thereby reducing the risk of cardiovascular complications (Gachet 2001; Ahmad et al. 2011). Nevertheless, there was no significant association between platelet inhibition and clopidogrel pretreatment types or loading regimen.

Furthermore, the sizes of the different patient groups according to their types of clopidogrel pretreatment or loading regimen were not equal. Only 20 patients were given clopidogrel 75 mg daily for ≤ 3 days; while only 12 patients received a single loading dose of 300 mg clopidogrel with or without a subsequent 75 mg daily dose prior to PCI. The small sample size of these patient groups may explain the absence of significant association between ADP-PA levels and clopidogrel pretreatment types or loading regimen.

Poor clopidogrel response among our clopidogrel-treated patients could be influenced by the genetic variation in CYP2C19 isoenzyme, which contributes significantly in the two sequential oxidative steps in the biotransformation of clopidogrel into active metabolites (Brandt et al. 2007; Kazui et al. 2010). These active metabolites will inhibit ADP receptors to
prevent ADP-PA (Savi et al. 2000; Ding et al. 2003). The three studied SNPs were CYP2C19 *2, *3 and *17 variant alleles. Both CYP2C19 *2 and *3 alleles are LOF variant alleles which are associated with decreased functional metabolic activity. The genetic defect of CYP2C19 *2 is caused by the 681 G>A substitution in exon 5, resulting in a splice-defective site; while CYP2C19 *3 has a point mutation of 636 G>A in exon 4, leading to premature stop codon and truncated protein (Ieiri et al. 1996; Yin et al. 2004). Conversely, CYP2C19 *17 GOF allele is a polymorphism in the 5’ flanking region of the gene which is associated with increased CYP2C19 gene transcription (Sim et al. 2006; Li et al. 2010). Hence, CYP2C19 *2 and *3 carriers (PMs) will experience decreased antiplatelet effect of clopidogrel and may have increased risk of thrombotic events (de Morais et al. 1994), whereas CYP2C19 *17 (UMs) carriers will experience increased CYP2C19 expression which might lead to risk of bleeding (Sim et al. 2006; Sibbing et al. 2010).

Harmsze et al. (2010) and Sukasem et al. (2013) had reported that CYP2C19 *2 and *3 LOF variant alleles were associated with decreased antiplatelet effect of clopidogrel. However, our study demonstrated that there was no significant impact of the LOF variant alleles in clopidogrel response. Our collected data showed low prevalence of poor clopidogrel responders among the PMs. A recent study conducted on healthy Malaysian subjects by Sani et al. (2013) also concluded that CYP2C19 genotype information cannot adequately predict the clopidogrel responsiveness among the patients. Besides, there were poor clopidogrel responders who were NMs. This further explained that this could be due to patients’ non-compliance, inadequate clopidogrel absorption or other cardiovascular independent risk factors (Nguyen et al. 2006; Feher et al. 2007).

Moreover, our study also observed that most of the Chinese clopidogrel-treated patients were PMs. There was a significant difference in ADP-PA levels among the Chinese clopidogrel-treated PMs (p = 0.031), but not among the other ethnic group patients. This is consistent with the studies reported by
Zou et al. (2013) and Yang et al. (2013), which concluded that Chinese CYP2C19 *2 and *3 carriers were significantly associated with ADP-PA levels.

Gladding et al. (2008) found that individuals that carried CYP2C19 *17 GOF allele had significantly reduced clopidogrel responsiveness. Sibbing et al. (2010) also observed that CYP2C19 *17 carriers displayed enhanced antiplatelet effect of clopidogrel, measured by MEA assays. Our study showed that UMs had lower mean ADP-PA levels and none of the UMs were poor clopidogrel responders.

5.6 Association between CYP2C19 Polymorphism and Clinical Outcomes

A meta-analysis by both Mega et al. (2010) and Hulot et al. (2010) had reported that carriers of LOF variant alleles had increased potential risk of MACE compared to non-carriers. Bonello et al. (2008) explained that adjusting the loading dose of clopidogrel based on the measurement of platelet function or genotyping test may significantly help to improve the clinical outcomes after PCI for patients with CAD.

