Pharmacogenetics in Clopidogrel

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To my Dad, my pillar and my guiding light, who instilled in me the virtues of perseverance and commitment and relentlessly encouraged me to strive for excellence.

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Abstract

The cytochrome P450 (CYP) 2C19 loss-of-function *2 allele is associated with reduced clopidogrel bioactivation, increasing the risk of atherothrombotic complications after percutaneous coronary intervention (PCI). In-stent restenosis (ISR) is a complication that limits the long-term prognosis of PCI.

The aim of the research was to investigate the association between *CYP2C19*2* and the incidence of ISR within one year after PCI in patients prescribed dual antiplatelet therapy (DAPT) with aspirin and clopidogrel.

A retrospective matched case-control study design with prospective follow-up was adopted. All (N=2,908) patients who underwent PCI with stent implantation between January 2014 and December 2018 were screened using the Cardiovascular Information Management System at the Department of Cardiology at Mater Dei Hospital. Patients with angiographically-confirmed drug eluting stent (DES)-ISR within 1 year when on DAPT with aspirin and clopidogrel were identified (Cases), and patients with no documented ISR post-PCI in the study period (Controls) were case-matched for age, gender, diabetes mellitus and estimated glomerular filtration rate (eGFR). Cases and controls were invited by the cardiologist for *CYP2C19*2* genotyping. After obtaining informed written patient consent, a data collection form was completed, an EDTA-blood sample was collected, and genomic DNA extraction was performed. *CYP2C19*2* genotyping of cases and controls was undertaken with the Autoimmun Diagnostika GmbH kits using gradient polymerase chain reaction and reverse hybridisation. The association between *CYP2C19*2* and incidence of coronary ISR was analysed using the

Fisher's Exact test (univariate analysis) and binary logistic regression (multivariate analysis); $p<0.05$ considered statistically significant.

Eighty-one patients with angiographically-confirmed DES-ISR within one-year post-PCI while on clopidogrel were identified, of whom 13 patients passed away, 7 refused enrolment into the study, and 1 was on haemodialysis, and these were excluded. Sixty cases (mean age 65±9.86 years, 51 male, 30 diabetics, mean eGFR 77±20.29 mL/min/1.73m^2) and 60 matched controls were enrolled. Twenty-six (43.3%) cases and 5 (8.3%) controls were carriers of *CYP2C19*2.* The association between *CYP2C19*2* carrier status and ISR within one-year post-PCI was statistically significant ($p<0.001$) in both the univariate and multivariate analysis. Univariate analysis showed an odds ratio of ISR occurrence in *CYP2C19*2* carriers of 8.4, which increased to 22.6 in the multivariate analysis.

The proportion of *CYP2C19*2* carriers who presented with ISR within one-year post-PCI while on clopidogrel was significantly higher compared to patients with no documented ISR. Previous revascularisation, heart failure and active smoking were other variables observed to be significantly associated with the incidence of ISR. The study indicates that *CYP2C19*2* genotyping may be used as a tool together with consideration of non-genetic factors for precision antiplatelet therapy to decrease the risk of ISR.

Keywords: Clopidogrel - *CYP2C19*2* - In-stent restenosis - Percutaneous coronary intervention - Pharmacogenetic testing - Precision antiplatelet therapy

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List of Abbreviations

(*)	Star allele name / Haplotype
ACC	American College of Cardiology
ACS	Acute Coronary Syndrome
ADR	Adverse Drug Reaction
AHA	American Heart Association
BMI	Body Mass Index
BMS	Bare Metal Stent
CABG	Coronary Artery Bypass Graft
CCS	Cardiac Catheterisation Suite
CPIC	Clinical Pharmacogenetics Implementation Consortium
CV	Cardiovascular
CVD	Cardiovascular Disease
CVIS	Cardiovascular Information Management System
CYP2C19	Cytochrome P450 2C19
DEB	Drug-Eluting Balloon
DES	Drug-Eluting Stent
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
DPWG	Royal Dutch Association for the Advancement of Pharmacy-Pharmacogenetics Working Group
EDTA	Ethylenediaminetetraacetic acid
eGFR	Estimated Glomerular Filtration Rate
EM	Extensive Metaboliser
EMA	European Medicines Agency
ESC	European Society of Cardiology
FDA	Food and Drug Administration
HF	Heart Failure
HPR	High Platelet Reactivity

IHD	Ischaemic Heart Disease
IM	Intermediate Metaboliser
ISR	In-Stent Restenosis
LoF	Loss-of-Function
LPR	Low Platelet Reactivity
LVEF	Left Ventricular Ejection Fraction
MACE	Major Adverse Cardiovascular Events
MDH	Mater Dei Hospital
MDU	Molecular Diagnostics Unit
NHS	National Health Service
NSTEMI	Non-ST-Elevation Myocardial Infarction
PCI	Percutaneous Coronary Intervention
PM	Poor Metaboliser
PPI	Proton Pump Inhibitor
ST	Stent Thrombosis
STEMI	ST-Elevation Myocardial Infarction
SSRI	Selective Serotonin Reuptake Inhibitor
TCA	Tricyclic Antidepressant
UM	Ultra-rapid Metaboliser
USA	United States of America

Chapter One
Introduction

1.1 Dual antiplatelet therapy

In accordance with European and American cardiology guidelines, dual antiplatelet therapy (DAPT), comprising aspirin and a $P2Y_{12}$ inhibitor (clopidogrel, ticagrelor or prasugrel), is recommended as the cornerstone therapy in patients with acute coronary syndrome (ACS) or chronic coronary syndrome, after percutaneous coronary intervention (PCI) with stent implantation to prevent atherothrombotic complications (Amsterdam et al, 2014; Levine et al, 2016a,b; Roffi et al, 2016; Ibanez et al, 2018; Valgimigli et al, 2018; Neumann et al, 2019; Knuuti et al, 2020).

In patients with ACS undergoing PCI with stent deployment, the European Society of Cardiology (ESC) recommends DAPT with aspirin and a $P2Y_{12}$ inhibitor, such as clopidogrel, for at least 12 months. In patients with chronic coronary syndrome, DAPT after drug-eluting stent (DES) implantation is to be continued for at least 6 months, provided there is no bleeding risk, and clopidogrel is the $P2Y_{12}$ inhibitor of choice. In both ACS and chronic coronary syndrome, a 3-month DAPT duration is recommended only for patients considered at high risk of bleeding (Valgimigli et al, 2018; Knuuti et al, 2020).

1.2 Pharmacology of clopidogrel and variability in patient response

Clopidogrel is a second-generation thienopyridine prodrug and its active metabolite inhibits platelet aggregation and activation through selective and irreversible adenosine diphosphate binding (Angiolillo et al, 2007; Jiang et al, 2015). Clopidogrel is the most frequently prescribed $P2Y_{12}$ inhibitor post-PCI due to its lower cost and fewer reported

bleeding events compared to the other $P2Y_{12}$ inhibitors (Mahoney et al, 2010; Theidel et al, 2013; Zhuang et al, 2014; Basra et al, 2018; Dayoub et al, 2018; Claassens et al, 2020b,c). Clopidogrel requires hepatic activation by several CYP450 enzymes to achieve its antiplatelet effect, principally the CYP2C19 enzyme (Gurbel et al, 2003; Brandt et al, 2007; Kazui et al, 2010; Scott et al, 2012; Scott et al, 2013; Jiang et al, 2015).

The effectiveness of clopidogrel has been reported in several studies demonstrating the benefits of incorporating clopidogrel with aspirin to reduce the risk of recurrent ischaemic cardiovascular (CV) events in patients with ACS and those undergoing PCI, such as myocardial infarction (MI) and stent thrombosis (ST) (Mehta et al, 2001; Yusuf et al, 2001; Steinhubl et al, 2002; Chen et al, 2005; Sabatine et al, 2005; Angiolillo et al, 2007; Eshaghian et al, 2007; Rollini et al, 2012; Squizzato et al, 2017). Clopidogrel is also reported to be usually well-tolerated with respect to bleeding adverse events (Eshaghian et al, 2007; DiNicolantonio et al, 2013; Yun et al, 2019).

However, despite treatment with clopidogrel, some patients still persist to experience recurrent CV episodes (Aradi et al, 2015; Spiliopoulos & Pastromas, 2015; Winter et al, 2015). A decreased response to clopidogrel has been associated with patient variability in several studies (Angiolillo et al, 2007; Gurbel & Tantry, 2007; Shuldiner et al, 2009; Combescure et al, 2010; Dahl & Gunes, 2010; Hochholzer et al, 2010; Perry, 2011; Würtz & Grove, 2012; Golukhova et al, 2015). The effectiveness and safety profile of clopidogrel can be influenced by several factors, such as concomitant drugs, diabetes mellitus, age, weight, gender, smoking and genetic factors (Siller-Matula et al, 2008; Collet et al, 2010; Hobson et al, 2009; Siller-Matula et al, 2009; Harmsze et al, 2011;

Frelinger et al, 2012; Scott et al, 2012; Frelinger et al, 2013; Siller-Matula et al, 2014; Rouby et al, 2018).

Approaches which have been adopted to personalise clopidogrel therapy with the goal to improve clopidogrel efficacy after PCI, include platelet reactivity testing to assess the degree of on-treatment platelet reactivity (Price et al, 2008; Price et al, 2011a; Sibbing et al, 2017), and antiplatelet therapy decisions guided by *CYP2C19* genotype (Cavallari & Owusu-Obeng, 2017; Empey et al, 2018; Moon et al, 2018; Claassens & Ten Berg, 2020).

Patients treated with standard doses of clopidogrel have demonstrated reduced platelet aggregation inhibition due to interpatient pharmacodynamic and pharmacokinetic variability (Gurbel et al, 2003; Angiolillo et al, 2004; Matetzky et al, 2004; Nguyen et al, 2005; Serebruany et al, 2005; Von Beckerath et al, 2005; Angiolillo et al, 2007; Shuldiner et al, 2009; Ma et al, 2011a; Pettersen et al, 2011; Price et al, 2012; Ferreiro et al, 2019). High platelet reactivity (HPR) while on clopidogrel therapy post-PCI has been associated with a higher risk of recurrent ischaemic events, ST, restenosis, unstable angina (UA) and mortality (Matetzky et al, 2004; Price et al, 2008; Marcucci et al, 2009; Aradi et al, 2010; Breet et al, 2010; Zou et al, 2020). A significantly lower rate of CV death, MI and ST were observed in patients on clopidogrel therapy with low platelet reactivity (LPR) compared to patients with HPR (Aradi et al, 2010; Mshelbwala et al, 2020).

In patients with HPR while on antiplatelet therapy, the most common clinical presentation reported is ACS, including UA and MI (Nayak et al, 2006; Tornyos et al, 2017; Paramasivam et al, 2019), and increased occurrence of DES-ISR and ST (Nayak et al, 2006; Stone et al, 2007; Appleby et al, 2011; Paramasivam et al, 2019). HPR while on clopidogrel was observed to be an independent predictor of ST and MI after DES implantation (Stone et al, 2013). Patients had a significantly higher incidence of major adverse cardiac events (MACE), ischaemia, ST, and restenosis (Mshelbwala et al, 2020; Zou et al, 2020) and HPR was not overcome when patients were switched to alternative $P2Y_{12}$ inhibitors (Mshelbwala et al, 2020).

Ferreiro et al., (2019) compared HPR and LPR in ACS patients undergoing PCI with stenting and prescribed DAPT at days 1 and 30. The study reported higher interpatient variability and increased HPR in patients administering clopidogrel compared to prasugrel and ticagrelor. Prasugrel has been reported to have the lowest on-treatment HPR, while ticagrelor had the highest platelet inhibition (Sweeny et al, 2017; Ferreiro et al, 2019).

HPR is often observed in patients with diabetes mellitus, insulin resistance and deficiency, hyperlipidaemia, obesity, and patients taking concomitant protein pump inhibitors (PPIs), regardless of clopidogrel therapy (Angiolillo et al, 2005; Schneider, 2009; Mshelbwala et al, 2020; Zou et al, 2020). Compared to non-diabetic patients, patients with diabetes, particularly those requiring insulin therapy, have more

pronounced HPR when on clopidogrel therapy due to altered $P2Y_{12}$ pathways (Angiolillo et al, 2006).

With respect to genetics, a multivariate analysis of the Pharmacogenomics of Antiplatelet Intervention (PAPI) study indicated a 12% inter-individual variability in clopidogrel treatment response due to CYP2C19 genetic polymorphisms (Shuldiner et al, 2009). CYP2C19 genetic polymorphisms have been widely reported to be an attributing factor for clopidogrel resistance (Hulot et al, 2006; Angiolillo et al, 2007; Shuldiner et al, 2009; Mega et al, 2010a,b; Holmes et al, 2011; Ma et al, 2011a; Pettersen et al, 2011; Scott et al, 2012; Scott et al, 2013; Saydam et al, 2017).

These observations have encouraged the use of CYP2C19 genotyping in patients undergoing PCI to identify decreased metabolisers who could potentially benefit from the use of alternative $P2Y_{12}$ inhibitors than clopidogrel, as part of personalised medicine programs (Roberts et al, 2012; Shuldiner et al, 2014; Cavallari & Owusu-Obeng, 2017; Cavallari et al, 2018a).

1.3 Pharmacogenetic implications of clopidogrel resistance

The CYP2C19 enzyme is highly polymorphic, and over 25 single nucleotide polymorphisms have been identified (Scott et al, 2012). The **1*, or wild-type allele, is responsible for functional CYP2C19-mediated metabolism. The CYP2C19 **2* and **3* alleles are mutant, reduced or loss-of-function (LoF) alleles, of which the **2* allele is the

more common and well-researched allele. The frequency of the **2* allele ranges from 29 to 35% in the Asian population and is reported as 15% in Africans and Caucasians. Individuals are classified according to CYP2C19 genotype into four metaboliser phenotypes with respect to clopidogrel, namely, extensive metabolisers (EMs), ultra-rapid metabolisers (UMs), intermediate metabolisers (IMs) and poor metabolisers (PMs) (Table 1.1) (Scott et al, 2013).

Table 1.1: CYP2C19 genotypes and corresponding phenotypes

Genotype	Phenotype	CYP2C19 enzyme activity
Carriers of at least one gain of function allele (*1/*17, *17/*17)	Ultra-rapid Metaboliser (UM)	Normal or increased
Carriers of two functional alleles (*1/*1)	Extensive Metaboliser (EM)	Normal
Carriers of one loss-of-function allele (*1/*2, *1/*3, 2/*17,*3/*17)	Intermediate Metaboliser (IM)	Intermediate
Carriers of two loss-of-function alleles (*2/*2,*2/*3,*3/*3)	Poor Metaboliser (PM)	Absent or Low

Adopted from: Scott SA, Sangkuhl K, Stein CM, Hulot JS, Mega JL, Roden DM, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for CYP2C19 genotype and clopidogrel therapy: 2013 update. Clin Pharmacol Ther. 2013;94(3):317-23.

The Summary of Product Characteristics of clopidogrel approved by the European Medicines Agency (EMA) was updated in July 2019 and reports an association between the CYP2C19 genotype and the antiplatelet effect of clopidogrel which differs according to patient genotype. Section 4.4 'Special warnings and precautions for use' states under 'Cytochrome P450 2C19 (CYP2C19)', that PMs form a reduced amount of active

metabolite when clopidogrel is prescribed at the recommended dose, achieving reduced platelet function and effect. Section 5.2 'Pharmacokinetic properties' states under 'Pharmacogenetics' that the *CYP2C19*2* and *CYP2C19*3* LoF alleles are responsible for the majority of reduced function alleles in Caucasians and Asians.[1]

The United States Food and Drug Administration (FDA) drug label of clopidogrel, revised in June 2017, contains a 'boxed warning' related to CYP2C19 alleles, which was first included in March 2010. The 'boxed warning' states that there may be a reduction in the effectiveness of clopidogrel in subjects genotyped as PMs and advises healthcare professionals to consider alternate antiplatelet agents. The label suggests an alternative to clopidogrel in ACS patients and patients undergoing PCI identified as PMs due to an increased chance of CV events.[2,3]

Section 5 'Warnings and precautions' states under 'Diminished antiplatelet activity in patients with impaired CYP2C19 function', that the metabolism of clopidogrel may be impaired in patients with genetic variations of the CYP2C19 enzyme, affecting the desired effect. In the 'Medication guide' section of the drug label, patients are advised

[1] Electronic Medicines Compendium (eMC). Clopidogrel 75mg film-coated Tablets - Summary of Product Characteristics (SmPC) [Internet]. UK: eMC; 2019 [cited 2020 Mar 28]. Available from: https://www.medicines.org.uk/emc/product/4755/smpc

[2] Food and Drug Administration (FDA). PLAVIX (clopidogrel bisulfate) tablets Labeling Revision [Internet]. USA: FDA; 2010 [cited 2020 Mar 28]. Available from: http://www.accessdata.fda.gov/drugsatfda_docs/label/2011/020839s055lbl.pdf

[3] Food and Drug Administration (FDA). FDA Drug Safety Communication: Reduced effectiveness of Plavix (clopidogrel) in patients who are poor metabolizers of the drug [Internet]. USA: FDA; 2017 [cited 2020 Mar 28]. Available from: https://www.fda.gov/drugs/postmarket-drug-safety-information-patients-and-providers/fda-drug-safety-communication-reduced-effectiveness-plavix-clopidogrel-patients-who-are-poor

that clopidogrel may not work if they have a genetic variation, making them more susceptible to have decreased formation of clopidogrel metabolite, further advising doctors that pharmacogenetic testing may need to be performed. [4]

1.4 *CYP2C19*2* and response to clopidogrel therapy

The *CYP2C19 *2* allele has been widely reported to significantly decrease the concentration of the active metabolite of clopidogrel, resulting in the reduction of platelet inhibitory activity and increasing the risk of platelet aggregation (Hulot et al, 2006; Brandt et al, 2007; Kim et al, 2008; Collet et al, 2009; Mega et al, 2009; Shuldiner et al, 2009; Hulot et al, 2010; Gong et al, 2012; Lewis et al, 2013; Scott et al, 2013; Saydam et al, 2017; Song et al, 2018; Yu et al, 2020). HPR while on antiplatelet therapy has also been reported in *CYP2C19*2* carriers undergoing PCI (Yang et al, 2020).

Furthermore, the presence of the *CYP2C19 *2* allele has been shown in multiple studies to negatively impact therapeutic response to clopidogrel, leading to poorer prognosis after ACS and PCI due to an increased risk for MACE, including ST (Collet et al, 2009; Mega et al, 2009; Sibbing et al, 2009; Simon et al, 2009; Shuldiner et al, 2009; Harmsze et al, 2010; Mega et al, 2010a,b; Wallentin et al, 2010; Holmes et al, 2011; Sawada et al, 2011; Sibbing et al, 2011; Price et al, 2011b; Delaney et al, 2012; Price et al, 2012; Zabalza et al, 2012; Scott et al, 2013; Claessen et al, 2014; Sorich et al, 2014; Cavallari et al, 2015; Niu et al, 2015; Sun et al, 2016; Khalil et al, 2016; Cavallari et al, 2017a,b,

[4] DailyMed. Clopidogrel. Drug label information [internet]. USA: FDA; 2017 [cited 2020 Apr 21]. Available from:https://dailymed.nlm.nih.gov/dailymed/lookup.cfm?setid=2ed86bc8-8ea5-4ffa-a762-4fc266c1e620

Cavallari et al, 2018a,b; Hokimoto et al, 2018; Lee et al, 2018a; Tahara et al, 2018; Ayesh et al, 2019; Dávila-Fajardo et al, 2019; Yu et al, 2020). Details of these studies are compiled in Appendix 1.

Interindividual variability, due to CYP2C19 genetic polymorphisms, has not been reported with prasugrel or ticagrelor (Wallentin et al, 2010; Scott et al, 2013; Cavallari et al, 2018a). Genetic analysis of the 'Trial to Assess Improvement in Therapeutic Outcomes by Optimizing Platelet Inhibition With Prasugrel–Thrombolysis in Myocardial Infarction 38' (TRITON-TIMI 38) and 'Platelet Inhibition and Patient Outcomes' (PLATO) trials, reported no impact of CYP2C19 genetic polymorphisms on clinical outcomes with both prasugrel and ticagrelor (Mega et al 2009; Wallentin et al, 2009; Mega et al, 2010a; Sorich et al, 2010; Wallentin et al, 2010; Wiviott et al, 2015; Yu et al, 2020).

1.5 CYP2C19 genotype-guided antiplatelet therapy

The Clinical Pharmacogenetics Implementation Consortium (CPIC) and the Royal Dutch Association for the Advancement of Pharmacy-Pharmacogenetics Working Group (DPWG) are entities which publish pharmacogenetics-based drug dosing guidelines (Dávila-Fajardo, 2019). Both entities recommend actioning according to CYP2C19 genotype by avoiding clopidogrel in PMs and IMs and advocate the use of alternative $P2Y_{12}$ inhibitors in these patients if there is no contra-indication (Table 1.2, Table 1.3) (Swen et al, 2011; Scott et al, 2013).[5]

[5] The Royal Dutch Association for the Advancement of Pharmacy-Pharmacogenetics Working Group (DPWG). The DPWG pharmacogenomic guidelines [Internet]; the Netherlands: DPWG; 2019 [cited 2020 Mar 28]. Available from:

Table 1.2: CPIC clopidogrel recommendations according to CYP2C19 genotype

Phenotype (Genotype)	Implications for clopidogrel	Therapeutic recommendation	Level of evidence
EM (*1/*1)	Platelet Inhibition: Normal	Clopidogrel: Label recommended dose	Strong
UM (*1/*17, *17/*17)	Platelet Inhibition: Increased		
IM (*1/*2, *1/*3, *2/*17, *3/*17)	Platelet Inhibition: Reduced Increased risk of CV adverse events	Alternative $P2Y_{12}$ inhibitor, if no contra-indications (prasugrel or ticagrelor)	Moderate
PM (*2/*2, *2/*3, *3/*3)	Platelet Inhibition: Significantly reduced Increased risk of CV adverse events		Strong

CV: Cardiovascular; **EM**: Extensive metaboliser; **IM**: Intermediate metaboliser; **PM**: Poor metaboliser; **UM**: Ultra-rapid metaboliser

Adopted from: Scott SA, Sangkuhl K, Stein CM, Hulot JS, Mega JL, Roden DM, et al. Clinical Pharmacogenetics Implementation Consortium guidelines for CYP2C19 genotype and clopidogrel therapy: 2013 update. Clin Pharmacol Ther. 2013;94(3):317-23.

Table 1.3: DPWG clopidogrel recommendations according to CYP2C19 genotype [5]

Phenotype (Genotype)	CV Risk	Recommendation
EM (*1/*1)	None	Clopidogrel: Label dose
UM (*1/*17, *17/*17)		
IM (*1/*2, *1/*3, 2/*17, *3/*17)	Increased	Consider alternative antiplatelet (prasugrel or ticagrelor), if no contra-indication
PM (*2/*2, *2/*3, *3/*3)	Increased; Serious	Avoid clopidogrel Consider alternative antiplatelet (prasugrel or ticagrelor), if no contra-indication

EM: Extensive metaboliser; **IM**: Intermediate metaboliser; **PM**: Poor metaboliser; **UM**: Ultra-rapid metaboliser; **CV**: Cardiovascular

https://www.knmp.nl/patientenzorg/medicatiebewaking/farmacogenetica/pharmacogenetics-1/pharmacogenetics

Studies have demonstrated that *CYP2C19*-genotype-guided antiplatelet therapy in ACS patients undergoing PCI, including the implementation of point-of-care (POC) pharmacogenetic testing, results in better predictability of therapeutic response to clopidogrel and reduces the incidence of adverse cardiac outcomes (Roberts et al, 2012; Scott et al, 2013; Reese et al, 2012; So et al, 2016; Sánchez-Ramos et al, 2016; Jiang & You, 2017; Cavallari et al, 2018a; Lee et al, 2018a; Fragoulakis et al, 2019). Several studies also report that CYP2C19-genotype-guided antiplatelet therapy is cost-effective in ACS patients undergoing PCI (Reese et al, 2012; Kazi et al, 2014; Mitropoulou et al, 2016; Jiang & You, 2017; Lee et al, 2018a; Fragoulakis et al, 2019).

The ESC guidelines do not recommend the implementation of routine CYP2C19 pharmacogenetic testing to tailor DAPT. CYP2C19-genotype-guided antiplatelet therapy is suggested to be reserved for specific high-risk populations, such as patients with recurrent cardiac adverse events (Valgimigli et al, 2018). The American Heart Association (AHA) and the American College of Cardiology (ACC) in their joint guidelines for PCI, state that CYP2C19 genotype-guided antiplatelet therapy may be considered in patients considered high-risk, however, routine CYP2C19 pharmacogenetic testing for patients undergoing PCI is not recommended (Levine et al, 2016a).

1.6 In-stent restenosis: Prevalence, risk factors, and treatment

In-stent restenosis (ISR) is a complication that may arise post-PCI with stent placement and limits the long-term prognosis of the PCI. ISR is defined as the gradual re-narrowing of the stented coronary vessel diameter by ≥50%, which is determined via follow-up

coronary angiography (Mehran et al, 1999; Stone et al, 2005; Dangas et al, 2010). Clinical restenosis requires the presence of ISR ≥50% and any of the following characteristics, namely; ischaemia, recurrent angina with changes in electrocardiography, intravascular ultrasound minimum cross-sectional diameter <4mm^2, fractional flow reserve <0.80, or stenosis ≥70% with or without the presence of symptoms (Kuntz & Baim, 1993; Cutlip et al, 2004; Byrne et al, 2015).

The use of DES has reduced the incidence of clinical ISR from 20% to 35% with bare-metal stents (BMS) to between 5% and 10% with DES (Scott, 2006; Kim & Dean, 2011; Cassese et al, 2014). DES reduces ISR occurrence compared to BMS due to the release of antiproliferative mediators by DES that aid in preventing neointimal hyperplasia (Farooq et al, 2011). Although trials initially reported almost undetectable rates of ISR following implantation of the newer DES, short-and long-term follow-up, as well as real-world cases, have shown a 5% to 10% incidence of DES-ISR (Holmes et al, 2004; Morice et al, 2007; Ellis et al, 2009; Weisz et al, 2009; Mauri et al, 2010). A lower ISR rate with the use of second-generation everolimus and zotarolimus DES compared to first-generation paclitaxel and sirolimus DES has been reported (Guerra, 2014; Xu et al, 2014; Cho, 2017; Watanabe et al, 2017). Yet, ISR persists as a challenge post-PCI even after the introduction of newer DES (Dangas et al, 2010; Minha et al, 2013; Alfonso et al, 2014; Goel et al, 2016; Alraies et al, 2017).

Patients with multiple risk factors have shown an increased risk of lumen loss and ISR (Mishkel et al, 2007). Comorbidities and social risk factors such as diabetes mellitus, chronic kidney disease, hypertension, dyslipidaemia, heart failure, tobacco use, and history of ISR, have been independently associated with ISR (Agema et al, 2004; Singh et al, 2004; Fröbert et al, 2009; Hochholzer et al, 2010; Jukema et al, 2011; Latib et al, 2011; Magalhaes et al, 2014; Taniwaki et al, 2014; Kang et al, 2015; Eljery et al, 2016; Cho, 2017; Kundi et al, 2017).

Technical predictors include the presence of gaps between stents, type of stent, stent dimensions, and geographical miss (Van Mieghem et al, 2006; Gonzalo et al, 2009; Latib et al, 2011). Mechanistic factors contributing to ISR include acute or subacute disruption of plaque, vessel wall elastic recoil, length of the lesion, neointimal hyperplasia, vessel size, constrictive remodelling, and neo-atherosclerosis (Byrne et al, 2015). Genetic background has also been reported as a risk factor for ISR (Jukema et al, 2011; Byrne et al, 2015; Cassese et al, 2018).

The outcome of ISR is the unsuccessful maintenance of coronary vessel patency by the stent, which may result in the recurrence of signs and symptoms of ischaemia or ACS (Alfonso et al, 2014). Few studies report that 60% to 70% of patients with DES-ISR present with stable angina or were asymptomatic (Lee et al, 2008; Latib et al, 2011). Other studies found that the most common clinical presentation of DES-ISR was UA (78%), and 17% and 4% of cases presented with non-ST-elevation myocardial infarction (NSTEMI) and ST-elevation myocardial infarction (STEMI), respectively. Chronic kidney

disease was associated with worse one-year outcome in ACS compared to non-ACS presentations (Appleby et al, 2011; Paramasivam et al, 2019).

The treatment of ISR continues to be an unresolved challenge in today's practice. There are several options for the treatment of ISR, such as medical treatment, balloon angioplasty, brachytherapy, restenting with DES, or the deployment of a drug-eluting balloon (DEB) (Her & Shin, 2018). A DEB that can deliver an antiproliferative agent coated on a balloon to the restenosed artery or stent is being used for the treatment of ISR, circumventing the need to use extra layers of stent (Indermuehle et al, 2013; Gao et al, 2016). Based on clinical evidence, DEB and DES are presently the treatment options recommended by clinical guidelines for ISR (Class IA) (Neumann et al, 2019).

A recent meta-analysis conducted to evaluate the clinical efficacy of DES versus DEB in the treatment of DES-ISR reported that patients treated with a DEB were associated with a higher risk of MACE. One-year clinical results and angiographic outcomes were also reported to be better with DES than DEB when treating DES-ISR (Gao et al, 2019). Several trials were conducted to compare the clinical efficacy between the use of DES and DEB in ISR. However, conflicting findings were reported, and the question of the ideal treatment remains open for debate (Alfonso et al, 2014; Alfonso et al 2015; Pleva et al, 2016; Baan et al, 2018; Wong et al, 2018; Peng et al, 2020).

1.7 *CYP2C19*2* and in-stent restenosis

Very few studies have been conducted to explore the association between *CYP2C19*2* and ISR in patients receiving clopidogrel, and conflicting findings were reported. Lin et al., (2014) identified *CYP2C19*2* as a significant predictor of the development of vertebral ISR and a study by Guo et al., (2014) found that the incidence and degree of restenosis in patients with peripheral artery disease undergoing endovascular treatment was higher in carriers of *CYP2C19*2* compared to non-carriers.

With regards to coronary ISR, Nozari et al, (2015) and Hokimoto et al (2018) showed a higher frequency of ISR in carriers of *CYP2C19*2*, however, the correlation was not statistically significant. Conversely, Ruedlinger et al., (2017), reported a lower incidence of ISR among carriers of *CYP2C19*2*. In a previous local study, a higher incidence of ISR within one year of PCI was observed in carriers of *CYP2C19*2* in comparison to non-carriers, but there was no statistical significance (Wirth, 2015; Wirth et al, 2018). These studies concluded that the findings could be attributed to a small sample size and recommended further analysis with larger sample cohorts. A very recent study by Zhang et al., (2020) demonstrated that a significantly higher proportion of carriers of one CYP2C19 LoF allele (IMs) experienced ISR compared to non-carriers when multivariate analysis was conducted.

1.8 Research question, aim, and objectives

The research question was: Is the incidence of ISR within one-year post-PCI when on DAPT with aspirin and clopidogrel significantly higher in *CYP2C19*2* allele carriers compared to non-carriers?

The aim of the research was to investigate the pharmacogenetic implications in clopidogrel use.