In this present study, a majority of the PMs had either BMS or DES placement. Nevertheless, only two clopidogrel-treated patients experienced poor clinical outcomes within 12 months after the DES placement. The two patients were NMs. No PM experienced MACE or bleeding within one month of follow up after the BMS placement. Hence, our study determined that clinical outcomes were not significantly associated with clopidogrel antiplatelet therapy and CYP2C19 genotypes. Most of the patients in our study cohort were low-risk stable patients and not all clopidogrel-treated patients had undergone PCI with stent placement. Thus, this might explain the low MACE rate among our patients.
Likewise, both GRAVITAS (Price et al. 2011) and TRIGGER-PCI studies (Trenk et al. 2012) showed no significant differences of clinical outcomes in patients with high platelet reactivity. The GRAVITAS randomized trial demonstrated that the risk of MACE did not decrease among the patients with high platelet reactivity even with the use of high dose or standard dose of clopidogrel (Price et al. 2011). TRIGGER-PCI study also reported low rate of MACE among patients with high on-treatment platelet reactivity, even after switching from clopidogrel to prasugrel (Trenk et al. 2012).
Chapter 6 Research Limitations

6.1 Study Design

The main limitation in this study was the relatively small sample size. For this reason, the findings were underpowered and were inadequate to determine the significant association between CYP2C19 genotypes, platelet inhibition and clinical outcomes. Besides, the sizes of the different patient groups (types of pretreatment or loading regimen and ethnic groups) were not equal. Hence, larger and equal numbers of different patient groups might provide better and significant findings to evaluate the relationship between CYP2C19 genotypes, platelet inhibition and clinical outcomes among different types of pretreatment or loading regimen and ethnic groups. The size and homogeneity of the sample limit the generalizability of this study. In other words, this study was unable to reach statistical significance.

6.2 Alternative Loading Dose of Clopidogrel

Our study did not investigate and determine the optimal pretreatment and loading dose of clopidogrel, especially among the PMs (high risk patients). FDA had highlighted in March 2010 the existence of patients who are PMs. PMs have low levels of active metabolites of clopidogrel and hence will experience reduced antiplatelet effect of clopidogrel. Therefore, alternative dosing strategies for the PMs should be considered to improve their clopidogrel efficacy (FDA 2014).

Von Beckerath et al. (2005) reported that patients prescribed with 600 mg of clopidogrel had higher levels of active metabolites and lower ADP-PA levels compared to patients who received 300 mg of clopidogrel. This study concluded that 600 mg of clopidogrel was recommended to efficiently suppress platelet aggregation. Bonello et al. (2010) also observed that higher dosage (600 mg) of clopidogrel can effectively help to enhance the antiplatelet effect of clopidogrel in CYP2C19 *2 LOF allele carriers.
Mega et al. (2011) evaluated that CYP2C19 *2 heterozygous given increased clopidogrel dosage showed similar levels of platelet reactivity compared to non-carriers who received the standard dosage of clopidogrel. However, CYP2C19 *2 homozygous did not show similar levels of platelet inhibition even with higher dosage of clopidogrel. In addition, Collet et al. (2011) also reported that increased dosage of clopidogrel was effective in CYP2C19 *2 heterozygous but not in homozygous carriers.

To date, an appropriate and established loading dose regimen for PMs still remains unclear and requires further investigation and more clinical trials (FDA 2014).

6.3 Alternative Antiplatelet Agents and Therapies

Alternative antiplatelet agents and therapies are recommended to overcome the clinical resistance toward clopidogrel among PMs (Gurbel et al. 2012; FDA 2014). The potential antiplatelet agents are prasugrel, ticagrelor, cilostazol, ticlopidine and dipyridamole. The pharmacodynamic response of these antiplatelet drugs is not affected by the CYP2C19 pathway (Krishna et al. 2012). Prasugrel, ticagrelor and cilostazol had been reported by various studies to have better antiplatelet effect in high risk CAD patients with stent placement.