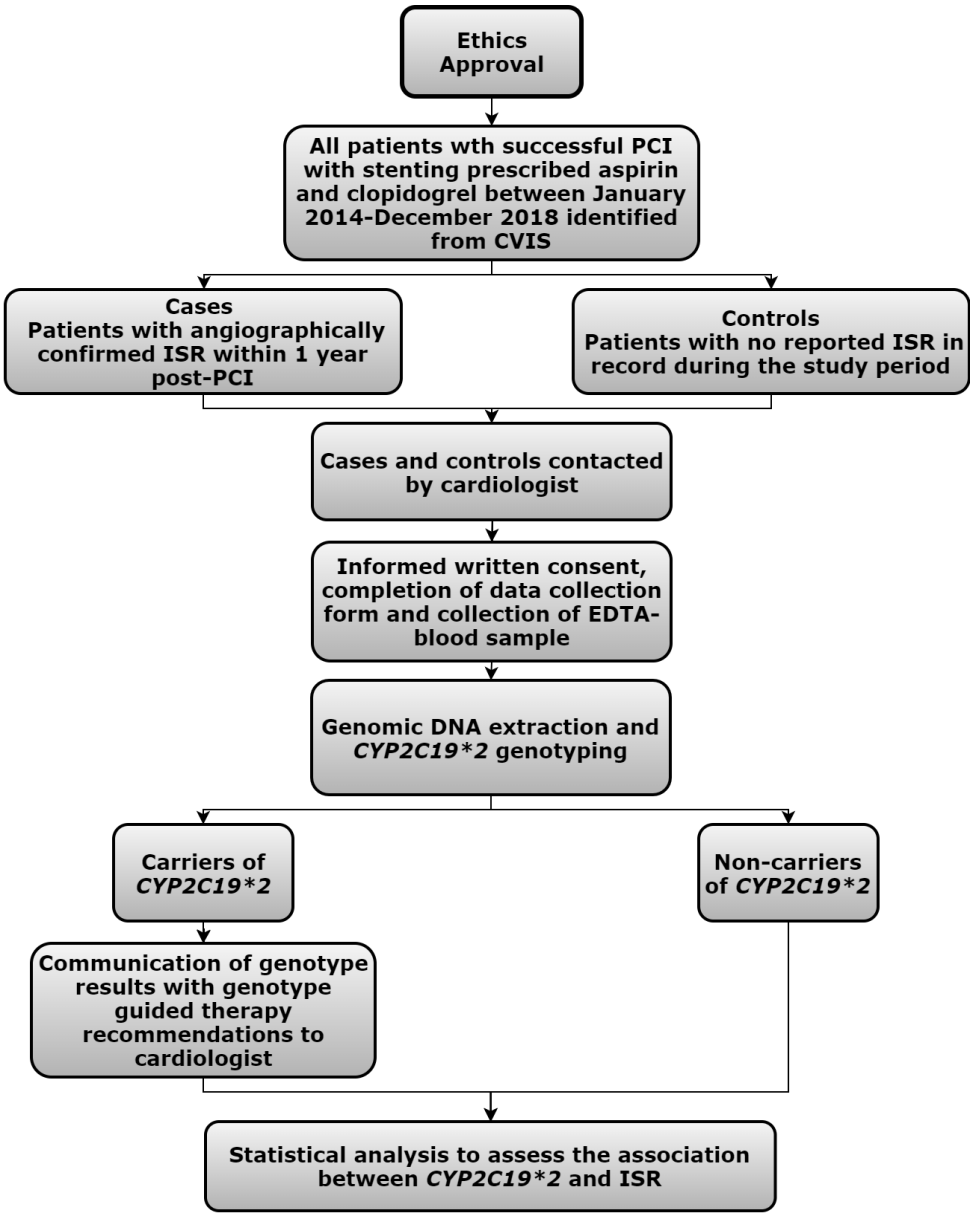
The objectives were to:

- Assess the association between *CYP2C19*2* and incidence of coronary ISR within one-year post-PCI in patients prescribed DAPT with aspirin and clopidogrel
- Identify significant predictors of coronary ISR
- Estimate the direct cost of repeat PCI due to ISR

Chapter Two
Methodology

2.1 Study design

A retrospective matched case-control study design was adopted in this research. A prospective approach was applied for patients who underwent PCI from January to December 2018, who were followed-up for any ISR occurrence until December 2019. The methodology flowchart is shown in Figure 2.1.



CVIS: Cardiovascular Information Management System; **DES:** Drug-Eluting Stent; **DNA:** Deroxyriboneuclic Acid; **EDTA:** Ethylenediaminetetraacetic Acid; **ISR**: In Stent Restenosis

Figure 2.1: Methodology Flowchart

2.2 Study Setting

The study was carried out at the Department of Cardiology and the Department of Pathology at Mater Dei Hospital (MDH).

Patient were recruited and followed-up using the Cardiovascular Information Management System (CVIS), from the Cardiac Catheterisation Suite (CCS) and cardiology wards. CVIS is a software used at the Department of Cardiology to document and manage patient clinical records; it includes information such as patient history, risk factors, medications prescribed before or after procedures, and angiographic reports. CVIS is used to record videos and images taken during coronary intervention procedures (Wirth, 2015). *CYP2C19*2* genotyping was performed at the Molecular Diagnostics Unit.

The total number of coronary angiograms, PCIs, and CABG surgeries performed at MDH between 2014 and 2018 are shown in Table 2.1.

Table 2.1: Cardiac procedures performed at MDH [6] [7] [8] [9] [10]

	Number Per Year				
Procedure	**2014**	**2015**	**2016**	**2017**	**2018**
Coronary Angiogram	2,010	1,646	1,571	1,692	1,707
PCI (with stenting)	843	866	727	927	881
1 stent	511	509	447	542	497
2 stents	192	186	151	203	216
3 stents	44	46	42	50	46
4 stents	7	9	10	5	15
5 stents	2	3	2	1	2
6 stents	0	0	0	0	1
PCI (Ballooning only)	87	113	75	126	104
CABG	205	190	153	154	141

CABG: Coronary Artery Bypass Graft; **PCI:** Percutaneous Coronary Intervention

2.3 Patient recruitment

A total of 15,787 procedures were performed in the CCS between January 2014 and December 2018. The list of procedures was screened; procedures other than PCI, and patients who were non-residents of Malta and could not be recruited for genotyping or

[6] Janulova L. Surgical operations/interventions at operating theatres: Annual report 2014 operations statistics. Malta (MDH): Medical Administrator's Office; 2015.
[7] Janulova L. Surgical operations/interventions at operating theatres: Annual report 2015 operations statistics. Malta (MDH): Medical Administrator's Office; 2016.
[8] Janulova L. Surgical operations/interventions at operating theatres: Annual report 2016 operations statistics. Malta (MDH): Medical Administrator's Office; 2017.
[9] Janulova L. Surgical operations/interventions at operating theatres: Annual report 2017 operations statistics. Malta (MDH): Medical Administrator's Office; 2018.
[10] Janulova L. Surgical operations/interventions at operating theatres: Annual report 2018 operations statistics. Malta (MDH): Medical Administrator's Office; 2019.

followed-up were eliminated. From the identified list of PCIs, duplicate patients and patients who passed away and could not be recruited for genotyping or followed-up were not considered.

All patients who underwent PCI between January 2014 and December 2018 were further screened in CVIS. The inclusion criteria for the study were patients ≥18 years, PCI with DES, prescribed DAPT with aspirin and clopidogrel for 12 months, any gender, any ethnicity. Exclusion criteria were PCI with ballooning only or with bare-metal stenting (BMS), DAPT less than 12 months, patients with severe liver impairment, and patients with renal impairment (eGFR ≤30 mL/min/1.73m^2).

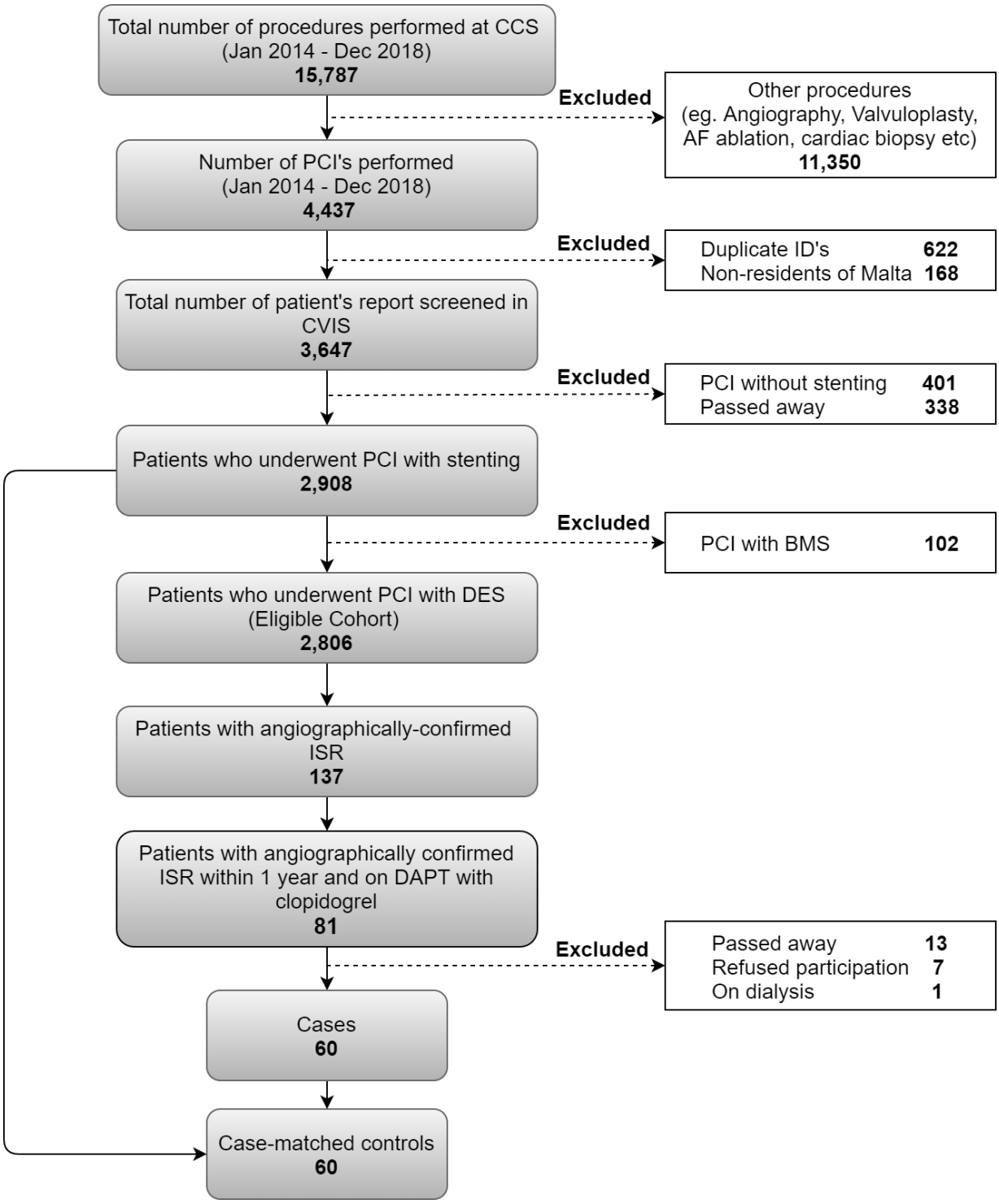
Patients who underwent PCI with BMS were excluded from the study as BMS is reported as an independent strong predictor of ISR in various students (Sim et al, 2011; Marino et al, 2015; Zbinden et al, 2017). The incidence of BMS-ISR is reported to be up to 35%, while the incidence of DES-ISR is lower (up to 10%) (Kuntz & Baim, 1993; Mehran et al, 1999; Cassese et al, 2014). Over the years, several studies have demonstrated the effectiveness of DES compared to BMS in reducing ISR rates (Morice et al, 2002; Grube et al, 2003; Colombo et al, 2003; Ardissino et al, 2004; Stone et al, 2004; Stone et al, 2005; Beijk et al, 2007; Spaulding et al, 2007; Steinberg et al, 2007; Sim et al, 2011; Zbinden et al, 2017).

Patients (n=137) with angiographically-confirmed ISR were identified and narrowed down to 81 patients with angiographically-confirmed ISR within 1-year post-PCI and on

clopidogrel. Patients who passed away after this screening process, patients who refused participation in the study, and a patient on haemodialysis, were excluded at this stage.

A total of 120 patients; 60 cases, and 60 case-matched controls were included as the study population. Cases were patients with angiographically-confirmed ISR within 1 year of DES placement while on aspirin and clopidogrel therapy, and controls were patients with no history of angiographically-documented ISR post-PCI, and case-matched for age, gender, diabetes mellitus and estimated glomerular filtration rate (renal function).

Diabetes mellitus was selected as a condition for matching since it is the most consistently reported risk factor for ISR (Hoffman & Mintz, 2000; Carson et al, 2002; Gilbert et al, 2004; Halkin et al, 2006; Hassani et al, 2006; Daemen et al, 2007; Fröbert et al, 2009; Rathore et al, 2010; Ma et al, 2011b; Qin et al, 2013; Cho, 2017; Wang et al, 2018; Cheng et al, 2019). The occurrence of ISR was reported to be up to 20% in diabetic patients (Wang et al, 2018), and compared to non-diabetics, patients with DM were shown to have an increased ISR risk of 30-40% (Mathew et al, 2004; Daemen et al, 2007). Renal failure patients receiving haemodialysis were reported to be at a higher risk of ISR and renal failure is reported to be an independent ISR predictor (Halkin et al, 2006; Hassani et al, 2006; Rathore et al, 2010; Hayano et al, 2013). Hence, renal function was selected as another parameter for matching. Figure 2.2 shows a flowchart of the patient recruitment process.



BMS: Bare-metal stent; **CCS**: Cardiac Catheterisation Suite; **CVIS**: Cardiovascular Information Management System; **DAPT**: Dual antiplatelet therapy; **DES**: Drug-Eluting Stent; **ISR:** In stent Restenosis **PCI**: Percutaneous Coronary Intervention

Figure 2.2: Patient recruitment flowchart

2.4 Development and validation of patient data collection form

A patient data collection form was developed based on the validated data collection form from the previous study by Wirth (2015) and after literature review. An updated data collection form, including angiographic characteristics and investigations such as LVEF, was developed. The form was re-validated for face and content validity by the Chair of the Department of Cardiology, two consultant cardiologists, and two pharmacists in academia, and no further amendments were suggested. The data collection form used in this study comprised six sections (Table 2.2).

Table 2.2: Sections of the patient data collection form

Section		Description
1	Patient information	Age, gender, ethnicity
2	Cardiac risk factors and social history	Family history of IHD, smoking history, previous MI and/or revascularisation, BMI, alcohol consumption
3a. 3b.	Relevant comorbidities and investigations	Comorbidities (Hypertension, dyslipidaemia, DM, renal impairment, HF chronic liver disease); investigations (glycated haemoglobin, creatinine, eGFR) and LVEF
4	Angiographic factors	Month ISR occurred, ISR presentation/reason for PCI, type of PCI performed, number of stents deployed, number of stents stenosed, stent dimensions, vessel/s stented
5	Current medications	Generic name, dose, dosage regimen
6	CYP2C19 genotype results (phenotype)	*1/*1 homozygous wild-type, *1/*2 heterozygous, *2/*2 homozygous variant; Phenotype (Normal, Intermediate or Poor metaboliser of clopidogrel)

BMI: Body Mass Index; **DM:** Diabetes Mellitus; **HF**: Heart Failure; **eGFR:** Estimated Glomerular Filtration Rate**; IHD**: Ischaemic Heart disease; **ISR**: In stent restenosis; **LVEF:** Left Ventricular Ejection Fraction; **PCI:** Percutaneous Coronary Intervention

Information from CVIS and patient interviews were used to complete sections 1, 2, 3.a and 5. Laboratory investigations in section 3.b were completed using iSoft Clinical Manager and LVEF was obtained from the transthoracic echocardiogram result in CVIS. Investigations for the cases were recorded at the time of ISR presentation or the closest date to the ISR presentation which was available in the patient records. For the controls, the investigations at the time of PCI were recorded. Angiographic reports present in CVIS were used to complete section 4 for both cases and controls, except information about ISR (such as the time of the event, presentation), which was only completed for cases. After *CYP2C19*2* genotyping, the result was documented in section 6 of the form (Appendix 2).

2.5 Study approvals

Approvals were attained from the Chair of the Department of Cardiology, consultant cardiologists, Chair of the Department of Pathology, Chief Executive Officer, and Data Protection Officer of MDH. Approval from the Faculty of Medicine and Surgery Research Ethics Committee was granted (Appendix 3).

2.6 Data collection

Patients who met the study criteria were invited via telephone by the cardiologist responsible for the patients or a physician delegate, between August and December 2019 to present at the CCS for *CYP2C19*2* genotyping. A brief description of the research study and what was expected from the patient was provided, and a date and time for a meeting with the investigator was set if they agreed to participate. When meeting the

investigator, patients were provided with a detailed patient information sheet about the research formulated in English and Maltese (Appendix 4). Patients who agreed to participate were asked to provide informed written consent by completing a consent form, also available in English and Maltese (Appendix 4). At the time of recruitment, the investigator completed the data collection form for each patient through patient interview and with information from CVIS. Each patient was provided with a unique study number at the time of recruitment, which was used only for the purpose of the study.

From each patient, a 5ml blood sample was collected by a physician or phlebotomist in a purple-top ethylenediaminetetraacetic acid (EDTA) vacutainer labelled with the patient's study number. The vacutainers were stored at the Molecular Diagnostics Unit (MDU) between 2 and 8°C prior to extraction of genomic DNA (gDNA).

2.7 Genomic DNA extraction

The extraction of gDNA was performed using the QIAamp® DNA Mini QIAcube Kit (Qiagen®) on the QIAcube® robotic workstation. Each kit consisted of collection tubes (2ml), proteinase K for binding and lysis, buffer AE for genomic DNA elution, ethanol and buffer AL for lysis, buffer AE for elution of the gDNA, and buffer AW1 and AW2 concentrate for washing (Wirth, 2015). [11]

[11] Qiagen. QIAamp DNA Mini Blood Mini Handbook - EN [Internet]. Germany: Qiagen; 2020 [cited 2020 May 12]. Available from: https://www.qiagen.com/ch/resources/download.aspx?id=62a200d6-faf4-469b-b50f-2b59cf738962&lang=en

Extraction was performed from a 200µL sample of whole blood collected from each patient, which yields 3 to 12µg of gDNA. The automated QIAcube® allows safe management of samples by averting cross-contamination between samples. Ninety minutes is the approximate time taken per run from preparation to extraction for a maximum of 12 samples in each run. The extracted gDNA sample was stored at -20 °C until genotyping (Wirth, 2015).[11] Training with respect to gDNA extraction took place at the MDU with a medical laboratory scientist and included an observation and a hands-on session (2 hours each session).

2.8 *CYP2C19*2* genotyping

Genotyping for the *CYP2C19*2* allele was performed for cases and controls with gradient polymerase chain reaction (PCR) using the Eppendorf mastercycler® gradient, and reverse hybridisation using the Autoimmun Diagnostika GmbH RDB 2070X and RDB 2071X *CYP2C19*2* genotyping kits. These techniques and kits were used to categorise patients into *CYP2C19*2* carriers, which included carriers of one or two *2 alleles, and non-carriers of *CYP2C19*2*.

The *CYP2C19*2* genotyping kits included denaturing agents, DNA extraction solution, hybridisation buffer, stringent wash buffer, 5X concentrated rinse solution, concentrated conjugate solution, substrate, conjugate buffer, incubation trays, and nitrocellulose test strips. Other materials required and were not present in the kit included thermostable *Taq* DNA polymerase and buffer $MgCl_2$, which were purchased from the same supplier of the genotyping kits, reaction tubes, and pipette tips with

filters. The *Taq* DNA polymerase was stored between -10 and -20°C. Each PCR run was manually set, and a 25-µl mixture consisting of the reagents in Table 2.3 was used for each amplification mixture (Wirth, 2015).[12]

Table 2.3: Components of gradient PCR mixture

Component	Volume (µl)
Primer nucleotide mix	15
$MgCl_2$ solution	2.5
Thermostable *Taq* DNA polymerase	0.2
10X polymerase buffer	2.5
gDNA	3
H_2O (Distilled)	1.8
Total	**25**

The thermocycling conditions used for the gradient PCR are shown in Table 2.4.

Table 2.4: Thermocycling conditions for gradient PCR

Time	Temperature (°C)	Cycles
5 min	95	1
30 sec 2 min	95 60	10
10 sec 30 sec 30 sec	95 55 72	20
8 min	72	1
Hold	3	∞

[12] Autoimmun Diagnostika GmbH - Kits: Method [Internet]. Germany: AID; 2020 [cited 2020 May 12]. Available from: https://www.aid-diagnostika.com/en/kits/molecular-biologic-assay/method

Reverse hybridisation was the next step. The components of the genotyping kits and the amplicon were brought to room temperature, except the hybridisation buffer and stringent wash, which were warmed to 47 °C in an incubator. The incubator trays were marked at the edge according to the number given to each PCR amplicon. 20 µl labelled amplicon obtained from the PCR was used per single determination and was added to each marked well by a pipette, changing the tip between each sample. This was followed by placing a 20 µl denaturing agent in each well and then incubated at room temperature for 5 minutes. 1 ml of pre-warmed mixed hybridisation buffer was added to each well using a pipette (Wirth, 2015).[12]

After placing each strip into a well of the incubation tray using forceps and ensuring that they were submerged in the mixture with the coated/numbered side facing upward, 30 minutes of incubation at 47 °C of the tray in a thermoshaker was ensured. The strips were washed for 1 minute twice at room temperature using the pre-warmed stringent solution after discarding the hybridisation buffer. After ensuring the complete removal of the stringent solution, 1ml of the pre-warmed stringent solution was added to each well and incubated in a thermoshaker at 47°C. After discarding the solution, the strips were washed twice for 1 minute with 1ml dilute rinse solution each time. 1ml of the prepared conjugate was added to the wells, and the tray was incubated at room temperature for 30 minutes on a thermoshaker. After removing the conjugate, each well was washed 3 times using 1ml of diluted rinse solution for 1 minute each time, shaking lightly. 1ml of substrate was added to each well and incubated for 10 to 20 minutes on a horizontal shaker. 1ml of distilled water was added to stop the reaction by washing the strips twice after discarding the substrate. The strips were removed from the wells

and dried, and results were interpreted using the evaluation sheet provided in each kit (Wirth, 2015).[12]

Each strip consisted of three internal control zones; Conjugate, Specificity and Sensitivity control zones that show efficient DNA isolation, hybridisation, and amplification, and two gene probe zones; *CYP2C19*1* allele and *CYP2C19*2* allele. The conjugate control band depicts efficient conjugate binding, the specificity control band only appears if the temperature is too low indicating improper hybridisation, and the sensitivity control band serves as an amplification control (Figure 2.3) (Wirth, 2015).[12]

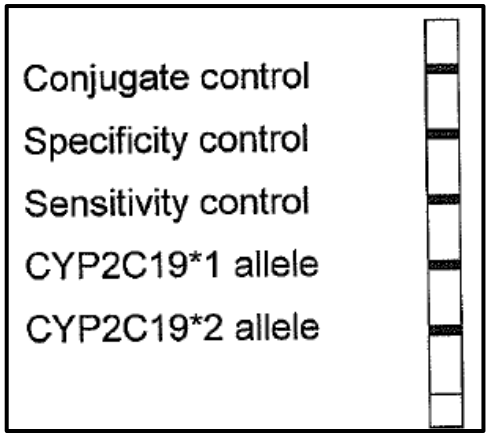


Figure 2.3: Nitrocellulose strip zones

If only the homozygous (*1/*1) allele was present, only the *CYP2C19*1* allele band formed. The *CYP2C19*2* band appeared alone without the *CYP2C19*1* allele band for homozygous (*2/*2) patients. Both the *CYP2C19*1* and *CYP2C19*2* bands developed for heterozygous (*1/*2) subjects. The conjugate control and sensitivity control must appear for all tests, and the specificity control should not appear for the test to be

considered positive. Figure 2.4 shows the different band patterns and possible corresponding genotypes (Wirth, 2015).[12]

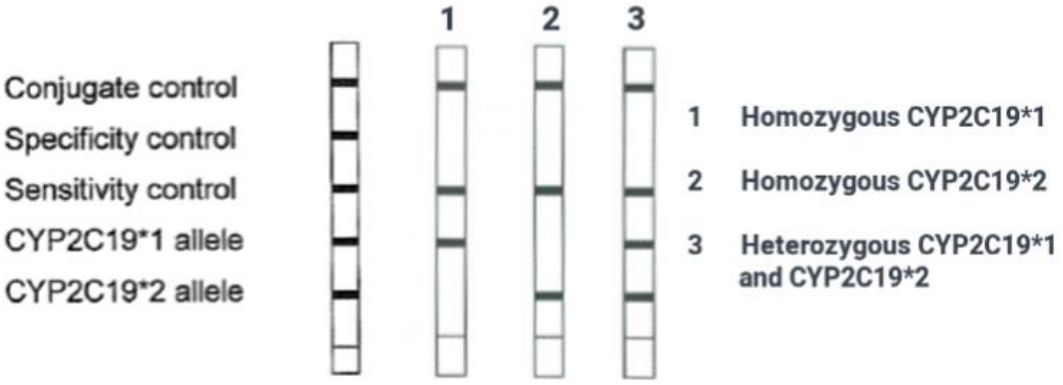


Figure 2.4: Possible band patterns and corresponding genotypes

2.9 Action taken after genotyping

Genotype results were communicated to the respective consultant cardiologist. Thirty-one letters (26 cases and 5 controls who were carriers of the *CYP2C19*2* allele) were presented to six consultant cardiologists. The letters included the patient's identity, genotype result with genotype-guided antiplatelet recommendations based on the CPIC guidelines for CYP2C19 genotype and clopidogrel therapy (Scott et al, 2013) (Appendix 5). The decision to switch from clopidogrel therapy to prasugrel, if recommended, was left to the cardiologist's discretion.

2.10 Patient follow-up

One-year follow up for angiographically confirmed ISR was carried out at months 1, 6, 9, and 12 post-PCI until December 2019. CVIS was used to check angiography and catheterisation reports, as well as patient clinical records.

2.11 Statistical analysis

Data analysis was carried out using IBM SPSS® version 22.0. Continuous data (age, height, weight, BMI, eGFR) are presented as mean (±95% Confidence Interval, CI) and Standard Deviation (SD), and categorical data was presented as frequency and percentage (%). For univariate analysis, the z-score calculator was used to find the difference between two proportions for categorical variables, and the two-tailed *t-test* was used for continuous variables, with a p-value <0.05 considered statistically significant (p-value at 95% CI).

Fisher's exact test reported with an odds ratio (OR) with 95% CI and p-value was used to analyse the association between *CYP2C19*2* and ISR. A p-value <0.05 implied proportions were significantly different, and a p-value >0.05 implied the proportions were not significantly different. Multivariate analysis using binary logistic regression was used to determine significant risk factors (predictors) associated with the occurrence of ISR and were reported as OR with 95% CI and p-value. Binary logistic regression analysis was used because the dependent variable (Group) is categorical and has two categories (Cases, Controls).

2.12 Compilation of costs for genotyping and repeat PCI for in-stent restenosis

The cost of all items required for repeat PCI due to ISR was obtained as of November 2019 from the in-charge nurse at the CCS. Items were divided into 'essential' (always used) and 'non-essential' (may be used) items, after discussion with the Chair of the Department of Cardiology. The direct cost (in Euro) of repeat PCI due to ISR was

estimated based on the deployment of one DEB or one DES. The cost of the gDNA extraction kits (2019) was obtained from the medical laboratory scientist at the MDU, and the cost of the genotyping kits was attained from the procurement invoice from the supplier (2019). The direct cost of genotyping was calculated.

Chapter Three
Results

3.1 Patient characteristics

One hundred and twenty patients divided into two groups, 60 cases and 60 matched controls comprised the study population.

The mean age of the patients in both groups was 65 years, ranging from 39 to 84 years for the cases, and 41 to 82 years for the control group (t-value = 0.207, p>0.05). In both groups there were 51 males (85%) and 9 females (5%) (z-score = 0, p>0.05). There was an equal number (n=30) of patients with diabetes in both groups (z-score = 0, p>0.05). Mean eGFR was 77 ml/min/1.73m^2 in both groups, ranging from 39 to 127 ml/min/1.73m^2 in cases and 31 to 127 ml/min/1.73m^2 in controls (t-value = -0.0712, p>0.05) (Table 3.1).

Table 3.1: Case-control matching (N=120)

	Cases (n=60)	**Controls (n=60)**	**p-value**
Mean age in years (± SD)	65 ±9.8	65 ±9.4	0.835
Male gender	51	51	1.000
Diabetes mellitus	30	30	1.000
Mean eGFR in mL/min/1.73m^2 (± SD)	77 ±20.0	77 ±19.0	0.934

eGFR: estimated Glomerular Filtration Rate

With regards to ethnicity, 59 cases and 59 controls were Caucasian (98.3%), and 1 case and 1 control were Asian (1.7%) (z-score = 0, p>0.05).

3.2 Cardiac risk factors and social history

Mean body mass index (BMI) in the cases was 30 ±4.7 kg/m^2 and 31 ±5.0kg/m^2 in the controls and there was no significant difference between the groups (t-value = -0.655, p = 0.256). Most patients in each group were classified in 'Obesity class I' (n=23, 38.3% cases; n=21, 35% controls), followed by 'Pre-obesity' (n=19, 31.6% cases; n=18, 30% controls). There was no significant difference between cases and controls in the different BMI categories (p>0.05) (Table 3.2).

Table 3.2: BMI classification (N=120)

BMI Classification (kg/m^2)	**Cases** (n=60)	**Controls** (n=60)	**z-score**	**p-value**
Normal weight (18.5-24.99)	10 (16.6%)	11 (18.3%)	-0.240	0.810
Pre-obesity (25-29.99)	19 (31.7%)	18 (30%)	0.197	0.841
Obesity Class I (30-34.99)	23 (38.3%)	21 (35%)	0.378	0.703
Obesity Class II (35-39.99)	7 (11.6%)	6 (10%)	0.293	0.771
Obesity Class III (>40)	1 (1.7%)	4 (6.7%)	-1.370	0.170

BMI: Body Mass Index

Positive family history of IHD was prominent in both groups. In the cases, 47 (78.3%) patients had a positive family history of IHD, either a parent (n=38, 81%) or a sibling (n=9, 19%). In the control group, 42 (70%) patients had a positive family history, either a parent (n=35, 85.4%) or a sibling (n=7, 14.6%). There was no significant difference between the groups (z-score = 1.042, p = 0.290).

Thirty-two patients (53.3%) in the cases group were active smokers compared to 19 (31.7%) patients in the control group. The difference between groups was statistically significant (z-score = 2.400, p = 0.016). Eighteen patients (30%) were ex-smokers in the cases group, and 7 (11.7%) patients were ex-smokers in the control group. The difference between groups was not statistically significant (z-score = 2.472, p=0.135).

With regards to alcohol consumption, a significantly higher proportion of patients in the cases group (n=30, 50%) were current consumers of alcohol compared to the control group (n=14, 23%) (z-score = 3.030, p=0.002). Eighteen (30%) patients in the cases group consumed alcohol occasionally compared to 23 (38.3 %) patients in the control group. The difference between groups was not statistically significant difference (z-score = -0.962, p = 0.337).

3.3 Comorbidities

The most common comorbidities were hypertension and dyslipidaemia. A significantly higher proportion of patients with hypertension and dyslipiaemia was observed in the control group compared to the cases group ($p<0.05$). A significantly higher proportion of patients with heart failure with left ventricular ejection fraction (LVEF) ≤50% was observed in the cases group compared to the control group ($p<0.05$) (Table 3.3).

The mean LVEF ±SD in the cases was 59 ±10% and 73 ±14% in the controls and the difference was statistically significant (t-value = -6.395, $p<0.001$).

Table 3.3: Comorbidities (N=120)

Comorbidity	Cases (n = 60)	Controls (n = 60)	z-score	p-value
Dyslipidaemia	22 (36.6%)	47 (78.3%)	-4.616	< 0.001
Heart failure	15 (25%)	2 (3.3%)	3.403	0.007
Hypertension	37 (61.6%)	48 (80 %)	-2.209	0.027
Diabetes mellitus	30 (50%)	30 (50%)	0	1.000
Renal Impairment (eGFR < 60 mL/min/1.73m^2)	10 (16.6%)	10 (16.6%)	0	1.000

eGFR: estimated Glomerular Filtration Rate; **LVEF**: Left Ventricular Ejection Fraction

3.4 Clinical presentation for PCI

The majority of cases (n=40, 66.7%) and controls (n=27, 45%) were undergoing PCI due to IHD. The difference between groups was statistically significant ($p<0.05$). A significantly higher proportion of controls compared to cases were undergoing PCI following STEMI ($p<0.05$), and there was no significant difference between groups for NSTEMI presentation ($p>0.05$).

The majority of cases (n=31, 52%) and controls (n=35, 58.3%) were undergoing emergency/primary PCI. The difference between groups with respect to the type of PCI was not statistically significant ($p>0.05$).

Table 3.4: PCI presentation and type of PCI (N=120)

	Cases (n=60)	**Controls** (n=60)	**z-score**	**p-value**
Reason for PCI				
IHD	40 (66.6%)	27 (45%)	2.389	0.016
NSTEMI	16 (26.7%)	13 (21.7%)	0.639	0.522
STEMI	4 (6.7%)	20 (33.3%)	-3.651	< 0.001
Type of PCI				
Emergency/Primary	31 (51.7%)	35 (58.3%)	-0.734	0.465
Elective	29 (48.3%)	25 (41.7%)	0.734	0.465

IHD: Ischaemic Heart Disease; **NSTEMI:** Non-ST-Elevation Myocardial Infarction; **STEMI**: ST-Elevation Myocardial Infarction

The majority of the cases (n=31, 51.7%) and controls (n=41, 68.3%) had one DES deployed per PCI. The difference between groups with respect to the number of stents implanted per PCI was not statistically significant (p>0.05). Twenty-nine (48.3%) cases and 19 controls (31.7%) underwent PCI with >1 stent and the difference was not statistically significant (z-score = -1.863, p = 0.062) (Table 3.5).