Park et al. (2011) demonstrated that the addition of cilostazol to DAPT (aspirin and clopidigrel) can aid in decreasing platelet reactivity in carriers of CYP2C19 LOF alleles. Moreover, Alexopoulos et al. (2011) observed that prasugrel is a more potent antiplatelet drug compared to clopidogrel, and is effective in inhibiting platelet aggregation in CYP2C19 *2 carriers. Wallentin et al. (2010) determined that ticagrelor is a better alternative P2Y12 receptor blocker compared to clopidogrel. Although many studies have proven the efficacy of these alternative antiplatelet drugs, clopidogrel still remains the dominant drug in clinical use, especially when the affordability and accessibility
of these newer P2Y12 inhibitors have to be taken into consideration (Chan et al. 2012; Sani et al. 2013).

In addition, the absolute benefits of dual antiplatelet therapy (aspirin plus clopidogrel) or monotherapy (aspirin or clopidogrel alone) for CAD patients still remain unclear. Both Mehta et al. (2001) and Sabatine et al. (2005) had demonstrated that the addition of clopidogrel to aspirin monotherapy can help to reduce MACE and ischemic complications. However, CHARISMA trial observed that DAPT with clopidogrel and aspirin was not more effective than aspirin monotherapy in reducing or preventing the rate of myocardial infarction, stroke and cardiovascular complications (Bhatt et al. 2006). Therefore, more investigations and assessments are necessary to determine the significant association between both DAPT and monotherapy with antiplatelet response and clinical outcomes.

6.4 Genetic Variations of Other Genes

In this present study, we studied only the genetic polymorphism of the CYP2C19 isoenzyme, which play an important role in metabolism of clopidogrel. CYP2C19 contributes significantly in the two sequential oxidative steps in the biotransformation of clopidogrel into active metabolites. These active metabolites then inhibit ADP receptors to prevent ADP-PA (Brandt et al. 2007; Kazui et al. 2010). However, the genetic variations of other genes involved in absorption (ABCB1 gene) and metabolism (other CYP450 isoenzyme genes such as CYP1A2, CYP2B6, CYP2C9 and CYP3A4) may also influence the pharmacokinetic and pharmacodynamic response of clopidogrel (Taubert et al. 2006; Simon et al. 2009; Kazui et al. 2010).

The oral bioavailability and intestinal absorption of clopidogrel is mediated by the ABCB1 gene. Hence, genetic variation in the ABCB1 gene may influence clopidogrel drug transport and efficacy. The commonly studied variant alleles are ABCB1 C1236T, G2677T/A and C3435T (Fung et al. 2009;
Harmsze et al. 2010). There were studies reported that carriers of \textit{ABCB1} LOF variant alleles had lower levels of active metabolites and might have higher risk of cardiovascular complications when compared to non-carriers (Taubert et al. 2006; Simon et al. 2009). Simon et al. (2009) also observed that individuals who carried both \textit{CYP2C19} and \textit{ABCB1} LOF variant alleles may have the highest risk of MACE. The \textit{ABCB1} variant alleles alone showed no significant independent effects, but both \textit{CYP2C19} and \textit{ABCB1} showed complementary and significant findings.