Table 3.5: Number of stents implanted per PCI (N=120)

Number of stents deployed per PCI	**Cases** (n = 60)	**Controls** (n = 60)	**z-score**	**p-value**
1	31 (51.7%)	41 (68.3%)	-1.863	0.062
2	17 (28.3%)	13 (21.7%)	0.843	0.400
3	10 (1.7%)	6 (10%)	1.074	0.284
4	2 (3.3%)	0	1.426	0.152

Fifty-four (90%) patients in the cases group underwent previous PCI compared to 24 (40%) patients in the control group, which was statistically significant (z-score = 5.741, p<0.001). A significantly higher proportion of cases (n=16, 29.7%) compared to controls (n=7, 29%) had previous coronary artery bypass graft surgery (z-score = 2.087, p = 0.036). Previous MI was reported in 29 (48.3%) cases and 19 (25%) controls. The difference was not statistically significant (z-score = 2.652, p= 0.062).

3.5 CYP2C19 enzyme-drug interactions

Five drug classes that either influence or are metabolised by the CYP2C19 enzyme and were co-administered with clopidogrel were observed in the cases, mostly proton pump inhibitors - omeprazole (n=52, 86%) (Table 3.6).

Table 3.6: CYP2C19 enzyme-drug interactions for cases (n = 60)

Class	Drug	Effect on CYP2C19 enzyme (Scott et al, 2012)[13]	Number (%) of cases
PPI	Omeprazole	Inhibitor/Substrate	52 (86%)
Anticoagulant	Warfarin	Substrate	5 (6%)
SSRI	Fluoxetine	Inhibitor	4 (6%)
Antiepileptic	Phenytoin	Inducer/Substrate	2 (3%)
TCA	Amitriptyline	Inhibitor/Substrate	2(3%)

PPI: Proton Pump Inhibitor; **SSRI:** Selective Serotonin Reuptake Inhibitor; **TCA:** Tricyclic Antidepressant

[13] Food and Drug Administration (FDA). Drug Development and Drug Interactions: Table of Substrates, Inhibitors and Inducers [Internet]. USA: FDA; 2020 [cited 2020 May 5]. Available from: https://www.fda.gov/drugs/drug-interactions-labeling/drug-development-and-drug-interactions-table-substrates-inhibitors-and-inducers

3.6 Angiographic characteristics

The mean time ±SD in months from PCI to the presentation of ISR was 8 ±1 month, with 10-12 months being the most common (n=22) (Table 3.7).

Table 3.7: Time of presentation of in-stent restenosis (n=60)

Month	Number (%) of cases
> 1-3	5 (8.3%)
4-6	13 (21.7%)
7-9	20 (33.3%)
10-12	22 (36.7%)

The most commonly affected coronary vessel due to ISR, which required repeat PCI, was the left anterior descending artery (n=21, 33.3%) (Table 3.8).

Table 3.8: Coronary vessels with in-stent restenosis (n = 60)

Coronary vessel	Number of cases (%)
Left anterior descending artery	21 (33.3%)
Right coronary artery	12 (19%)
Circumflex artery	10 (16%)
Grafts	7 (11%)
Obtuse marginal artery	5 (8%)
Left main artery	3 (4.8%)
Diagonal artery	2 (3.2%)
Intermediate artery	2 (3.2%)
Posterior descending artery	1 (1.5%)

The most common stent diameters of the restenosed stent were 2.5 mm (n=20, 33%) and 2.75 mm (n=18, 30%), and the most common stent lengths were 18 mm (n = 9, 15%), 15 mm (n=8, 13%) and 12 mm (n=8, 13%). The mean stent length ±SD was 18.02 ± 7.10 mm and the mean ±SD stent diameter was 2.78 ±0.40 mm.

Seventeen different PCI operators were observed for the cases. The highest number of procedures performed by the same operator was 10 (17%), followed by 7 (12%) and 6 (10%). The rest were all ≤ 5 PCI procedures by the same operator.

The majority of cases (n=58, 96.6%) had ISR in only one stent requiring repeat PCI, one patient had ISR in 2 stents and was a carrier of *CYP2C19*2* and one patient had ISR in 3 stents and was a non-carrier of *CYP2C19*2*. Zotarolimus eluting stents (second-generation) showed the most ISR. The majority of the cases (n=34, 56.7%) cases had ISR in a zotarolimus-eluting stent (Figure 3.1).

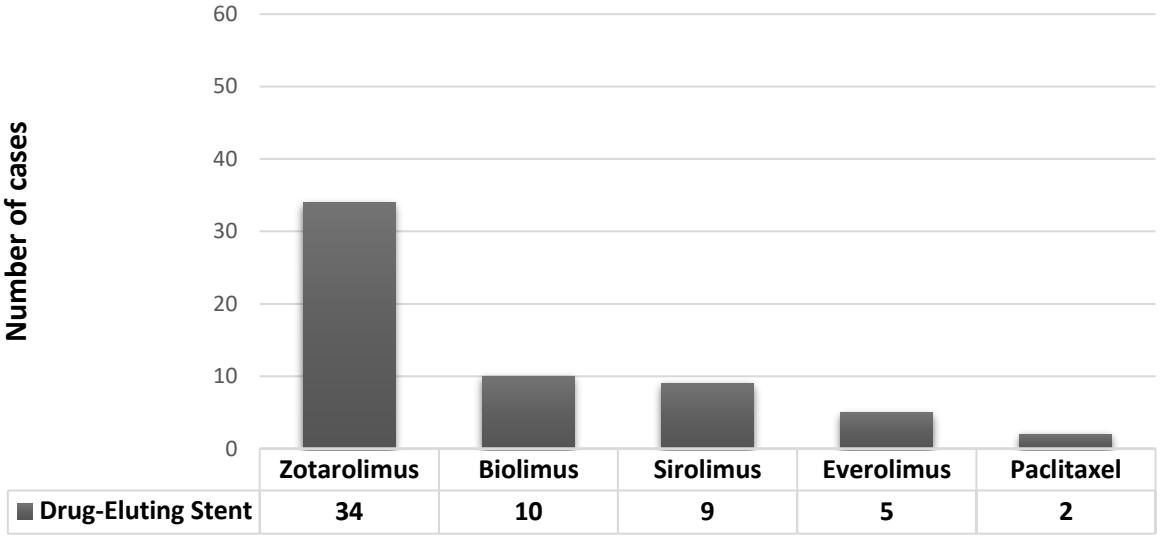


Figure 3.1: Types of drug-eluting stent with in-stent restenosis (n=60)

Nineteen cases (31%) with second-generation (zotarolimus or everolimus) DES implanted presented with MI (Table 3.9).

Table 3.9: DES generation and in-stent restenosis presentation (n=60)

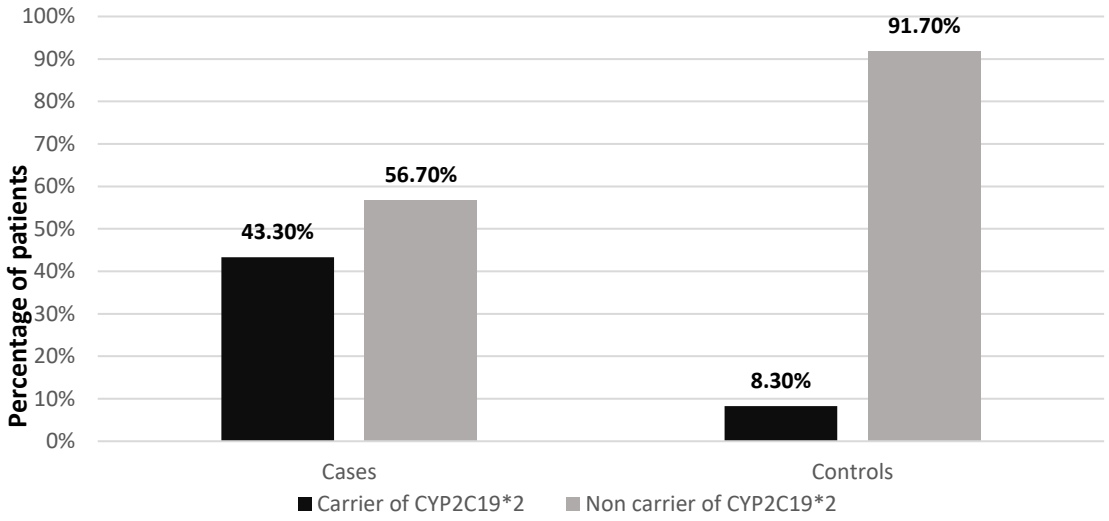
DES generation	STEMI	NSTEMI	UA
First-generation (paclitaxel, sirolimus)	3 (5%)	1 (2%)	7 (12%)
Second-generation (zotarolimus, everolimus)	11 (18%)	8 (13%)	20 (33%)
Third-generation (biolimus)	1 (2%)	4 (7%)	5 (8%)

DES: Drug Eluting Stent; **NSTEMI**: Non-ST-Elevation Myocardial Infarction; **STEMI**: ST-Elevation Myocardial Infarction; **UA**: Unstable Angina

3.7 *CYP2C19 *2* allele carrier status and in-stent restenosis

Out of the 120 patients, 89 (74%) patients were non-carriers of the *CYP2C19*2* (homozygous*1/*1) and 31 (25.8%) patients were carriers of the *CYP2C19*2* allele. Thirty (25%) patients were genotyped as heterozygous *1/*2, and 1 patient was genotyped as homozygous *2/*2 and belonged to the cases group.

A significantly higher proportion of cases (n=26, 43.3%) were carriers of the *CYP2C19*2* allele compared to controls (n=5, 8.3%) (z-score=4.3796, p <0.001). Using univariate analysis, the association between *CYP2C19*2* carrier status and coronary ISR within one-year post PCI was statistically significant (p<0.001, OR 8.4). Carriers of the *CYP2C19*2* allele were 8.4 times more likely to develop ISR than non-carriers (Figure 3.2).



p < 0.001 (Fisher's Exact Test) **Odds Ratio 8.4** (95% CI 2.95-24)

Figure 3.2: Correlation between *CYP2C19*2* and in-stent restenosis within one-year post-PCI

3.8 Multivariate analysis

A 10-predictor binary logistic regression model was used for multivariate analysis to identify and analyse independent predictors for ISR. When the 10 independent variables that were not matched between cases and controls were analysed, previous revascularisation, carrier of *CYP2C19*2,* heart failure, active smoking and dyslipidaemia were observed to be significant predictors ($p<0.05$) (Table 3.10).

Table 3.10: Variables assessed by binary logistic regression analysis

Variable	-2 Log Likelihood	Likelihood Ratio Tests		
		Chi-Square	Df	p-value
Intercept	64.927	0.000	0	.
Previous Revascularisation	97.316	32.389	1	0.000
Carrier of *CYP2C19*2*	**78.016**	**13.090**	**1**	**0.000**
Heart Failure	77.438	12.511	1	0.000
Active smoking	72.739	7.812	1	0.005
Dyslipidaemia	70.637	5.711	1	0.017
Hypertension	68.641	3.714	1	0.054
≥1 stent implantation	67.887	2.960	2	0.228
BMI ≥30 kg/m^2	66.719	1.792	1	0.181
Positive IHD Family History	65.134	0.207	1	0.649
Current Alcohol Intake	65.133	0.207	1	0.649

Nagelkerke Pseudo R-Square value = 0.727

A forward entry procedure was used to identify the parsimonious binary logistic regression model, which solely includes positive significant predictors of ISR i.e. where there was a significantly higher number of cases compared controls for the particular variable and not vice versa. This model retained 4 predictors (Table 3.11). Previous

revascularisation is the best predictor of ISR, followed by carrier of *CYP2C19*2*, heart failure and active smoking

Table 3.11: Parsimonious Model - Forward Entry

Variable	-2 Log Likelihood	Effect Selection Tests		
		Chi-Square	df	p-value
Intercept	145.640			
Previous Revascularisation	110.024	35.616	1	0.000
Carrier of *CYP2C19*2*	**87.219**	**22.805**	**1**	**0.000**
Heart Failure	76.772	10.447	1	0.001
Active Smoking	71.454	5.318	1	0.021

Nagelkerke Pseudo R-Square value = 0.615

The odds ratios (OR) of previous revascularisation, carrier of *CYP2C19*2*, heart failure, and active smoker are all >1 implying that ISR within 1-year post-PCI while on DAPT with aspirin and clopidogrel is more likely to occur if a patient is an active smoker, has heart failure, had previous revascularisation, and is a carrier of *CYP2C19*2* (Table 3.12).

Table 3.12: Odds ratio of significant predictors of in-stent restenosis

	Parameter Estimates				
	B	Standard Error	df	p-value	Odds ratio (OR)
Intercept	-3.979	0.869	1	0.000	
Previous Revascularisation=Yes	3.654	0.823	1	0.000	38.621
Carrier of *CYP2C19*2*=Yes	**3.118**	**0.900**	**1**	**0.001**	**22.612**
Heart Failure=Yes	2.875	1.146	1	0.012	17.717
Active Smoker=Yes	1.250	0.563	1	0.026	3.489

3.9 Cost of repeat PCI due to in-stent restenosis

The estimated direct cost for repeat PCI due to ISR ranges from € 1,126 to €2,474 (Table 3.13).

Table 3.13: Estimated cost of repeat PCI due to in-stent restenosis

	Always required (cost in €)	Not always required (cost in €)
Items	Drug-eluting balloon**: 295.00**	Drug-eluting stent**: 158.00**
	Angio-seal: **86.74**	Intravascular ultrasound catheter**: 900.00**
	Pre- and post-dilation balloon: **88.00**	1-night inpatient stay**: 256.23**
	Contrast Dye: **30.00**	Aspirin 75 mg tablets/500 mg injection **0.080** (4 tablets) **0.83** (intravenous)
	Aspiration catheter: **302.43**	Clopidogrel 75 mg tabs **0.48** (4 tabs); **0.96** (8 tabs)
	Balloon pre-dilation and Balloon post-dilation: **28.00 x2**	Isosorbide dinitrate 1 mg/ml in 10 ml injection**: 2.86**
	Medrad Consumables: **75.00**	Midazolam 10 mg/5 ml injection**: 1.10**
	Indeflator: **25.46**	Diamorphine 5 mg injection**: 2.52**
	Manifold**: 7.23**	Adrenaline minjet**: 9.18**
	Introducer sheath**: 14.50**	Atropine 600 mcg injection or minijet**: 8.05**
	Guiding catheter**: 29.50**	Plasma expander**: 4.14**
	Standard wire**: 3.23**	Trans-radial Band**: 6.70**
	Guide wires (Hi-Torque balance): **77.00**	
	Heparinised saline**: 4.08**	
	Unfractionated heparin 5000 units/5 ml or enoxaparin 6000 IU injection **2.22, 6.88**	
	Lidocaine 1% injection**: 0.46**	
	Cardiac angiographic pack**: 25.00**	
Cost (€)	**1,126**	**1,348**
Total Direct Cost (Range): **€ 1,126 – € 2,474**		
Other costs (Indirect) **Personnel - Cardiologist, 2 nurses, radiographer, ECG technician** **Cardiac catheterisation suite recovery ward**		

3.10 Antiplatelet therapy changes due to in-stent restenosis

Six patients were switched from clopidogrel to prasugrel after developing ISR; 3 patients were carriers of *CYP2C19*2* and 3 patients were non-carriers.

Chapter Four
Discussion

4.1 In-stent restenosis: Analysis of predictors

Findings from this research demonstrated a significant assosciation between the presence of the *CYP2C19*2* allele and ISR within one-year post-PCI in both the univariate (OR 8.4, p<0.001) and multivariate analysis (OR 22.6, p=0.001). The risk of developing ISR within one-year post-PCI on clopidogrel therapy was shown to be significantly higher in *CYP2C19 *2* carriers than in non-carriers and the signal observed in the previous study by Wirth et al. (2018) was confirmed. A recent study by Zhang et al, (2020) carried out in China also supports these findings, where significantly higher ISR rates were observed in carriers of the *CYP2C19* loss-of-function alleles (*1/*2, *1/*3) on standard dose clopidogrel compared to non-carriers. Further to *CYP2C19*2* carrier status, the multivariate analysis in the present study identified a significant association between the non-genetic factors previous revascularisation, heart failure and active smoking and incidence of ISR.

Previous revascularisation was observed to have the most significant association with ISR occurrence (OR 38.6, p<0.001). This finding is in accordance with three previous studies, where history of PCI was identified as an independent predictor of DES-ISR (Singh et al, 2004; Taniwaki et al, 2014; Wang et al, 2018). Heart failure was also identified to be significantly associated with ISR (OR 17.7, p=0.012), which was reflected in two previous studies reporting a significant association between heart failure and ISR (Singh et al, 2004; Kang et al, 2015). In the study by Singh et al., (2004), a significantly higher number of patients with ISR had heart failure compared to non-ISR patients in

the univariate analysis. However, heart failure was shown to be a non-significant predictor of ISR in the multivariate analysis.

Conflicting evidence on the effect of smoking on ISR is reported. Similar to the present study, where active smoking was identified to be significantly associated with the incidence of ISR (OR 3.5, p=0.026), smoking was observed to be a significant predictor of ISR in two studies (Ma et al, 2011b, Kundi et al, 2017), while in three other studies no association between smoking and ISR was observed (Mohan & Dhall, 2010; Hu et al, 2015; Cassese et al, 2018). Conversely, it has been reported that smoking may have a 'protective effect' contributing to decreased HPR on clopidogrel therapy and enhanced clinical benefit of clopidogrel in smokers compared to non-smokers, a phenomenon described as the ''smoker's paradox'' (Hasdai et al, 1997; Cohen et al, 2001; Singh et al, 2004; Gurbel et al, 2013).

A higher number of cases compared to controls in the present study underwent PCI with multiple stenting, however there was no statistically significant association between ISR and a higher number of stents implanted. This finding contrasts with other studies which demonstrated that the number of stents deployed was an independent predictor of ISR (Kang et al, 2015; Tocci et al, 2016; Wan et al, 2016; Qian et al, 2018; Tang et al, 2019). This association can be explained since as the number of stents increases, the probability of vessel trauma causing intimal hyperplasia increases (Byrne et al, 2015; Lee et al, 2018b). Initiation of the inflammation cascade may be precipitated, causing the recruitment of platelets, neutrophils and fibrin, along with the proliferation of smooth

muscle and fibroblasts, leading to the development of ISR (Mercado et al, 2001; Wasser et al, 2011; Kucukseymen, 2017). A positive correlation has also been observed between the number of stents and the risk of stent thrombosis (Palmerini et al, 2012; Thayssen et al, 2012), and this increase in thrombus load and production may eventually result in ISR (Bulum et al, 2012; Miyake et al, 2013).

There was no statistically significant association found in the present study between BMI and ISR incidence. Conflicting evidence is reported on this association, where two studies have demonstrated patients with higher BMI to have a significantly higher risk of ISR (Mercedo et al, 2001; Mohan & Dhall, 2010), and conversely, a study by Wan et al., (2016) found lower BMI to be a significant predictive factor of ISR.

A significantly higher number of controls with dyslipidaemia compared to cases were observed in the present study. This finding is similar to a few studies that reported a significantly higher number of patients with dyslipidaemia in non-ISR patients compared to patients with ISR (West et al, 2004; Wattanbe et al, 2017; Zbinden et al, 2017), and to two studies which showed no association between dyslipidaemia and ISR (Eljery et al, 2016; Cheng et al, 2019). A study by Kundi et al., (2017) showed that the triglyceride/high-density lipoprotein-cholesterol ratio was independently associated with the presence of ISR.

As with dyslipidaemia, a significantly higher number of controls with hypertension compared to cases were observed in the present study. This finding contrasts with other studies that report hypertension to be a risk factor for ISR (Agema et al, 2004; Tocci et al, 2016). These studies explain that this association may be attributed to several mechanisms by which high blood pressure may promote ISR, such as endothelial dysfunction and the increased prevalence of concomitant risk factors (Cercek et al, 1991; Tashiro et al, 2001; Schwartz & Henry, 2002; Scott, 2006; Kibos et al, 2007).

Fifty percent of the cases in the present study had diabetes mellitus. Diabetic patients are at a higher risk of developing atherothrombotic events than non-diabetics and diabetes mellitus is a main cause of mortality in CVD.[14] Type 2 diabetes mellitus, especially when uncontrolled, has been shown to cause platelet dysfunction leading to accelerated atherosclerosis and an increased risk for atherothrombotic complications, morbidity, and mortality (Tschoepe et al, 1991; Mak et al, 1997; Abizaid et al, 1998; Vinik et al, 2001; Colwell & Nesto, 2003; Almdal et al, 2004; Véricel et al, 2004; Angiolillo et al, 2005; Samoš et al, 2014; Schuette et al, 2015). The pivotal role of diabetes mellitus in ISR predisposition has been well-established (Daemen et al, 2007; Ma et al, 2011b; Kim et al, 2013; Qin et al, 2013; Wang et al, 2018). Patients with diabetes mellitus have been reported to be two to four times more susceptible to developing ISR than non-diabetic patients (Qin et al, 2013), and insulin resistance was associated with higher rates of ISR compared to patients without insulin resistance (Zhao et al, 2015).

[14] International Diabetes Federation (IDF). Diabetes and cardiovascular disease report [Internet]. Brussels: IDF; 2016 [cited 2020 Apr 07]. Available from: https://www.idf.org/our-activities/advocacy-awareness/resources-and-tools/90:diabetes-and-cardiovascular-disease-report.html

Reduced clopidogrel responsiveness in diabetics is widely reported (Gurbel et al, 2003; Müller et al, 2003; Angiolillo et al, 2004; Angiolillo et al, 2005; Geisler et al, 2007; Samoš et al, 2014; Schuette et al, 2015; Sweeny et al, 2017), however the effect on clopidogrel response with diabetes mellitus was not observed with ticagrelor response in diabetics (Sweeny et al, 2017).

Eighty-seven percent of the cases were on at least one medication known to influence the metabolism of clopidogrel, predominantly the proton pump inhibitor (PPI) omeprazole; of whom 38% were carriers of the loss-of-function *CYP2C19 *2* allele. PPIs are prescribed with $P2Y_{12}$ inhibitors to diminish the risk of gastrointestinal side-effects, especially bleeding (Bouziana & Tziomalos, 2015; Roubi et al, 2018). PPIs inhibit the CYP2C19 enzyme to varying degrees depending on the type of PPI (Abraham et al, 2010; Shah et al, 2012; Scott et al, 2013), where the highest reduction of clopidogrel antiplatelet effect has been reported with omeprazole compared to other PPIs (Gilard et al, 2008; Ferreiro et al, 2010; Siller-Matula et al, 2010; Frelinger et al, 2012; Yamane et al, 2012). These studies suggest the use of alternative PPIs to omeprazole, such as pantoprazole, to reduce the negative effect on clopidogrel efficacy.

A study by Hu et al., (2018) reported a significantly higher risk of MACE in patients with stent implantation administering PPIs with clopidogrel. Results from two studies have also supported these findings, reporting a significantly higher risk of MACE in patients administering clopidogrel with PPIs (Gupta et al, 2010; Huang et al, 2012). More recent studies have reported a significantly lower mortality, revascularisation, and fewer

MACE, MI, and ST among patients on clopidogrel therapy who did not administer PPIs, with omeprazole having the highest effect on clopidogrel metabolism (Bundhun et al, 2017; Hu et al, 2018). However, the evidence is not consistent and the ESC guidelines state that a PPI with DAPT is presently recommended for gastroprotection (Class I Level of evidence B) (Valgimigli et al, 2018).

4.2 *CYP2C19*2* genotyping for precision antiplatelet therapy prescribing

Over the past decade several papers regarding the clinical implementation of pharmacogenetics for personalised medicine have been published. Clinical decision-making with respect to antiplatelet therapy in high-risk populations undergoing PCI considering *CYP2C19* genotype and non-genetic risk factors has been implemented in various institutions, predominantly in the USA. This is a result of the increasing reports of improved clinical and economic outcomes, access to guidance from entities such as the CPIC, availability of alternative antiplatelet agents to clopidogrel, and availability of rapid *CYP2C19* genotyping (Lesko & Zineh, 2010; Crews et al, 2011; Pulley et al, 2012; Gottesman et al, 2013; Johnson & Cavallari, 2013; Hoffman et al, 2014; O'Donnell et al, 2014; Shuldiner et al; 2014; Weitzel et al, 2014; Caudle et al, 2015; Cavallari et al, 2015; Dunnenberger et al, 2015; Lee et al, 2015; Cavallari et al, 2016; Peterson et al, 2016; Cavallari et al, 2017a,b; Harada et al, 2017; Luzum et al, 2017; Cavallari et al, 2018a,b; Empey et al, 2018; Lee et al, 2018a; Notarangelo et al, 2018; Claassens et al, 2019; Gurbel et al, 2019; Black et al, 2020; Claassens & Ten Berg, 2020; Hulot et al, 2020).

The use of CYP2C19 genotyping in patients undergoing PCI to guide antiplatelet therapy prescribing is reported to result in better platelet inhibition and decreased adverse cardiac outcomes compared to patients who did not undergo antiplatelet adjustments, in whom significantly poorer outcomes were observed (Cavallari et al, 2015; Cavallari et al, 2018a,b; Lee et al, 2018a; Notarangelo et al, 2018; Claassens et al, 2019; Hulot et al, 2020).

These papers showed improved outcomes for patients, however, none of the evidence-base resulted from large prospective clinical trials. As a result, the AHA/ACC and ESC guidelines do not presently recommend implementation of routine *CYP2C19* pharmacogenetic testing to tailor DAPT (Levine et al, 2016a; Valgimigli et al, 2018).

A very recent, large, multisite trial, TAILOR-PCI, undertaken to study the effectiveness of using *CYP2C19* genotyping to guide antiplatelet treatment, narrowly missed the primary endpoint of demonstrating a 50% reduction at one-year in the combined rate of CV death, MI, stroke, severe recurrent ischemia and ST. However, the results of this trial are still very promising and provide a signal supporting the benefit of *CYP2C19* genotype-guided antiplatelet therapy, since approximately 34% fewer adverse events were observed in patients who received genotype-guided treatment compared to those who did not.[15]

[15]American College of Cardiology (ACC). TAILOR-PCI: Genotype-guided Antiplatelet Therapy Post PCI Misses Mark [Internet]. USA: ACC; 28 March 2020 [cited 2020 May 19]. Available from: www.acc.org/latest-in-cardiology/articles/2020/03/24/16/41/sat-9am-tailor-pci-clinical-implementation-clopidogrel-pharmacogenetics-acc-2020

Another large, randomized trial, POPular genetics, aimed to investigate the benefit of genotype-guided selection of a $P2Y_{12}$ inhibitor in patients undergoing PCI compared to standard therapy with ticagrelor or prasugrel (Claassens et al, 2020). The trial has showed that the genotype-guided group was non-inferior compared to standard therapy with regards to thrombotic events ($p<0.001$) with a reduction in thrombotic events in the genotype-guided group and a lower incidence of bleeding ($p=0.04$) and ishchaemia (Claassens et al, 2020).

Twenty-six percent of the cohort in the present study were carriers of one or two *CYP2C19 *2* alleles. These patients had an 'actionable' genotype with regards to clopidogrel and were eligible for *CYP2C19* genotype-guided intervention according to guidance from the CPIC and the DPWG, which recommend carriers of *CYP2C19*2* to be prescribed alternative $P2Y_{12}$ inhibitors (prasugrel or ticagrelor) instead of clopidogrel, if there is no-contraindication (Swen et al, 2011; Scott et al, 2013).

Six patients were switched from clopidogrel to prasugrel after developing ISR; 3 patients were genotyped as carriers of *CYP2C19*2,* and 3 patients were genotyped as non-carriers. The direct cost of genotyping in the present study was determined as approximately €16 per test compared to the direct cost of repeat PCI with one DEB or one DES, which amounts to between €1,126 to €2,474.

Clopidogrel is the only $P2Y_{12}$ inhibitor available on the Maltese National Health Service (NHS) formulary. Prasugrel (Effient®) is the only alternative $P2Y_{12}$ inhibitor available in

Malta. It is not presently approved on the NHS and is only available on the private market for out-of-pocket purchase, at an approximate cost of €80 per month. The patient for Effient® expired in October 2017, and a generic form of prasugrel (Mylan) was approved by the EMA through a centralised procedure in March 2018.[16] Ticagrelor (Brilinta®) is not available in Malta, neither on the Maltese NHS or on the private market. The patent for Brilinta® expired in August 2018.[17] Inaccessibility, along with the price of alternative antiplatelet therapy, may cause prescription hesitancy among physicians which may be addressed by the addition of alternate $P2Y_{12}$ inhibitors to the NHS formulary in Malta.

Prasugrel and ticagrelor are newer generation $P2Y_{12}$ inhibitors and are alternatives to clopidogrel showing superiority in preventing CV events in patients with ACS, as demonstrated in the TRITON-TIMI 38 and PLATO trials (Wiviott et al, 2006; Wallentin et al, 2009), and in other studies (Roe et al, 2012; Wiviott et al, 2015; Almendro-Delia et al, 2017; Khayata et al, 2017; Krishnamurthy et al, 2019).

When compared to clopidogrel, prasugrel has a predictable and greater antiplatelet effect and is not susceptible to CYP2C19 enzyme drug interactions or the effect of *CYP2C19* reduced function genetic polymorphisms (Sorich et al, 2010; Ferri et al, 2013;

[16] European Medicines Agency (EMA). Prasugrel Mylan: EPAR – Public assessment report [Internet]. UK: EMA; 2018 [cited 2020 Apr 29]. Available from: https://www.ema.europa.eu/en/medicines/human/EPAR/prasugrel-mylan

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Almendro-Delia et al, 2017). Ticagrelor, unlike clopidogrel and prasugrel, is an active drug that does not require hepatic activation; it has a faster onset of action and more prominent platelet inhibition. However, it has been reported to have a higher rate of discontinuation when compared to clopidogrel due to its side-effects (Kowalczyk et al, 2009; Wallentin et al, 2009; Ferri et al, 2013; Oprea & Popescu et al, 2013; Zhang et al, 2015). Compared to the other $P2Y_{12}$ inhibitors, ticagrelor has the most predictable and constant platelet inhibition in adherent patients on maintenance dosing. Genetic analysis of several studies reported a null-effect of *CYP2C19* genetic polymorphisms on clinical outcomes with both prasugrel and ticagrelor (Mega et al 2009; Wallentin et al, 2009; Mega et al 2010a; Small et al, 2010; Sorich et al 2010; Wallentin et al, 2010; Wiviott et al, 2015; Cavallari et al, 2018a).

Prasugrel and ticagrelor have been associated with increased bleeding incidence in ACS patients undergoing PCI compared to clopidogrel (Wiviott et al, 2006; Siller-Matula et al, 2009; Wallentin et al, 2009; Wiviott et al, 2015; Bacquelin et al, 2016; Lattuca et al, 2016; Khayata et al, 2017; Siller-Matula et al, 2017; Claassens et al, 2020b,c; Yu et al, 2020). However, some studies reported no difference in bleeding (Montalescot et al, 2009; James et al, 2011; Brener et al, 2014; Krishnamurthy et al, 2019; Turgeon et al, 2020), and no difference in MACE (Roe et al, 2012; Velders et al, 2016; Vercellino et al, 2017; Claassens et al, 2020a) between the different $P2Y_{12}$ inhibitors.

In ACS patients undergoing PCI, prasugrel has shown superior efficacy in the reduction of adverse CV outcomes, such as MI and ST (Montalescot et al, 2009; Brener et al, 2014; Khayata et al, 2017; Watti et al, 2017), compared to other $P2Y_{12}$ inhibitors. Olier et al., (2018) reported a significantly lower mortality rate with prasugrel with no difference between clopidogrel and ticagrelor. Similarly, Krishnamurthy et al., (2019) reported a statistically significant lower incidence of mortality with prasugrel versus ticagrelor. A post-hoc analysis reported ticagrelor to be superior to clopidogrel in efficacy and safety, where CV events occurred less frequently with ticagrelor (James et al, 2011). Conversely, a study by Mahaffey et al, (2011) demonstrated decreased efficacy of ticagrelor compared to clopidogrel, and in a recent study ticagrelor showed no statistical superiority in lowering the risk of MACE in ACS patients undergoing PCI compared to clopidogrel (Turgeon et al, 2020).

CYP2C19 genotype-guided antiplatelet therapy prescribing may be useful to optimise therapy effectiveness and reduce adverse events. However, for such precision therapy to be clinically possible and applicable it is of the utmost importance that alternative antiplatelet drugs become readily available and accessible in Malta. Ideally, this initiative would be administered by the NHS, and provided for high-risk patients undergoing PCI. Patients at an elevated risk of adverse outcomes, namely ISR, which were identified in the present study include carriers of the *CYP2C19 *2* allele, patients with a history of previous revascularisation, heart failure, active smokers, renal impairment and diabetics.