Besides \textit{CYP2C19} isoenzyme, other CYP450 isoenzymes (\textit{CYP1A2}, \textit{CYP2B6}, \textit{CYP2C9} and \textit{CYP3A4}) involved in clopidogrel metabolism should be evaluated too. The role of \textit{CYP1A2}, \textit{CYP2B6} and \textit{CYP2C19} in the formation of 2-oxo-clopidogrel was 35.8%, 19.4% and 44.9% respectively; while the role of \textit{CYP2B6}, \textit{CYP2C9}, \textit{CYP2C19} and \textit{CYP3A4} in the formation of the active thiol metabolites was 32.9%, 6.8%, 20.6% and 39.8% respectively (Kazui et al. 2010). According to the contributions from \textit{CYP2C19} and \textit{CYP3A4}, Kazui et al. (2010) indicated that genetic variations of both \textit{CYP2C19} and \textit{CYP3A4} have a more significant impact in influencing the antiplatelet effect of clopidogrel.
6.5 Non-genetic Risk Factors

Shuldiner et al. (2009) reported that the variability of pharmacokinetic and pharmacodynamic response of clopidogrel is affected by both non-genetic and genetic factors. The non-genetic factors, which include age, body mass index, triglyceride levels and high-density lipoprotein cholesterol levels, have about less than 10.0% of impact towards clopidogrel response. CYP2C19 *2 genotype (genetic factor) can only explain approximately 12.0% of clopidogrel response variability. Similarly, Hochholzer et al. (2010) observed that CYP2C19 *2 alone affects only 5.2% of the clopidogrel antiplatelet effect. Other major independent factors are age, body mass index and diabetes mellitus. The combined genetic and non-genetic factors contribute to about 11.5% of clopidogrel response. Geisler et al. (2008) also concluded that increased age, ACS, diabetes mellitus, renal failure and reduced left ventricular function have significant roles in poor platelet inhibition. A recent study had demonstrated that there is a wide variability of clopidogrel response even with exclusion or control of factors such as genetic polymorphisms, non-compliance, concomitant medications (proton-pump inhibitors, statins), diet, smoking and other demographic factors (Frelinger et al. 2013). Therefore, both genetic and non-genetic factors should be investigated to determine the significant relationship between those risk factors and platelet inhibition.
Chapter 7 Conclusions
7.1 Summary of Research Findings

When compared with North American National Cardiovascular Disease Registry (Peterson et al. 2010) and Melbourne Interventional Group Registry (Yan et al. 2009), our CAD patients in Malaysia had a younger mean age. Most of our patients had higher prevalence of multiple cardiovascular risk factors which increased the risk of cardiovascular complications. The majority of the patients in our study cohort were male patients.

CYP2C19 *2 carriers, but not CYP2C19 *3 and *17 carriers, are highly prevalent in Malaysians. There was a wide inter-ethnic diversity of CYP2C19 polymorphism in our multiethnic Malaysian population. Most of the CYP2C19 *2 LOF allele carriers were Chinese patients. In this study, the allele frequencies of CYP2C19 *3 and *17 variant alleles were relatively small and were quite equally distributed among the ethnic groups. This study showed more Chinese PMs compared to other ethnic groups (Malay, native Iban and other races).

All genotype distributions and allele frequencies in this study were in Hardy-Weinberg equilibrium. Our linkage disequilibrium analysis identified that both SNPs of CYP2C19 *17 (-3402C>T and -806C>T) were in complete linkage disequilibrium with each other.

In our study, DAPT (aspirin and clopidogrel) was a more preferable antiplatelet therapy. The most common pretreatment or loading regimen was clopidogrel 75 mg daily for ≥ 4 days. However, there was no significant influence of clopidogrel pretreatment types or loading regimen on platelet inhibition. In addition, our study determined that LOF variant alleles have no significant impact in clopidogrel response. This study also showed low prevalence of poor clopidogrel responders among the PMs. Nevertheless, there was a significant difference in ADP-PA levels among the Chinese PMs, but not among other ethnic groups. None of the UMs in this study were poor clopidogrel responders. Besides, our study demonstrated that clinical outcomes were not
significantly associated with clopidogrel antiplatelet therapy as well as CYP2C19 genotypes.