4.3 Study limitations

The lower number of cases than controls with dyslipidaemia identified in clinical records did not match the patients' medication history (statin therapy). This could be due to statins being prescribed for secondary prevention post-PCI and not to treat diagnosed dyslipidaemia or due to underreporting, causing discrepancies in the data collected. Low-density lipoprotein cholesterol and triglyceride levels were not recorded; hence this discrepancy could not be verified. Moreover, the correlation between lipid profile parameters and ISR would have been interesting to explore if recorded. Another limitation was the inability to follow-up with the cardiologists for any action taken post-genotyping after being presented with the CPIC recommendations due to the COVID-19 pandemic which resulted in restrictions for students to access the hospital for research purposes. Cardiologists' responsiveness and collaboration are essential for successful clinical implementation of genotype-guided therapy. Adherence to clopidogrel was not evaluated in this study and could be another factor that affects predisposition to ISR.

4.4 Recommendations for clinical practice improvement and further study

Several institutions have successfully implemented *CYP2C19* guided antiplatelet therapy highlighting its positive impact on patient clinical outcomes (section 4.2). These institutions reserved CYP2C19 genotyping for pre-set patient criteria, depending on the institution, for example, undergoing left heart catheterisation, undergoing PCI with high-risk anatomical findings, high-risk of bleeding. In the present study, *CYP2C19*2* was identified as a significant predictor of ISR in both the univariate and multivariate analyses. *CYP2C19*2* genotyping to achieve precision antiplatelet therapy and reduce

the risk of ISR may be proposed for high-risk patients undergoing PCI with DES, including patients with previous revascularisation, active smoking and heart failure, where a significant association was identified, together with diabetics and patients with renal impairment, which are well-documented risk factors of ISR. The number of stents implanted should also be considered as a risk factor according to results from previous studies despite no significant association being observed in the present study.

A recommendation for further study to further explore the clinical utility of *CYP2C19*2* genotyping with regards to antiplatelet therapy personalisation would be a two-armed study identifying high-risk patients undergoing PCI with antiplatelet therapy selection guided by *CYP2C19*2* genotyping in one arm versus a control group on standard therapy with no genotyping and assessing the occurrence of ISR and other MACE at different time points within one-year post-PCI. Analysis of the cost-effectiveness of *CYP2C19*2* guided antiplatelet therapy prescribing is recommended. The previous study by Wirth et al, (2016) demonstrated that compared to the same laboratory-based PCR and reverse hybridisation assay used in the present study, POC genotyping accurately and reliably identified carriers of the *CYP2C19*2* allele (97% agreement in genotype results). The POC genotyping assay has the advantages of providing rapid results, is user-friendly, requires minimal training, and is portable enabling testing at the patient's bedside compared to the laboratory assay, encouraging a more preemptive treatment approach rather than a reactive one, however testing is more expensive. Further exploration of the accessibility and use of rapid POC *CYP2C19*2* genotyping in the local setting for appropriate and timely antiplatelet prescribing is warranted.

Despite numerous advances in the implementation of pharmacogenetics into clinical practice in the past decade, progress of implemetenation to clinical practice is still reported to be slow. Barriers such as lack of training and limited knowledge among healthcare professionals may be serving as barriers and impeding significant strides forward (Johansen & Dickinson, 2014; Klein et al, 2017; Lee et al, 2018c; Owusu-Obeng et al, 2018; Borden et al, 2019). Accordingly, further training on the clinical usefulness of precision therapy using pharmacogenetic testing is important.

Adherence to DAPT is a chief predictor of poor outcomes post-stent implantation (Larkin et al, 2016; El-Toukhy et al, 2016; Luu et al, 2019), and may be a potential area for the application of pharmacist-led education to optimise clinical outcomes.

4.5 Conclusion

This study demonstrated and confirmed a previously observed signal that the *CYP2C19*2* allele was significantly associated with incidence of ISR. The findings show that the risk of developing ISR within one-year post-PCI on clopidogrel therapy is significantly higher in *CYP2C19 *2* carriers than in non-carriers. Other significant associations identified to increase the risk of ISR were previous revascularisation, heart failure and active smoking. *CYP2C19*2* genotyping may be used as a tool together with non-genetic risk factors, including previous revascularisation, heart failure, active smoking, diabetes and renal impairment, for precision antiplatelet therapy in patients undergoing PCI with DES implantation and prescribed DAPT to decrease the risk of ISR.

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Appendices

Appendix 1
Studies describing the association between
CYP2C19*2 **and adverse cardiac outcomes**

Reference	Objective	Country	Population	N	Results
Yu et al, 2020	Safety and efficacy of clopidogrel vs. ticagrelor in PCI patients and investigate association with *CYP2C19* polymorphisms	China	CAD patients who underwent PCI with stenting	971	1. *CYP2C19*2* carriers had higher incidence of MACE (p<0.001) 2. No difference in occurrence of MACE between clopidogrel and ticagrelor group 3. Ticagrelor showed a higher incidence of bleeding (p< 0.001)
Zhang et al, 2020	Assess impact of *CYP2C19* polymorphisms and dosing of clopidogrel on ISR	China	Patients who underwent PCI with stenting and on DAPT and genotyped for *CYP2C19*2*	111	Higher proportion of carriers of CYP2C19 loss of function allele had ISR (p=0.008)
Ayesh et al, 2019	Determine prevalence of *CYP2C19*2*/*3 on patients on DAPT who underwent PCI and determine their association to MACE	Palestine	Post-PCI patients	110	Higher incidence of MACE occurred in patients with *CYP2C19*2* allele (p=0.001)
Claassens et al, 2019	Assess genotype guided antiplatelet therapy and incidence of bleeding and thrombotic risk	Netherlands Belgium, Italy	Patients ≥21 years, with signs and symptoms of STEMI lasting 3-13 hours and underwent PCI with stenting	2,488	Use of clopidogrel in genotyped individuals resulted in a lower risk of bleeding when compared to standard treatment (Prasugrel or ticagrelor) p<0.05

Reference	Objective	Country	Population	N	Results
Zhang et al, 2019	Assess the relationship between *CYP2C19*2*/*3 polymorphisms and development of CAD	China	Suspected CAD candidates undergoing PCI	231	Higher proportion of *CYP2C19*2* allele carriers had CAD and coronary events compared to non-carriers (p=0.025)
Cavallari et al, 2018a	Multisite investigation of clinical implementation of CYP2C19 genotype-guided antiplatelet therapy post-PCI	Chicago, Florida, North Carolina, Indiana, Birmingham	All patients ≥18 years, underwent PCI and CYP2C19 genotyping and received DAPT post-PCI	1,815	Higher risk/event rate of MACE in loss of function-Clopidogrel group vs loss of function - prasugrel/ticagrelor group (p=0.013)
Hokimoto et al, 2018	Assess the impact of CYP2C19 polymorphisms on ISR	Japan	Patients that underwent PCI with DES stent implantation	113	Carriers of CYP2C19 *2 and *3 LoF allele had a higher rate of ISR (p>0.05)
Idrissi et al, 2018	Investigate association between *CYP2C19*2* and clopidogrel resistance	Morocco	ACS patients undergoing PCI	75	Non-significant association between *CYP2C19*2* and clopidogrel resistance (p>0.05). Most ACS presentations were carriers of *CYP2C19*2* compared to other LoF alleles (p < 0.001)
Rytkin et al, 2018	Analyse correlation between CYP2C19 gene polymorphisms on stent implantation complications	Moscow	Patients with ACS and underwent PCI with stenting	76	Non-significant association between CYP2C19 polymorphism and stent thrombosis (p=0.262)

Reference	Objective	Country	Population	N	Results
Tahara et al, 2018	Analyse association between CYP2C19 and incidence of polymorphism and MACE and ST	Japan	Patients who underwent PCI on DAPT	247	Incidence of stent thrombosis ($p = 0.04$) and MACE ($p < 0.01$) was highest in poor metabolizers
Wirth et al, 2018	*CYP2C19*2* allele and ISR	Malta	Patients who underwent PCI and on DAPT	82	Although higher percentage of *CYP2C19*2* carriers exhibited ISR within 1 year compared to non-carriers, association between *CYP2C19*2* allele and ISR was not statistically significant ($p=0.067$)
Yang et al, 2018	Compare and assess bleeding and clinical outcomes between prasugrel and ticagrelor in patients with type-2 DM post-PCI	China	Randomized/non-randomized trials comparing post-PCI clinical and bleeding outcomes in type-2 DM	2,004	Difference in mortality, MI, MACE, and bleeding were not significantly different
Almendro-Delia et al, 2017	Compare efficacy and safety of ticagrelor and prasugrel versus clopidogrel	Spain	Patients with ACS receiving clopidogrel, ticagrelor or prasugrel at the time of hospital discharge or in-hospital death	2,906	Total mortality ($p<0.0001$), non-fatal thrombotic events ($p=0.05$) and ST ($p=0.025$) was lower in prasugrel and ticagrelor compared to clopidogrel with no difference in bleeding events
Gosling et al, 2017	Analyse and compare effect of ticagrelor, prasugrel and clopidogrel on all-cause mortality and ST	United Kingdom	ACS patients undergoing coronary angiography	10,973	Ticagrelor was associated with a lower all-cause mortality ($p=0.01$) compared to clopidogrel; Ticagrelor and prasugrel were associated with lower mortality compared to clopidogrel ($p<0.001$)

Reference	Objective	Country	Population	N	Results
Jiang and You, 2017	Assess cost-effectiveness of CYP2C19 genotype-guided antiplatelet therapy	China	Patients with ACS undergoing PCI	16,086	Genotype guided antiplatelet therapy was less expensive compared to universal $P2Y_{12}$ inhibitor and universal alternative $P2Y_{12}$ inhibitor ($p< 0.001$)
Ruedlinger et al, 2017	Impact of *CYP2C19*2* polymorphism on ISR	Chile	CAD patients who underwent successful PCI	163	1. No association between *CYP2C19*2* and ISR ($p=0.06$) 2. *CYP2C19*2* carriers on DAPT developed less ISR than non-carriers ($p = 0.05$)
Saydam et al, 2017	Demonstrate impact of *CYP2C19*2* on clopidogrel response	Turkey	Patients with CAD undergoing PCI with stent implantation	1,180	Significant association between *CYP2C19*2* and clopidogrel responsiveness ($p<0.001$)
Deiman et al, 2016	Explore clinical outcomes and cost-effectiveness in CYP2C19 poor metabolizers treated with either clopidogrel or prasugrel post-PCI	Netherlands	All patients scheduled for elective PCI except patients with STEMI	3,260	Higher number of ST ($p=0.003$) and CV events ($p=0.003$) was recorded in poor-metaboliser clopidogrel group vs prasugrel group
Motovska et al, 2016	Compare efficacy and safety of ticagrelor vs prasugrel in patients with MI undergoing PCI	Czech Republic	Patients with acute MI indicated for primary PCI	1,230	No difference in CV death, stroke, MI, ischaemic attacks, and bleeding events

Reference	Objective	Country	Population	N	Results
Sánchez-Ramos et al, 2016	Analyse if CYP2C19 genotype guided strategy reduces CV events and bleeding rates	Spain	Patients ≥ 18 years, diagnosed with CAD, underwent PCI with stenting	719	Genotype-guided antiplatelet therapy reduces the rate of CV death, ACS, or stroke without differences in bleeding during 12 months after PCI vs control (p=0.03) Rates of ST did not differ between groups (p=0.87)
Konishi et al, 2015	Association between CYP2C19 loss of function and ST	Japan	ACS patients on DAPT underwent PCI	196	No difference in death and ST among different CYP2C19 loss of function alleles (p = 0.002)
Niu et al, 2015	Systematic review and meta-analysis of CYP2C19 polymorphism and clinical outcomes in patients treated with clopidogrel	China	Patients with CAD treated with clopidogrel and tested for reduced function CYP2C19 and followed up for at least 1 year	25,564	Carriers of reduced function CYP2C19 allele had an increased risk of MACE (p=0.001)
Nozari et al, 2015	Impact of *CYP2C19*2* polymorphism on ISR	Iran	All patients who underwent PCI on DAPT	100	Prevalence of ISR post-PCI was higher in *CYP2C19*2* carriers with a non-significant association (p = 0.273)

Reference	Objective	Country	Population	N	Results
Wei et al, 2015	Analysis of the correlation of CYP*2C19*2* mutation with clopidogrel resistance	China	ACS patients undergoing PCI on DAPT	100	Incidence of recurrent angina, MI and ST was higher in *CYP2C19*2* ($p < 0.05$). *CYP2C19*2* carriers had higher clopidogrel resistance when compared to CYP2C19 wild-type allele ($p = 0.009$)
Namazi et al, 2012	Assess pharmacogenetic response variability with clopidogrel use	Iran	All patients who undergoing PCI	112	No difference in clopidogrel responsiveness between *CYP2C19*2*,*3 or *1 ($P > 0.05$)
Price et al, 2012	Assess genetic determinants of clopidogrel response	USA	Patients with stable angina/ischemia or NSTE-ACS undergoing PCI with DES	1,028	CYP2C19 is a significant determinant of pharmacodynamic effect of clopidogrel

Reference	Objective	Country	Population	N	Results
Holmes et al, 2011	Analyse association between CYP2C19 and clopidogrel response	-	Studies that compared subjects with loss-of-function CYP2C19 carriers with non-carriers	42,016	1. Statistically significant association between CYP2C19 genotype and response to clopidogrel ($p < 0.05$) 2. No significant association between genotype status and CV outcomes ($p > 0.05$)
Jeong et al, 2011	Evaluation of effect of CYP2C19 polymorphisms on clopidogrel pharmacodynamics and prognosis	Korea	All patients ≥ 18 years old, underwent coronary angiography or PCI	266	CYP2C19 loss of function allele carriers were associated with an increase in CV events when compared to non-carriers ($p=0.013$)
Nishio et al, 2012	Incidence of MACE and ST among the different CYP2C19 LOF groups and normal metabolisers	Japan	Patients who underwent PCI with DES implantation and were on DAPT	160	Higher incidence of MACE in poor and intermediate metabolisers ($p =0.005$). No difference in ST ($p = 0.79$) among the different groups

Reference	Objective	Country	Population	N	Results
Zabalza et al, 2012	Meta-analysis to assess association between CYP2C19 LoF and CV outcomes with clopidogrel	Spain	CYP2C19 LoF polymorphisms in CAD patients on clopidogrel therapy	8,686	*CYP2C19*2* was associated with an increased risk of ST ($p<0.001$)
Harmsze et al, 2010	Assess genetic variants of CYP2C19 (*2/*3) in patients on DAPT in cases and control	Holland	Case: Angiographically assessed ST patients on DAPT at time of incidence. Control: Patients who underwent PCI with stenting with no adverse CV events during 1 year follow up post-PCI	176	*CYP2C19*2* and *CYP2C19*3* associated with a 1.7 and 2.4-fold increase incidence of ST compared to control after PCI ($p = 0.013$)
Hulot et al, 2010	Assess association between LOF *CYP2C19*2* variant and ischaemic outcomes in patients administering clopidogrel (Meta-analysis)	France	Data reported from original studies, randomized or cohort and reported incidence of MACE or mortality of CAD patients treated with clopidogrel	11959 (10 studies)	*CYP2C19*2* carriers had a significantly higher risk of MACE and ST compared to non-carriers; majority of which was subacute happening within the first 30 days of stent implantation ($p<0.001$)

Reference	Objective	Country	Population	N	Results
Simon et al, 2008	Assess impact of CYP2C19 LOF allele on CV events during a 1-year follow up	France	Patients presenting with acute MI admitted to intensive care units	2208	Subjects with CYP2C19 LOF alleles had a higher rate of CV events (P <0.05)
Hulot et al, 2006	Investigate pharmacogenetics in clopidogrel	France	Healthy Caucasian males between ages of 18-35	29	Response to clopidogrel was influenced by CYP2C19 carrier status (p<0.030)
Mega et al, 2009	1. Analyse the association between CYP2C19 genetic variants, plasma concentration and platelet inhibition in response to clopidogrel 2. Determine association between LOF alleles and adverse CV outcomes	USA	1.Healthy patients 2. Patients with ACS with planned PCI to administer DAPT for up to 15 months (TRITON-TIMI 38)	162 1,477	Carriers of at least one LOF allele had lower plasma levels of active metabolite and diminished platelet inhibition, poor metabolizers being the lowest (P<0.001) Risk of MACE was higher and risk of ST was 3- fold higher in carriers of *CYP2C19*2* allele vs non carriers (p=0.020)

Appendix 2
Data Collection Form

Data Collection Form

Patient study no:	Consultant:	Date of PCI:

1. Patient Information

Age (in years)	Date of blood sample collection: _____
Gender	**O** Male **O** Female **O** Other
Ethnicity	**O** Caucasian **O** Other (North African, Black/African American, Asian, Middle Eastern)