Hence, regardless of high prevalence of PMs in our study cohort, the findings of this study showed that CYP2C19 polymorphism had less impact on both clopidogrel platelet inhibition and clinical outcomes after stent placement. Based on our study, CYP2C19 genotyping and platelet function test alone cannot accurately determine the patients’ levels of platelet reactivity and clinical outcomes. Furthermore, there is no clearly defined optimal dosage, timing and duration of antiplatelet treatment for patients with CAD.
7.2 Recommendations of Future Works

Our study was limited to a single-centre study in Sarawak, Malaysia. Thus, larger studies and trials to compare larger groups of patients from different states and ethnic groups are recommended to provide better and significant findings. Moreover, the impact of DAPT and aspirin monotherapy as well as different loading regimens of clopidogrel in antiplatelet effect of clopidogrel among PMs requires further observation. Besides CYP2C19 polymorphism, genetic variations of other genes involved in clopidogrel absorption (ABCB1 gene) and metabolism (other CYP450 isoenzyme genes such as CYP1A2, CYP2B6, CYP2C9 and CYP3A4) should be evaluated too. Furthermore, it is also essential to determine the influence of both genetic and non-genetic factors (age, body mass index, hypertension, hyperlipidemia, diabetes mellitus, diet and smoking history) in pharmacokinetic, pharmacodynamic and clinical efficacy of clopidogrel. In addition, the necessity of alternative antiplatelet drugs and therapies for poor clopidogrel responders warrants further investigation.

More studies are necessary to aim towards optimal test or treatment strategies to improve healthcare among patients with CAD. An ideal treatment strategy has to be cost effective and practical in a clinical setting and can help to accurately characterize low or high risk patients. These predictive treatment strategies have to be able to improve treatment outcomes significantly.
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Appendices

Appendix 1: Consent Form

Title: Platelet reactivity to Aspirin and Clopidogrel in patients undergoing coronary angiography: a multiethnic study in a Malaysian population.

Investigator: Dr Alan Fong
Sponsor: Department of Cardiology
         Sarawak General Hospital
         Jalan Tun Ahmad Zaidi Adruce
         93586 Kuching, Sarawak,
         Malaysia

Background and Purpose of this Study

You are invited to participate in this research study (the “Study”) because you are about to undergo a special test (procedure) called a coronary angiogram. If there is a blockage to one or more of the blood vessels that supply blood to the heart (coronary artery, arteries plural), we may be able to treat it with a procedure called angioplasty +/- with stent (percutaneous intervention, “PCI”). Angioplasty is using a small balloon to open up a blockage from within the artery, while a stent is a small metallic device to keep the blockage unblocked. In preparing for a coronary angiogram which may require a PCI, all patients, like you, have been started on blood thinning agents (“antithrombotics”) before this procedure.

Antithrombotic agents allow our research team (“us”; doctors) to perform PCI quite safely, in particular, when we have to use a stent during the procedure. Antithrombotics make stents less likely to block off suddenly due to a blood clot (“thrombus”, “thrombosis”). In some cases, these blood clots do occur (at an approximate rate of 2%/year), even during treatment with antithrombotics, and it is not yet fully understood why that occurs. Blood clots that suddenly occur within the stent can be dangerous, and may bring about a serious heart attack.

However, treatment with antithrombotics increases the risk of serious bleeding (e.g. stroke, abdominal wall and gut), (approximately 2% during treatment with antithrombotics) and why this occurs in some patients is also not fully understood.

This Study allows us to study the actions of the antithrombotics taken by you before your PCI. This study also allows us to better understand the process of thrombosis; hence, we hope that, in the future, we shall be able to treat our patients better, in particular those who require coronary procedures and PCI. In this Study, we do this by taking some blood from you and test for special markers (“biomarkers”) of thrombosis. Part of this also involves analyzing the blood for the level of the antithrombotics as well as the low your genes affect the thrombosis process.

A biomarker is a molecule found in blood, other body fluids, or tissues that is a sign of a normal or abnormal process, or of a condition or disease. A biomarker may be used to see how well the body responds to a treatment for a disease or condition. Some biomarkers are related to the expression of your genes. Therefore, genetic studies will also be performed on your blood as part of this research project.

In total, 100-150 patients will be enrolled into this study.
Approximately 20mls (2 tablespoonfuls) of your blood samples will be taken when you are admitted to hospital for an angiogram. No extra blood sample is required for the assessment.

Blood samples will be stored and processed for the selected biomarkers used in this research study only. [Please sign Informed Consent Form Section 1 ONLY]

In the future, there may be new biomarkers may be discovered that may help us to understand thrombosis even better. If you agree to allow us to store your blood in a way for analysis of selected biomarkers used in this Study AND also future analysis using newly discovered biomarkers, please kindly let us know. Your blood will be stored for no longer than 10 years from the date signed on your Consent Form. [Please sign Informed Consent Form Section 2 ONLY]

If you should disagree to participate in this Study, you will still be treated in the standard manner. Participation in this Study is absolutely voluntary. You may withdraw consent at any time without giving any reason, and blood samples taken from you disposed. There will not be any disadvantages for you or your treatment.

Confidentiality of your Information

If you agree to participate in this Study, personal information will be collected from your medical records. This data will be used and processed manually or by computer. The data collected is for medical research purposes, to be used within the limits specified in this Study. Any database containing information about you shall refer to a participant identification number which is unique to each participant and will not reveal your identity. You unconditionally accept that this data will also be made available to, or accessed by, the relevant regulatory authorities, the relevant Ethics Committees and monitor(s) and auditor(s) appointed by our organization for verification of the procedures of this Study. Such access shall not violate the confidentiality of your identity and personal information and shall be subject to the extent permitted by the applicable laws and regulations. It is likely that the results of this Study will be published in one or more medical journals. Any such publication will not identify you by name.

Problems or Questions

If you have further questions about this Study, you can contact any doctor working in our Department. (082-276666; ext 2222 or 5925)

If you have further questions regarding your rights as a participant in clinical research, please contact the Medical Research and Ethics Committee, c/o National Institutes of Health Secretariat. (03-22874032)
Informed Consent Form (SECTION 1)

I have read and understood the information of this Study. Before signing, information about the contents of this document and the Study was explained to me. My physician has answered all my questions regarding the procedure and the Study. I have had sufficient time to consider my participation in this Study and I am aware that participation in this Study is completely voluntary. I realize that I may decide to stop participation at any time without affecting the quality of healthcare provided.

I authorize and instruct my physician(s) and institution to release necessary personal information about me pertaining only to this Study. I understand that I am entitled to access the personal information collected about me and to have inaccuracies corrected.

I agree to participate voluntarily in this Study.

Patient (or if applicable, Legal Representative)

Name: ____________________________  Signature: ____________________________  Date: ____________________________

IC Number: ________________________

Witness

Name: ____________________________  Signature: ____________________________  Date: ____________________________

IC Number: ________________________

Investigator or delegated person by the investigator

Name: ____________________________  Signature: ____________________________  Date: ____________________________

IC Number: ________________________
Informed Consent Form (SECTION 2)

I have read and understood the information of this Study. Before signing, information about the contents of this document and the Study was explained to me. My physician has answered all my questions regarding the procedure and the Study. I have had sufficient time to consider my participation in this Study and I am aware that participation in this Study is completely voluntary. I realize that I may decide to stop participation at any time without affecting the quality of healthcare provided.

I authorize and instruct my physician(s) and institution to release necessary personal information about me pertaining only to this Study. I understand that I am entitled to access the personal information collected about me and to have inaccuracies corrected.

I agree to participate voluntarily in this Study.

I agree to allow my blood samples to be stored in a way to allow future testing of selected biomarkers for no longer than 10 years. Any testing or research will require permission from an approved Ethics Committee.