2. Cardiac risk factors and social history

O Family History of IHD	**O** Alcohol consumption
O Previous revascularisation	**O** Previous MI
O Smoking	**O** Active (No. of cigarettes/day _____) **O** Past (Date/year stopped _____) **O** Never
Weight (kg)	Height (m)
BMI (kg/m^2)	**O** Underweight (<18.5) **O** Normal weight (18.5-24.99) **O** Pre-obesity (25-29.99) **O** Obesity Class I (30-34.99) **O** Obesity Class II (35-39.99) **O** Obesity Class III (≥ 40)

3a. Relevant comorbidities

O Hypertension
O Diabetes
O Renal impairment (eGFR <60)
O Chronic liver disease
O Dyslipidaemia
O Heart failure

3b. Investigations

HbA1c	
eGFR	
Cr	
LVEF %	

4. Angiographic factors

In-Stent restenosis (ISR)	**O** Yes **O** No **O** Month 1 **O** Month 2-6 ___ **O** Month 7-12 ___

Reason for PCI	**O** STEMI **O** NSTEMI **O** UA **O** ISR
Type of PCI performed	**O** Elective (outpatient) **O** Emergency (Inpatient)
Number of stents deployed	**O** 1 **O** 2 **O** 3 **O** >3 (____)
Number of stents with stenosed	**O** 1 **O** 2 **O** 3 **O** >3 (____)
Stent dimensions (mm):	Stent type:
Vessel/s stented	**O** Right coronary (RCA) **O** Left anterior descending (LAD) **O** Left main (LM) **O** Obtuse marginal (OM) **O** Circumflex (CX) **O** Diagonal (D) **O** Posterior descending (PDA) **O** Intermediate **O** Grafts

5. Current Medications

	Drug generic name	Dose and dosage regimen
1	**Clopidogrel**	**75 mg OD**
2	**Aspirin**	**75 mg OD**
3		
4		
5		
6		
7		
8		
9		
10		

6. CYP2C19 genotype/phenotype

Carrier of *CYP2C19*2* allele	**Genotype (Phenotype)**
O Yes **O** No	**O** *1/*1 Homozygous wild type (Normal metaboliser) **O***1/*2 Heterozygous (Intermediate metaboliser) **O***2/*2 Homozygous variant (Poor metaboliser)

Appendix 3
Ethics approval

L-Università ta' Malta

Faculty of
Medicine & Surgery

University of Malta
Msida MSD 2080, Malta

Tel: +356 2340 1879/1891/1167
umms@um.edu.mt

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Ref No: **FRECMDS_1819_59**

Friday 24th May 2019

Ms Sara Osama
Yacht Marina Apartments,
Block 2 flat 3
Triq ix-xatt
Pieta. Malta.

Dear Ms Sara Osama,

Please refer to your application submitted to the Research Ethics Committee in connection with your research entitled:

Pharmacogenetics in clopidogrel use

The Faculty Research Ethics Committee granted ethical approval for the above mentioned protocol.

Yours sincerely,

Professor Pierre Mallia
Chairman
Research Ethics Committee

Appendix 4
Patient information sheets and Consent forms

PATIENT INFORMATION SHEET

I, Sara Osama, a Doctorate in Pharmacy student at the Department of Pharmacy, University of Malta, am currently undertaking a research project entitled 'Pharmacogenetics in clopidogrel use' under the supervision of Dr Francesca Wirth from the Department of Pharmacy, University of Malta, in collaboration with the Department of Cardiology and the Department of Pathology at Mater Dei Hospital.

You have been identified to participate in this research, which involves the following:

Aim of the research and how will you benefit?
Clopidogrel, a medicine you are taking after stent implantation, needs to be converted by liver enzymes to be effective to thin your blood and keep the stent open. The reduced functioning of these enzymes may cause clopidogrel to not work as efficiently to give its maximum protection. This research will determine the functioning of these enzymes so that your consultant cardiologist will be in a better position to prescribe a safer and more effective therapy according to your needs.

Your involvement

- Have a blood sample taken only once by a physician or nurse at the Cardiology Department at Mater Dei Hospital (today).
- Answer a few questions about your cardiac-health and medications.
- Be followed-up by your consultant cardiologist and myself for 6-12 months at your regular outpatient visits.

Other important information

- Participation in this research is entirely voluntary. The information gathered will be kept strictly confidential and used solely for the purpose of the research according to the Data Protection Act (General Data Protection Regulation (EU) 2016/679).
- Refusal to participate will in no way affect the treatment you receive as a patient at the Cardiology Department at Mater Dei Hospital.
- You may discontinue participation in the research at any time without any prejudice.
- Results of this research will not influence the routine treatment/service you receive.
- Your consultant cardiologist will communicate results of this research or any other incidental findings to you**.**

Kindly sign the attached consent form if you agree to participate in this research.

Thank you in advance for your cooperation.

Sara Osama
0183649A

INFORMAZZJONI GĦALL-PAZJENT

Jiena, Sara Osama, studenta ghad- Dottorat fid- Dipartiment tal-Farmaċija fl-Universita' ta' Malta, qed nagħmel proġett ta' riċerka ntitolat 'Pharmacogenetics in clopidogrel use' taħt is-sorveljanza ta' Dr Francesca Wirth mid- Dipartiment tal-Farmaċija fl-Universita' ta' Malta, b'kollaborazzjoni mad-Dipartiment tal-Kardjoloġija u d-Dipartiment tal-Patoloġija fl-Isptar Mater Dei.

Inti gejt maghżul/a biex tipparteċipa f'din ir-riċerka li tinvolvi dan li gej:

L-għan ta din ir-riċerka u kif ser tibbenefika?
Clopidogrel, hija mediċina li inti qed tieħu wara l- impjantazzjoni tal-molla (stent). Din il-mediċina taħdem permezz tal-enżimi tal-fwied biex traqqaq id-demm u ghalhekk iżżom il-molla (stent) miftuħa. Jekk il-funzjoni ta' dawn l-enżimi tonqos, il-mediċina Clopidogrel tista' ma taħdimx effiċenti u ma' tagħtix il-protezżjoni massima li hemm bżonn. F'din ir-riċerka, l-funzjoni ta l-enżimi ha tiġi determinata, u permezz tà dan, il-konsulent tal-Kardjoloġija i/tkun f-pożizzjoni aħjar biex j/tippreskrivi mediċina effiċenti għall-bżonnijiet tiegħek.

L-involviment tiegħek

- Jittieħed kampjun tad-demm darba biss minn tabib jew infermier fid-Dipartiment tal-Kardjoloġija fl-Isptar Mater Dei (li jssir illum).
- Twieġeb ftit mistoqsijiet dwar is-saħħa tiegħek relatati mal-mard tal-qalb u l-mediċini tiegħek.
- Tkun segwit/a mill-Konsulent tal-Kardjoloġija tiegħek u minni għal 6-12-il xahar fiż-żjarat regolari tal-outpatients tiegħek.

Informazzjoni oħra importanti

- Il-parteċipazzjoni f'din ir-riċerka hija kompletament volontarja. L-informazzjoni miġbura tinżamm strettament kunfidenzjali u tintuża biss għall-iskop tar-riċerka skond l-Att dwar il-Protezzjoni tad-Data (Regolament Ġenerali dwar il-Protezzjoni tad-Data (EU) 2016/679).
- Ir-rifjut ta' parteċipazzjoni bl-ebda mod ma jaffettwa t-trattament li tirċievi bħala pazjent fl-Isptar Mater Dei.
- Tistá twaqqaf il-parteċipazzjoni fir-riċerka fi kwalunkwè ħin mingħajr preġudizzju.
- Ir-riżultati ta' din ir-riċerka mhux se jinfluwenzaw it-trattament / servizz ta' rutina li tirċievi fl-Isptar Mater Dei.
- Il-Konsulent tal-Kardjoloġiku tiegħek ser j/tikkomunika lilek ir-riżultati ta' din ir-riċerka jew sejbiet inċidentali oħrajn.

Jekk jogħġbok iffirma l-formola tal-kunsens mehmuża jekk taqbel li tieħu sehem f'din ir-ricerka.
Grazzi bil-quddiem għall-kooperazzjoni tiegħek.
Sara Osama 0183649A

CONSENT FORM

I am a Maltese citizen and I am over eighteen (18) years of age. asked to participate in a research study entitled:

Pharmacogenetics in clopidogrel use.

The purpose and details of the study have been explained to me by **Sara Osama** and any difficulties which I have raised have been adequately clarified. I give my consent to the Principal Investigator to take the required samples and/or to make the applicable observations. I am aware of any inconveniences which this may cause.

I understand that the results of this study in which I am participating may be used for medical or scientific purposes and that the results of this study may be reported/published. However, I shall not be personally identified in any way, either individually or collectively, without my expressing written permission. Under the General Data Protection Regulation (GDPR) and national legislation that implements and further specifies the relevant provisions of the said Regulation, I have the right to obtain access to, rectify, and where applicable ask for the data concerning me to be erased.

I am under no obligation to participate in this study and am doing so voluntarily. I may withdraw from the study at any time, without giving any reason. This will not influence in any way the care and attention and treatment normally given to me**.** I understand that any complications or adverse effects which may arise during or as a consequence of the study will be recorded and that any treatment which this may entail will be given within the Government Health Services.

Access to patient records is limited to the Principal Investigator and supervisor. A randomly assigned study number will be used for each patient to maintain anonymity and confidentiality. All data collected will be securely disposed of at end of the study.

I am not receiving any remuneration for participating in this study.

In case of queries during the study I may contact: **Sara Osama**

Signature of participant	_____
Name of participant	_____
ID. Number of participant	_____
Contact number of participant	_____
Signature of Principal Investigator	_____
Name of Principal Investigator	Sara Osama
Email of Principal Investigator	Sara.Osama.17@um.edu.mt
Contact number of Principal Investigator	99695174
Name of Principal Supervisor	Dr. Francesca Wirth
Email of Principal Supervisor	Francesca.wirth@um.edu.mt
Contact number of Principal Supervisor	23402902/79266006
Date	_____

PROPOSTA GĦALL-FORMULA TAL-KUNSENS

Jien/a ċittadin/a Malti/ja u għalaqt tmintax-il sena.

Talbuni biex nieħu sehem fi studju ta' riċerka bl-isem ta': Pharmacogenetics in clopidogrel use

L-għanijiet u d-dettalji tal-istudju spejga(t)homli Sara Osama li wkoll iċċara(t)li xi mistoqsijiet li għamilt.

Nagħti l-kunsens tiegħi lill-persuna responsabbli għal din ir-riċerka biex j(t)ieħu l-kampjuni u/jew j(t)agħmel l-osservazjonijiet li hemm bżonn u nifhem li dan jista' jkun ta' skomdu għalija.

Jiena nifhem li r-riżultati ta' dan l-istudju jistgħu jintużaw għal skopijiet xjentifiċi u jistgħu jiġu ppubblikati, jekk isir hekk jiena b'ebda mod ma nista' nkun identifikat/a, individwalment jew bħala parti minn grupp, mingħajr il-kunsens tiegħi bil-miktub. Taħt ir-Regolament Ġenerali dwar il-Protezzjoni tad-Data (GDPR) u l-leġislazzjoni nazzjonali li timplimenta u tispeċifika aktar id-dispożizzjonijiet relevanti ta 'limsemmi Regolament, għandek id-dritt li tikseb aċċess għal, tikkoreġi, u fejn applikabbli titlob li d-data li tikkonċerna lilek titħassar.

Jiena m'għandi l-ebda dmir li nieħu sehem f'dan l-istudju u dan qiegħed/qiegħda nagħmlu minn rajja. Jiena nista' meta rrid ma nkomplix nieħu sehem f'dan l-istudju mingħajr ma' nagħti raġuni. Jekk nagħmel hekk xorta nibqa' nieħu l-kura li ssoltu tingħatali. Jiena nifhem li jekk ikun hemm xi kumplikazzjoniji jew effetti mhux mistennija waqt l-istudju, dawn jiġu mniżżla bil-miktub u jekk ikun hemm bżonn xi kura tiġi mgħotija mis-servizz nazzjonali tas-saħħa.

Aċċess għall-fajl tiegħi tal-isptar huwa permess biss għar-riċerkatriċi u superviżur tar-riċerka. Numru każwali jingħata lil kull pazjent biex tinżamm l-anonimità u l-kunfidenzjalità matul l-studju kollu. Kunfidenzjalità ta' data ser tinżamm matul ir-riċerka kollha u l-informazzjoni miġbura ser tiġi abolita b'mod sigur wara li tintemm ir-riċerka.

<u>Jiena mhux qed nitħallas</u> biex nieħu sehem f'dan l-istudju.

Jekk ikolli xi diffikulta' waqt l-istudju nista' nistaqsi għal: Sara Osama

Firma tal-parteċipant	_____
Isem tal-parteċipant	_____
Numru tal-identita	_____
Numru tat-telefon	_____
Firma tal-persuna responsabbli għal din ir-riċerka	_____
Isem tal-persuna responsabbli għal din ir-riċerka	Sara Osama
Email tal-persuna responsabbli għal din ir-riċerk	Sara.osama.17@um.edu.mt
Numru tal-mowbajl tal-persuna responsabbli għal din ir-riċerka	99695174
Isem tas-superviżur prinċipali	Dr. Francesca Wirth
Email tas-superviżur prinċipali	Francesca.wirth@um.edu.mt
Numru tat-telefon tas-superviżur prinċipali	23402902/79266006
Date	2.5.19

Appendix 5

CYP2C19 Genotyping and antiplatelet therapy recommendation for cardiologists

CYP2C19 Genotyping Test Result

ID Card Number:
Patient's Name: Date:

Attention: Doctor

Your patient was genotyped for the CYP2C19 loss-of-function *2 allele. Presence of the *2 allele is associated with reduced CYP2C19 activity which impairs clopidogrel metabolism into its active form, resulting in reduced platelet inhibition, increased residual platelet aggregation and an increased risk of adverse cardiovascular events, particularly ACS patients undergoing PCI.

RESULT of CYP2C19 genotype: Carrier of one*2 allele (*1/*2)

According to this test result the patient has predicted **impaired metabolism** of clopidogrel and predicted clopidogrel efficacy is **DECREASED** (Intermediatemetaboliser).

RECOMMENDATION (based on CPIC guidelines):
Consider prasugrel, unless contra-indicated*

According to prasugrel SPC:
*Contraindications: History of stroke or TIA, active pathological bleeding, hypersensitivity, severe hepatic impairment

Cautions (increased bleeding risk): Age ≥ 75 years (generally not recommended in these patients and should only be prescribed at a 5 mg maintenance dose following careful benefit/risk evaluation), with a propensity to bleed (recent trauma, recent surgery, recurrent GI bleeding, active PUD), body weight < 60 kg (use 5 mg maintenance dose in these patients), concomitant medications that may increase bleeding risk (warfarin, NSAIDs), renal impairment

Note: CYP2C19 genotype is one of several factors that influence clopidogrel efficacy and physician judgement should be exercised when considering antiplatelet therapy for a given patient.

Sincerely,
Sara Osama

CYP2C19 Genotyping Test Result

ID Card Number:
Patient's Name: Date:

Attention: Doctor
Your patient was genotyped for the CYP2C19 loss-of-function *2 allele. Presence of the *2 allele is associated with reduced CYP2C19 activity which impairs clopidogrel metabolism into its active form, resulting in reduced platelet inhibition, increased residual platelet aggregation and an increased risk of adverse cardiovascular events, particularly ACS patients undergoing PCI.

RESULT of CYP2C19 genotype: Carrier of two*2 alleles (*2/*2)

According to this test result the patient has predicted **significantly impaired metabolism** of clopidogrel and predicted clopidogrel efficacy is **DECREASED** (Poor metaboliser).

RECOMMENDATION (based on CPIC guidelines):

Consider prasugrel, unless contra-indicated*

According to prasugrel SPC:
*Contraindications: History of stroke or TIA, active pathological bleeding, hypersensitivity, severe hepatic impairment.

Cautions (increased bleeding risk): Age ≥ 75 years (generally not recommended in these patients and should only be prescribed at a 5 mg maintenance dose following careful benefit/risk evaluation), with a propensity to bleed (recent trauma, recent surgery, recurrent GI bleeding, active PUD), body weight < 60 kg (use 5 mg maintenance dose in these patients), concomitant medications that may increase bleeding risk (warfarin, NSAIDs), renal impairment

Note: CYP2C19 genotype is one of several factors that influence clopidogrel efficacy and physician judgement should be exercised when considering antiplatelet therapy for a given patient.

Sincerely,
Sara Osama