Patient (or if applicable, Legal Representative)

Name ___________________________ Signature ___________ Date ___________

IC Number ________________________

Witness

Name ___________________________ Signature ___________ Date ___________

IC Number ________________________

Investigator or delegated person by the Investigator

Name ___________________________ Signature ___________ Date ___________

IC Number ________________________

Total pages: 4
Appendix 2: Ethics Approval Letter from MREC

MEDICAL RESEARCH & ETHICS COMMITTEE
MINISTRY OF HEALTH MALAYSIA
c/o Institute for Health Management
Jalan Rumah Sakit, Bangsar
59000 Kuala Lumpur

Kami : (6)Jm.KKM/NIHSEC/08/0804/P09-316
Tarikh : 4 June 2010

Protocol Title :
Platelet Reactivity to Aspirin and Clopidogrel in Patients Undergoing Coronary
Angiography: a Multiethnic Study in a Malaysian Population

Principal Investigator : Dr Alan Fong Yean Yip
Jabatan Kardiologi
Hospital Umum Sarawak

Documents received and reviewed with reference to the above study:
1. Protocol,
2. Form JTP/KKM-3ver1.1
3. Informed Consent Form
   - English and Malay
4. Curriculum Vitae and GCP certificate of Investigator

The Medical Research & Ethics Committee, Ministry of Health Malaysia operates in accordance
to the International Conference of Harmonization Good Clinical Practice Guidelines.

Project Sites: Hospital Umum Sarawak

Decision by Medical Research & Ethics Committee:
( ) Approved
( ) Conditionally Approved
( ) Disapproved

Date of Decision : 4 June 2010

DATO' DR CHANG KIAN MENG
Chairman
Medical Research & Ethics Committee
Ministry of Health Malaysia
Appendix 3: Ethics Approval E-mail from SUHREC

From: Keith Wilkins [kwilkins@swin.edu.au]
Sent: Friday, July 06, 2012 4:01 PM
To: Siaw San Hwang
Cc: Ken Heskin; alanfong@crc.gov.my; Anatoli Vaikhgelt; Wallace ShungHui Wong; Dennis MouLing Wong; resethics@swin.edu.au
Subject: SUHREC Project 2012/091 Swinburne Ethics Clearance

To: Dr Hwang Siaw San, PhD
School of Engineering, Computing and Science, SUTL

Dear Siaw San Hwang,

SUHREC Project 2012/091 Platelet reactivity to Aspirin and Clopidogrel in patients undergoing coronary angiography: a multiethnic study in a Malaysian population
Dr Siaw San Hwang, Swinburne Sarawak; Ms Chua Jia Ni et al
Approved Duration: 6/07/2012 to 31/07/2013 (Interim)

I refer to the ethical review of the above Masters student-related project by delegates of Swinburne's Human Research Ethics Committee (SUHREC). Your submissions to date were as per your emails of 6 and 23 March 2012 with attachments. Feedback on these submissions were sent to you on 5 April 2012. Your further responses were emailed: on 11 May 2012 with attached letter of support from the Head of SUTL School of Engineering, Computing & Science and attached Masters project proposal; and 25 June 2012 with attached letter of support from the Head of Cardiology of Sarawak General Hospital. The Swinburne ethical review also proceeded significantly on the basis of the prior ethical review conducted by the Malaysia Ministry of Health Medical Research & Ethics Committee. Significantly, it was also noted that the existing participant consents for the original project approved by the Ministry’s Medical Research & Ethics Committee apparently allow for further research use of human blood samples collected.

I am pleased to advise the project has approval to commence in line with standard on-going ethics clearance conditions listed below (as applicable). However, this clearance is given on the understanding that, where applicable or required, the Malaysia Ministry of Health Medical Research & Ethics Committee has been or will have been properly notified by Swinburne Sarawak, if not also by Dr Alan Fong, of the Swinburne ethics clearance and Swinburne Sarawak involvement in relation to the project originally approved by the Ministry’s Medical Research & Ethics Committee. A copy of the notification sent to that Committee should be sent to my office for the record as soon as practicable. In the event of a
query or complaint relating to the original project, the notification may prove significant for and protective of Swinburne researchers.

- All human research activity undertaken under Swinburne auspices must conform to Swinburne standards, including external regulatory standards such as the current National Statement on Ethical Conduct of Human Research and with respect to secure data use, retention and disposal.

- The named Swinburne Chief Investigator/Supervisor remains responsible for any personnel appointed to or associated with the Swinburne student project being made aware of ethics clearance conditions, including research and consent procedures or instruments approved. Any change in chief investigator/supervisor requires timely notification and appropriate endorsement.

- The above project has been approved as submitted for ethical review by or on behalf of SUHREC. Amendments to approved procedures or instruments ordinarily require prior ethical appraisal/clearance. SUHREC must be notified immediately or as soon as possible thereafter of (a) any serious or unexpected adverse effects and any redress measures; (b) proposed changes in protocols; and (c) unforeseen events which might affect continued ethical acceptability of the project.

- At a minimum, an annual report on the progress of the project is required as well as at the conclusion (or abandonment) of the project.

- A duly authorised external or internal audit of the project may be undertaken at any time.

Please contact the Research Ethics Office at Swinburne Research if you have any queries about the Swinburne ethical review and if you need a signed ethics clearance certificate, citing the SUHREC project number. Copies of communication emails should be retained as part of project record-keeping.

Best wishes for the project, including to Ms Chua Jia Ni.

Yours sincerely

Keith Wilkins
Secretary, SUHREC

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Keith Wilkins
Research Ethics Officer
Swinburne Research (H68)
Swinburne University of Technology
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Appendix 4: Swinburne Ethics Extension E-mail from SUHREC

From: RES Ethics <resethics@swin.edu.au>
Date: April 1, 2014 4:44:22 AM GMT+08:00
To: 'SiawSan Hwang' <shwang@swinburne.edu.my>
Cc: RES Ethics <resethics@swin.edu.au>
Subject: RE: SUHREC Project 2012/091 Swinburne Ethics Modification/Extension (I)

To: Dr Hwang Siaw San, PhD
School of Engineering, Computing and Science, SUTL

Dear Siaw San Hwang

SUHREC Project 2012/091 Platelet reactivity to Aspirin and Clopidogrel in patients undergoing coronary angiography: a multiethnic study in a Malaysian population
Dr Siaw San Hwang, Swinburne Sarawak; Ms Chua Jia Ni et al
Approved Duration: 06/07/2012 to 31/07/2013 (Interim); extended to 31-012-2014 (April 2014)

I refer to your request for a simple extension of ethics clearance to complete the approved human research activity as per the report form received at Swinburne Research.

There being no change to the approved protocol as submitted to date, I am authorised to issue the clearance for the extension to 31/12/2014. The standard ethics clearance conditions previously communicated and reprinted below still apply.

Please contact the Research Ethics Office if you have any queries about on-going ethics clearance, citing the SUHREC project number. Copies of clearance emails should be retained as part of project record-keeping.

As before, best wishes for the project.

Yours sincerely,
Astrid Nordmann

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Dr Astrid Nordmann
Research Ethics Executive Officer
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Fax: +613 9214 5267
Appendix 5: Approved Final Report from SUHREC

Acknowledgement of Report for SUHREC Project - 2012/091

JiaNi Chua

Sent: Thursday, July 23, 2015 9:44 PM
To: JiaNi Chua

-----Original Message-----
From: resethics@swin.edu.au [mailto:resethics@swin.edu.au]
Sent: Thursday, 23 July, 2015 12:05 PM
To: Siau San Hwang
Cc: resethics@swin.edu.au
Subject: Acknowledgement of Report for SUHREC Project - 2012/091

Dear Siau San Hwang,

Re: Final Report for the project (Report Date: 23-07-2015)

2012/091 'Platelet reactivity to Aspirin and Clopidogrel in patients undergoing coronary angiography: a multiethnic study in a Malaysian population'

The Final report for the above project (Report Date: 23-07-2015) has been processed and satisfies the reporting requirements set under the terms of ethics clearance.

Research Ethics Team

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